

# Bloodlines: mammals, leeches, and conservation in southern Asia

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Running title: Mammals, leeches, and conservation

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Southern Asia is a biodiversity hotspot both for terrestrial mammals and for leeches. Many  
small-mammal groups are under-studied in this region, while other mammals are of known

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3 conservation concern. In addition to standard methods for surveying mammals, it has recently  
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5 been demonstrated that residual bloodmeals within leeches can be sequenced to find mammals in  
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7 a given area. While these invertebrate-parasite-derived DNA (iDNA) methods are promising,  
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9 most of the leech species utilized for this type of survey remain unevaluated, notwithstanding  
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11 that their diversity varies substantially. Here we examine approximately 750 individual leech  
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13 specimens in the genus *Haemadipsa* across a large range in southern Asia (Bangladesh,  
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15 Cambodia, and China), specifically reviewing the diversity of mammals they feed on and their  
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17 own genetic structuring. Leeches were found to feed on a considerable variety of mammals,  
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19 corroborating prior studies. Additionally, leeches were found to have fed both on bats and on  
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21 birds, neither of which has previously been recorded with this method. The genetic structuring of  
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23 the leeches themselves revealed 15 distinct clades of which only two corresponded to previously-  
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25 characterized species, indicating that much work is needed to finalize classifications in this  
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27 genus. Most importantly, with regards to mammal conservation, leeches in these clades appear to  
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29 feed on a broad range of mammals.  
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38 **Key words:** biodiversity, bloodmeal, conservation, *Haemadipsa*, iDNA, leech, mammal, survey  
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# Introduction

At least 25% of mammal species are threatened with extinction, but this number is almost surely higher: 18% are data deficient and thus of unknown vulnerability (Schipper et al., 2008). The need to increase efforts and develop innovative methods for determining vertebrate biodiversity is accordingly more important than ever. The paucity of data, even for well-surveyed groups of vertebrates, relates in part to biases and inefficiencies of traditional survey techniques for uncovering cryptic, rare, and secretive species (Schnell et al., 2012, 2015; Weiskopf et al., 2018). In contrast, sequencing iDNA from blood-sucking, feces-ingesting, and carrion-feeding invertebrates' stomach contents provides an efficient method for surveying vertebrate biodiversity (Brower & Judd, 2008; Calvignac-Spencer et al., 2013; Ngo & Kramer, 2003; Schnell et al., 2012, 2015; Townzen, 2008).

Terrestrial leeches (Haemadipsidae) appear to be particularly effective for conservation surveys of a variety of mammals (Schnell et al., 2012, 2015). Across their broad Indo-Pacific range, haemadipsids often are abundant, appear to prey on a wide variety of vertebrates, retain bloodmeal iDNA for long periods of time, and are easy to collect as they seek out humans (Borda & Siddall, 2010; Haeckel, 1883; Schnell et al., 2012). Schnell et al. (2012) identified six mammal species from 25 assayed leeches in Vietnam, including species that are cryptic, recently described, and likely threatened, as well as one previously unknown to the sampling area. Following up on this foundational work, Weiskopf et al. (2018) conducted a leech-based iDNA mammal survey in Bangladesh on ~200 leeches, compared the results to camera trap data, and found the iDNA method to be efficient and complementary to camera traps.

Leeches assayed in Schnell et al. (2012, 2015) and Weiskopf et al. (2018) manifested bloodmeals from a wide range of mammalian orders (Artiodactyla, Carnivora, Lagomorpha,

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3 Rodentia, Scandentia, and Primates), obviously with diverse behaviors, ecologies, and body  
4 sizes. The individual leeches assessed, however, were not identified to species in Schnell et al.  
5 (2012, 2015). Weiskopf et al. (2018) did use individual iDNA determinations from single  
6 bloodmeals corresponding to known leech genotypes, and found some differences between  
7 bloodmeals from different *Haemadipsa* species.  
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14 Terrestrial leeches, in their own right, are a major component of tropical Asian  
15 biodiversity and are notable human and livestock pests (Borda & Siddall, 2010). Yet few studies  
16 have been conducted on Haemadipsidae ecology, evolution, or even classification (Borda et al.,  
17 2008; Borda & Siddall, 2010; Tan, 2008; Tessler et al., 2016). Several recent species  
18 descriptions (e.g. a cave leech from Yunnan), range extensions (into Taiwan), and nomenclatural  
19 changes (recognition of Taiwanese species) further point to poor knowledge of Asian terrestrial  
20 leeches (Lai et al. 2011, Ngamprasertwong et al. 2007, Tong et al. 2009). These aspects of leech  
21 biology are important for a more precise understanding of this abundant group and are also  
22 necessary if these leeches are to stand as valuable and accurate tools for iDNA surveys of  
23 vertebrates.  
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38 Southern Asia is an excellent place for bloodmeal survey methods, as this is the principal  
39 range of *Haemadipsa*, one of the two most speciose genera of terrestrial leech (Borda & Siddall,  
40 2010; Richardson, 1975; Sawyer, 1986; Tessler et al., 2016). More importantly, southern Asia  
41 harbors many biodiversity hotspots for mammals. For example, China, particularly Yunnan  
42 province, is an important location for conservation surveys; it is phenomenally biodiverse, with  
43 many threatened and endangered species (Yang et al., 2004). In fact, Yunnan is one of the most  
44 biodiverse areas in the world, with 50% of Chinese mammals occurring in only 4% of China's  
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3 land area. Furthermore, Yunnan is a focal point for conservation efforts, as it contains 73% of  
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5 China's priority-protected wild animal species.  
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8 This study seeks to determine the following: 1) the extent to which bloodmeals are an  
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10 efficient tool for surveying and determining mammalian biodiversity in southern Asia, and 2) the  
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12 genetic diversity within the leeches reviewed.  
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## 14 15 16 17 **Materials and methods** 18

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20 Leeches were collected from a broad diversity of habitats (including farms, river edges, and  
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22 forests under different levels of protection) across Yunnan and Hainan provinces in China (2014)  
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24 and across Cambodia (2015). Additional leech specimens and data from Bangladesh were  
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26 incorporated from Weiskopf et al. (2018). Specimens were acquired using standard techniques of  
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28 walking through suitable habitat, such as humid forests and forest edges, collecting leeches from  
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30 the ground or as they attached to the investigators' clothing. Leeches found feeding on  
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32 investigators were not utilized for this study. Collected leeches were relaxed and fixed in either  
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34 95% ethanol or in RNAlater (Ambion). Leech specimens were then preliminarily identified to  
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36 morphogroups with stereomicroscopy.  
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40 Leech tissue was removed and further cut into pieces for proteolytic digestion and  
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42 extraction; specifically, tissue was taken from anterior of the posterior sucker to the mid-body (or  
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44 2.5 mm maximum for larger specimens). This dissection optimized chances of finding a  
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46 bloodmeal, as this is a principal area where blood is digested, while still maintaining voucher  
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48 specimens with all taxonomically important characters intact. DNA was then extracted from the  
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50 excised tissue using Qiagen DNeasy Blood and Tissue kits.  
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3 The bloodmeal iDNA was amplified for vertebrate-specific 16S rRNA (~294 bp) using  
4 700 µM of 16Scp primers (Caragiulo et al., 2014; Weiskopf et al., 2018), 0.3 µl BSA, 21.3 µl  
5 water, 2 µl of extracted template, and GE illustra PuReTaq Ready-To-Go PCR beads. This and  
6 thermocycler profiles followed Weiskopf et al. (2018). To additionally verify each leech's  
7 identity, cytochrome *c* oxidase subunit I (cox1) mtDNA was amplified using 500 µM of HHCO1  
8 (Tessler et al., n.d.) and LCO1490 primers (Folmer et al., 1994), 23.0 µl water, 1.0 µl of  
9 extracted template, and GE illustra PuReTaq Ready-To-Go PCR beads. Cox1 PCR protocols  
10 follow prior work on Haemadipsidae leeches (Borda et al., 2008). Amplification products were  
11 then cleaned using a 2:1 ratio of Agencourt AMPure XP (Beckman Coulter) to product. Products  
12 were cycle sequenced and ethanol precipitated prior to sequence determination via an ABI 3730  
13 (Applied Biosystems). Sequences had primers trimmed, were reconciled, and then edited for  
14 quality in Geneious version 6.1.8 (Biomatters).  
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30 Candidate mammal hosts were identified using NCBI's blastn function based on the  
31 match with the lowest e-value. It was noted whether or not the best match was at a 1% better  
32 match in terms of percent identity. This was assumed to be the case if the first 100 matches were  
33 the same species. If several matches had identical e-values, the identity was determined only to  
34 the lowest overlapping taxonomic rank (i.e. genus or family). Chi-squared tests were conducted  
35 in R to detect differences in the success rate of BLAST matches given the preservative used, as  
36 well as preliminary differences in host specificity that the leeches in a given country may have.  
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47 Our leech cox1 sequences were combined with cox1 data from prior phylogenetic studies  
48 of Haemadipsidae and GenBank (Borda et al., 2008; Borda & Siddall, 2010; Lai et al., 2011;  
49 Tessler et al., 2016; Won et al., 2014). Fully redundant sequences were then removed using the  
50 'fastx\_collapser' tool from the FASTX-Toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit](http://hannonlab.cshl.edu/fastx_toolkit)), as they  
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3 can have negative impacts on the topological stability of maximum likelihood reconstructions  
4 and ultimately do not provide any additional information. An appropriate model was fit to the  
5 data using jModelTest 2.1.4 (Darriba et al., 2012). A maximum likelihood phylogenetic  
6 reconstruction was then conducted using the best model (GTR+I+ $\Gamma$ ) with the non-redundant data  
7 using Garli 2.01 (Zwickl, 2006) via the CIPRES Science Gateway (Miller et al., 2010). Support  
8 for the most likely topology was calculated using 1,000 bootstrap pseudoreplicates in the same  
9 programme and summarized on the most likely phylogenetic reconstruction with SumTrees of  
10 the DendroPy library (Sukumaran & Holder, 2010).  
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## 24 **Results**

### 25 **Bloodmeal BLAST**

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27 Approximately 750 leech specimens were examined. Vertebrate species identified from iDNA  
28 (Table 1) represent one avian order (Galliformes; found in 10 leech samples) and a large number  
29 of mammalian orders (Artiodactyla, Carnivora, Chiroptera, Primates, Rodentia, Scandentia;  
30 found in 251 leech samples). Please note that using shorter fragments of iDNA could possibly  
31 amplify a higher percentage of bloodmeals. Artiodactyl species were those most commonly  
32 amplified, representing 45% of the sequences recovered. In total, 31 species in 16 families were  
33 identified; however, identities are preliminary, as definitive identification would often require  
34 additional loci and in larger databases and is beyond the scope of this work.  
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47 The most common wild mammals found were the Asian palm civet (*Paradoxurus*  
48 *hermaphroditus*; 19 samples) and the lion-tailed macaque (*Macaca silenus*; 17 samples). In  
49 addition to wild animals, iDNA was identified humans and domesticated animals (*Bos taurus*,  
50 *Bubalus bubalus*, *Canis lupus*, *Felis silvestris*, *Sus scrofa*, and *Gallus gallus*). We view these  
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3 human and domesticated animal identifications cautiously, as these taxa are sometimes lab  
4 contaminants; however, we believe our lab protocols should have mitigated such issues (see  
5 Weiskopf et al., 2018). These iDNA sequence results cannot differentiate between domesticated  
6 and wild forms of *S. scrofa* or *G. gallus* that co-occur in many of the areas reviewed.  
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12       Amplification success rates for iDNA were on average 34%, but varied substantially  
13 between sites. Most notably, iDNA sequences were successfully obtained in 62% of samples  
14 from the Tarap Hill Reserve Forest site in Bangladesh, while only 11% were recovered from  
15 bloodmeals from Seima in Cambodia. The iDNA was successfully amplified whether the leech  
16 specimen was preserved in ethanol (30 amplified) or RNAlater (231 amplified; no significant  
17 difference was found, with  $P = 0.9991$ ). Following sequence determination above, iDNA from  
18 265 specimens were reamplified and resequenced to achieve better quality. Of these, only two  
19 resulted in amplified products that had sequences not matching what was originally determined.  
20 In both cases, each isolate contained sequences corresponding both to *P. hermaphroditus* and *B.*  
21 *taurus*.  
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35       The species/clade of the leech did not seem to have notable host specificity or correspond  
36 to any obvious pattern in terms of vertebrate host found. However, sample sizes precluded all but  
37 the most rudimentary comparisons. Chi-squared tests were conducted using four leech clades and  
38 three vertebrate orders in China ( $P = 0.4561$ ) and two leech clades and three vertebrate orders in  
39 Bangladesh ( $P = 0.7512$ ), finding no relationship between these variables. These subsets were  
40 selected as they had a minimum frequency of 10 for each leech clade and each vertebrate order.  
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## Leech relationships



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3 A total of 730 leech cox1 isolates were successfully amplified and sequenced, and then  
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5 combined with 104 GenBank sequences. With redundant sequences removed, there were 333  
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7 unique sequences aligned in the final matrix. Leeches evaluated phylogenetically belonged to 15  
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9 clades of *Haemadipsa* (Fig. 1). These clades, including *H. trimaculosa* and *H. hainana*, were  
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11 generally well-supported; backbone relationships were not. Fully 107 specimens were  
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13 preliminarily identified morphologically as *H. sylvestris* and yet were recovered in disparate  
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15 branches of the phylogenetic reconstruction (i.e. Bangladesh Clade D and China clades F and G).  
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17 At present it is unclear which of these clades represents the 'true' *H. sylvestris*, and accordingly,  
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19 these clades were not named. Morphogroups generally corresponded to the 15 recovered clades.  
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21 Some sister clades that appeared morphologically similar showed evidence of being genetically  
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23 divergent (e.g. China clades C and D).  
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28 The most well-sampled clade, *H. trimaculosa*, was represented by individuals from three  
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30 major regions in Cambodia. These specimens had substantial genetic diversity, which was  
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32 strongly circumscribed by locality in Cambodia (Fig. 1). These regions are primarily separated  
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34 by distance and by major river drainages (e.g. the Mekong River and Tonle Sap). This  
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36 phylogenetic distance between geographically separate localities was reflected at other points in  
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38 the reconstruction (Fig. 1). Specifically, Bangladesh clades B and C and China clades C and D  
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40 were morphologically similar, sister, separated by meaningfully long branches, and  
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42 geographically adjacent. Data from prior studies (e.g. Lai et al., 2011), when combined and  
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44 analyzed here, sometimes showed a similar pattern for some (e.g. *H. picta*), but not other species  
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46 (e.g. *Haemadipsa rjukjuana* specimens from Taiwan were not a group nor were *H. interrupta*  
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48 from Thailand). Bangladesh sites harbored leech individuals from the same clade without any  
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50 phylogenetic distinction between adjacent sites. Bangladesh clade A comprised leech individuals  
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3 from all four sites. Bangladesh clades B and D had individuals from two sites and no  
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5 phylogenetic separation.  
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## 10 **Discussion**

### 11 **Bloodmeal iDNA survey**

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13 One of the most important findings of this bloodmeal iDNA survey (Table 1) is corroboration  
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15 that leeches feed on small mammals, such as rodents, that are often missed by other generalized  
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17 non-invasive methods commonly employed for mammal surveys. Camera traps are much less  
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19 likely to record small mammals compared to larger ones, as they often are not sensitive enough  
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21 to be triggered by small ground-dwelling species (Tobler et al., 2008). This advantage of the  
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23 iDNA method over the traditional camera trap method already has been shown directly in  
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25 Bangladesh by Weiskopf et al. (2018). This study expands on that finding, showing the ability of  
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27 iDNA surveys to find small mammals across a number of Asian tropical ecosystems.  
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33 Similarly, our broad survey combined with prior geographically constrained studies  
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35 (Schnell et al., 2012; Weiskopf et al., 2018) now makes it clear that species of *Haemadipsa* feed  
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37 on a large variety of mammalian orders (Artiodactyla, Carnivora, Chiroptera, Lagomorpha,  
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39 Rodentia, Scandentia, and Primates) at least somewhat indiscriminately across their range;  
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41 something of importance to the suitability of the iDNA method. The robustness of the iDNA  
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43 method is further strengthened by the fact that there are so few orders of wild mammals  
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45 potentially present for which iDNA was not recovered (e.g. Pholidota [pangolins] and  
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47 Proboscidea [elephants]). Pangolins, while potentially present, are rapidly decreasing across  
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49 much of their ranges (Trageser et al., 2017). Elephants were known from some localities in  
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3 Cambodia and were found using camera traps in Bangladesh (Weiskopf et al., 2018), but are  
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5 infrequent at best.  
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8 We recovered bloodmeals from three avian genera (*Coturnix*, *Gallus*, and *Lophura*) in the  
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10 Galliformes. At least one species of these ground-dwelling birds were recovered in leech  
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12 bloodmeals from each country surveyed, indicating that *Haemadipsa* species may regularly  
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14 target such birds. Members of the Haemadipsidae have only rarely been found on birds (Bhatai  
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16 & Bora, 1973; Sawyer, 1986). All other records are largely isolated and more anecdotal.  
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18 *Haemadipsa* leeches were discovered in one study on legs of wild pheasants (Bhatai & Bora,  
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20 1973). *Chthonobdella limbata* has been found on the leg of a juvenile yellow robin and *C.*  
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22 *bilineata* on the head of a black-backed magpie (Richardson, 1975). In Papua New Guinea and  
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24 New Britain, *C. novabritanniae* was found in the nasal chambers of cassowaries (Richardson,  
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26 1975). Our observation that leeches in this study fed on galliform birds and not passerines may  
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28 contribute to a lack of long distance dispersal for the leech.  
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33 One sample from Cambodia had a bloodmeal from a genus of leaf-nosed bat  
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35 (*Hipposideros*). Species of terrestrial leech previously known to feed on bats are specialized  
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37 cave-dwelling species: *H. cavatuses* from China (Yang et al., 2009) and *C. jawarerenis* from  
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39 Papua New Guinea (Ewers, 1974; Richardson, 1974). Prior to our record, generalist *Haemadipsa*  
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41 species have not been recorded from bats.  
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44 Many of the samples were found to have fed on domesticated animals, which is only to  
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46 be expected given the mixed-use nature of southern Asian forests. Yet, notwithstanding the  
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48 number of large domestic bovids (e.g. cows and domestic water buffalo), we were readily able to  
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50 distinguish a gaur (*Bos gaurus*) isolate. This species, also known as the Indian bison, is listed by  
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3 the International Union for Conservation of Nature and Natural Resources (IUCN) as a  
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5 vulnerable species on its Red List.  
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8 While the leeches generally appear to prefer ground-dwelling species, no pattern was  
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10 evident between leeches of a given clade and their prey. This lack of clade-specific host  
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12 specificity and relative similarity in efficiencies between different species suggests that the  
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14 iDNA technique may be robust for finding vertebrates in an area regardless of which leech  
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16 species is reviewed. However, future work should strive for larger samples sizes that could  
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18 rigorously test for differences in host specificity. Furthermore, this alone does not indicate the  
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20 site fidelity of a given leech species, which is equally important for the iDNA method and best  
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22 determined by the phylogenetic relationships between leech individuals of a given species.  
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## 28 **Leech relationships**

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30 Broad-scale site fidelity is perhaps best exemplified in *H. trimaculosa* individuals from sites in  
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32 Cambodia (Fig. 1). Schnell et al. (2015) have proposed that site-occupancy modelling can be  
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34 applied to iDNA surveys, which corrects host species distribution maps for the ubiquitous  
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36 problem of false negatives: i.e. when a host species is present in a site but not detected by the  
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38 census method. Site-occupancy models achieve this correction via multiple revisits to the same  
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40 site, so that detection probabilities can be estimated for each species. This is costly fieldwork, but  
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42 since haematophagous leeches are abundant, Schnell et al. (2015) have suggested that  
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44 subsamples of leeches from the same site could be used instead of multiple revisits. However, an  
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46 important assumption of site-occupancy models is ‘population closure,’ which in this context  
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48 amounts to the assumption that where a leech is collected is close to where the leech fell of its  
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50 previous host. This assumption seems reasonable, given that an engorged leech is unlikely to  
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52 travel far, but evidence so far is lacking. The correspondence between *H. trimaculosa* (as well as  
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3 Bangladesh clades B and C and China clades C and D) and geographic boundaries does not  
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5 provide evidence for population closure, but does leave open the possibility that further  
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7 examination may find this assumption to be met.  
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10 Several other clades do not show these patterns. Bangladesh clade A shows little  
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12 phylogenetic patterning within the clade and yet is distributed across four distinct localities in  
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14 Bangladesh. This points to the possibility that leeches in some clades are more mobile than  
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16 others and less accurate regarding the exact location of a leech's host. However, this finding is  
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18 preliminary and could be due to other factors. For example, the leeches in this clade, while  
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20 somewhat geographically disjunct (~30 km), may have contiguous population structure  
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22 throughout forested areas that connect in India.  
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26 In addition to the matter of site fidelity, previously, it had been argued that *Haemadipsa*  
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28 leeches are unlikely to have iDNA from more than one host (Schnell et al., 2012). Our Sanger  
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30 sequencing protocol finds that individual leeches do largely retain a single bloodmeal. Only two  
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32 of our surveyed leeches had conflicting nucleotide peaks that when reamplified and resequenced  
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34 (over 200 samples) for improved quality had BLAST matches to two separate species. However,  
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36 this is a rudimentary way to distinguish multiple hosts and surely misses additional cases of  
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38 mixed bloodmeals.  
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42 *Haemadipsa trimaculosa* was the species most frequently encountered in this study and is  
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44 the first records of this species (along with *H. interrupta*) in Cambodia. No prior *Haemadipsa*  
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46 records exist this country. The only other records for *H. trimaculosa* are from eastern (mostly  
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48 adjacent) parts of Thailand, where the species was first described (Ngamprasertwong et al.,  
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50 2007), and from adjacent and close provinces of Laos (Vongsombath et al., 2011).  
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3 Given what is known of the range of *H. trimaculosa*, the sampling presented here appears  
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5 to be the most thorough molecular sampling and evaluation ever conducted for any leech species,  
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7 except perhaps *Hirudo medicinalis*. Few species of leech have been molecularly compared using  
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9 more than two or three samples, and none of these studies focused on the terrestrial leeches that  
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11 are most useful for iDNA surveys. *Hirudo medicinalis* and *Hirudo verbana* have been  
12  
13 molecularly compared in a more thorough fashion than other leeches, with sampling occurring  
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15 over large portions of their range; however, at most around 60 wild caught specimens per species  
16  
17 are used in a given study (Siddall et al., 2007; Trontelj & Utevsky, 2012; Utevsky & Trontelj,  
18  
19 2015). With so few studies looking at the genetic structuring of leeches across any significant  
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21 geographic range, the geographically bound, genetically defined clades we found for *H.*  
22  
23 *trimaculosa* highlight just how little is known about leech diversity and biology.  
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28 Finally, *H. sylvestris* and *H. montana* appear to be polyphyletic and in clear need of  
29  
30 revision. This was also the case for many of our unnamed clades. Substantial further revisionary  
31  
32 work is needed on the group, but requires additional collections from type localities, especially  
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34 for those species originally described from India.  
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## References

- Bhatai, M. L., & Bora, S. S. (1973). Bionomics and distribution of the land leeches of Kumaon Hills, U.P. *Journal of the Bombay Natural History Society*, 70, 36–56.
- Borda, E., Ocegüera-Figueroa, A., & Siddall, M. E. (2008). On the classification, evolution and biogeography of terrestrial haemadipsoid leeches (Hirudinida: Arhynchobdellida: Hirudiniformes). *Molecular Phylogenetics and Evolution*, 46, 142–154.
- Borda, E., & Siddall, M. E. (2010). Insights into the evolutionary history of Indo-Pacific bloodfeeding terrestrial leeches (Hirudinida: Arhynchobdellida: Haemadipsidae). *Invertebrate Systematics*, 24, 456.
- Calvignac-Spencer, S., Merkel, K., Kutzner, N., Köhl, H., Boesch, C., Kappeler, P. M., ... Leendertz, F. H. (2013). Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. *Molecular Ecology*, 22, 915–924.
- Caragiulo, A., Dias-Freedman, I., Clark, J. A., Rabinowitz, S., & Amato, G. (2014). Mitochondrial DNA sequence variation and phylogeography of Neotropic pumas (*Puma concolor*). *Mitochondrial DNA*, 25, 304–312.
- Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772.
- Ewers, W. H. (1974). *Trypanosoma aunawa* sp. n. from an insectivorous bat, *Miniopterus tristis*, in New Guinea, which may be transmitted by a leech. *Journal of Parasitology*, 60, 172–178.
- Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan

- 1  
2  
3           invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294–299.
- 4  
5 Haeckel, E. (1883). *A Visit to Ceylon*. London, UK: Kegan Paul, Trench & Co.
- 6  
7  
8 Lai, Y.-T., Nakano, T., & Chen, J.-H. (2011). Three species of land leeches from Taiwan,  
9  
10           *Haemadipsa rjukjuana* comb. n., a new record for *Haemadipsa picta* Moore, and an  
11  
12           updated description of *Tritetrabdella taiwana* (Oka). *ZooKeys*, 139, 1–22.
- 13  
14  
15 Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for  
16  
17           inference of large phylogenetic trees. In *2010 Gateway Computing Environments*  
18  
19           *Workshop*. New Orleans, USA: IEEE Computer Society.
- 20  
21  
22 Ngamprasertwong, T., Thirakhupt, K., & Panha, S. (2007). Two new species of land leeches  
23  
24           from Thailand (Hirudiniformes: Haemadipsidae). *The Natural History Journal of*  
25  
26           *Chulalongkorn University*, 7, 155–159.
- 27  
28  
29 Ngo, K. A., & Kramer, L. D. (2003). Identification of mosquito bloodmeals using polymerase  
30  
31           chain reaction (PCR) with order-specific primers. *Journal of Medical Entomology*, 40, 215–  
32  
33           222.
- 34  
35  
36 Richardson, L. R. (1974). A new troglobitic quadrannulate land-leech from Papua (Hirudinoidea:  
37  
38           Haemadipsidae s. l.). *Proceedings of the Linnean Society of New South Wales. Linnean*  
39  
40           *Society of New South Wales*, 99, 57–68.
- 41  
42  
43 Richardson, L. R. (1975). A contribution to the general zoology of the land-leeches (Hirudinea:  
44  
45           Haemadipsoidea superfam. nov.). *Acta Zoologica Academiae Scientiarum Hungaricae*, 21,  
46  
47           119–152.
- 48  
49  
50 Sawyer, R. T. (1986). *Leech biology and behaviour: feeding biology, ecology, and systematics*.  
51  
52           Oxford, UK: Clarendon Press.
- 53  
54  
55 Schipper, J., Chanson, J. S., Chiozza, F., Cox, N. A., Hoffmann, M., Katariya, V., ... Young, B.
- 56  
57  
58  
59  
60



- 1  
2  
3 E. (2008). The status of the world's land and marine mammals: diversity, threat, and  
4  
5 knowledge. *Science*, 322, 225–230.  
6  
7  
8 Schnell, I. B., Sollmann, R., Calvignac-Spencer, S., Siddall, M. E., Yu, D. W., Wilting, A., &  
9  
10 Gilbert, M. T. P. (2015). iDNA from terrestrial haematophagous leeches as a wildlife  
11  
12 surveying and monitoring tool - prospects, pitfalls and avenues to be developed. *Frontiers*  
13  
14 *in Zoology*, 12, 24.  
15  
16  
17 Schnell, I. B., Thomsen, P. F., Wilkinson, N., Rasmussen, M., Jensen, L. R. D., Willerslev, E.,  
18  
19 ... Gilbert, M. T. P. (2012). Screening mammal biodiversity using DNA from leeches.  
20  
21 *Current Biology: CB*, 22, R262–3.  
22  
23  
24 Siddall, M. E., Trontelj, P., Utevsky, S. Y., Nkamany, M., & Macdonald, K. S. (2007). Diverse  
25  
26 molecular data demonstrate that commercially available medicinal leeches are not *Hirudo*  
27  
28 *medicinalis*. *Proceedings of the Royal Society B: Biological Sciences*, 274, 1481–1487.  
29  
30  
31 Sukumaran, J., & Holder, M. T. (2010). DendroPy: a Python library for phylogenetic computing.  
32  
33 *Bioinformatics*, 26, 1569–1571.  
34  
35  
36 Tan, E. (2008). Progress in the study of ecology, zoogeography, group, control repellent and  
37  
38 medical usage of Hirudinea in China. *Acta Ecologica Sinica*, 28, 6272–6281.  
39  
40  
41 Tessler, M., Barrio, A., Borda, E., Rood-Goldman, R., Hill, M., & Siddall, M. E. (2016).  
42  
43 Description of a soft-bodied invertebrate with microcomputed tomography and revision of  
44  
45 the genus *Chtonobdella* (Hirudinea: Haemadipsidae). *Zoologica Scripta*, 45, 552-565.  
46  
47  
48 Tessler, M., Siddall, M. E., & Ocegüera-Figueroa, A. Leeches from Chiapas, Mexico with a new  
49  
50 species of *Erpobdella* (Hirudinida: Erpobdellidae). *American Museum Novitates*, in press.  
51  
52  
53 Tobler, M. W., Carrillo-Percestequi, S. E., Leite Pitman, R., Mares, R., & Powell, G. (2008). An  
54  
55 evaluation of camera traps for inventorying large- and medium-sized terrestrial rainforest  
56  
57  
58  
59  
60

- 1  
2  
3 mammals. *Animal Conservation*, *11*, 169–178.
- 4  
5 Townzen, J. S., Brower, A. V. Z., & Judd, D. D. (2008). Identification of mosquito bloodmeals  
6  
7 using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences.  
8  
9  
10 *Medical and Veterinary Entomology*, *22*, 386–393.
- 11  
12 Trageser, S. J., Ghose, A., Faisal, M., Mro, P., Mro, P., & Rahman, S. C. (2017). Pangolin  
13  
14 distribution and conservation status in Bangladesh. *PloS One*, *12*, e0175450.
- 15  
16 Trontelj, P., & Utevsky, S. Y. (2012). Phylogeny and phylogeography of medicinal leeches  
17  
18 (genus *Hirudo*): fast dispersal and shallow genetic structure. *Molecular Phylogenetics and*  
19  
20 *Evolution*, *63*, 475–485.
- 21  
22  
23 Utevsky, S., & Trontelj, P. (2015). Phylogeography of the southern medicinal leech, *Hirudo*  
24  
25 *verbana*: a response to Živić et al. (2015). *Aquatic Ecology*, *50*, 97–100.
- 26  
27  
28 Vongsombath, C., de Boer, H. J., & Pålsson, K. (2011). Keeping leeches at bay: field evaluation  
29  
30 of plant-derived extracts against terrestrial blood-sucking leeches (Haemadipsidae) in Lao  
31  
32 PDR. *Acta Tropica*, *119*, 178–182.
- 33  
34  
35 Weiskopf, S. R., McCarthy, K. P., Tessler, M., Rahman, H. A., McCarthy, J. L., Hersch, R.,  
36  
37 Faisal, M. M., & Siddall, M. E. (2018). Using terrestrial haematophagous leeches to  
38  
39 enhance tropical biodiversity monitoring programs in Bangladesh. *Journal of Applied*  
40  
41 *Ecology*, in press.
- 42  
43  
44 Won, S., Park, B. K., Kim, B. J., Kim, H. W., Kang, J. G., Park, T. S., ... Chae, J. S. (2014).  
45  
46 Molecular identification of *Haemadipsa rjukjuana* (Hirudiniformes: Haemadipsidae) in  
47  
48 Gageo Island, Korea. *The Korean Journal of Parasitology*, *52*, 169–175.
- 49  
50  
51 Yang, T., Mo, X., & Wang, D. (2009). A new species of cavernous blood sucking land leech  
52  
53 (Hirudinea, Haemadipsidae) in the west of Yunnan Province, China. *Acta Zootaxonomica*  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 *Sinica/Dongwu Fenlei Xuebao*, 34, 125–129.  
4

5 Yang, Y., Tian, K., Hao, J., Pei, S., & Yang, Y. (2004). Biodiversity and biodiversity  
6 conservation in Yunnan, China. *Biodiversity and Conservation*, 13, 813–826.  
7

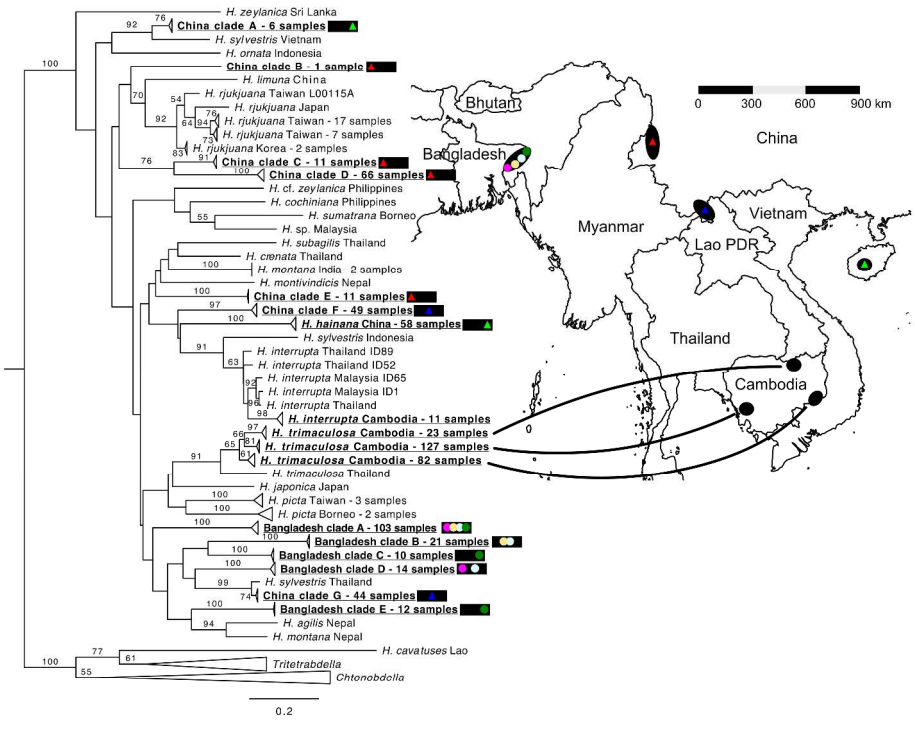
8  
9  
10 Zwickl, D. J. (2006). *Genetic algorithm approaches for the phylogenetic analysis of large*  
11 *biological sequence datasets under the maximum likelihood criterion*. Austin, USA: The  
12 University of Texas at Austin.  
13  
14  
15  
16  
17  
18  
19  
20  
21  
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23  
24  
25  
26  
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**Table 1.** Details on putative vertebrate taxa found in leech (*Haemadipsa*) bloodmeal surveys in southern Asia using the best match from BLAST searches on 16S DNA sequences. "ID %" is the percent identity found, while "1% gap" indicates whether or not the sequences were at least 1% better in their percent identity from the next best matching taxon while NA was given if only a higher taxon (e.g., genus) is listed, as these listings had multiple species of the same e-value and were accordingly considered equivalent matches. Range was determined using the IUCN Red List. Site names are as follows by country. Bangladesh: A = Atora Hill Reserve Forest, P = Patharia Hill Reserve Forest, R = Rajkandi Reserve Forest, and T = Tarap Hill Reserve Forest. Cambodia: C = Cardamom Mountains, P = Preah Vihear, and S = Seima. China: G = Gaoligong, H = Hainan, and X = XTBG.

Class	Order	Family	Taxon	E-value	ID %	1% gap	Clades of leeches with bloodmeal from vertebrate taxon	Bangladesh			Cambodia			China			Times found	In range
								A	P	R	T	C	P	S	G	H		
Aves	Galliformes	Phasianidae	<i>Coturnix japonica</i>	3E-108	99	Yes	<i>H. trimaculosa</i>					1				1	In China	
			<i>Gallus gallus</i>	1E-42 - 1E-140	87-100	Yes	Bangladesh A, D; China F; <i>H. hainana</i>			4				2	1	7	Yes	
			<i>Lophura nycthemera</i>	1E-125	96	Yes	China A						1		1	Yes		
			<i>Pucrasia or Lophura</i>	2E-39	90	NA	China F							1	1	Yes		
Mammalia	Artiodactyla	Bovidae	<i>Bos gaurus</i>	5E-133	99	Yes	<i>H. trimaculosa</i>					1				1	Yes	
			<i>B. taurus</i>	1E-34 - 3E-137	90-100	Yes	Bangladesh A, B, D; China D, G; <i>H. hainana</i> ; <i>H. interrupta</i> ; <i>H. trimaculosa</i>	2	6	5	10	3	2	2	9	3	42	Yes
			<i>Bubalus bubalis</i>	2E-25 - 1E-145	83-100	Yes	Bangladesh A; China F, G; <i>H. trimaculosa</i>	1				1			11	13	Yes	
		Cervidae	Cervidae	1E-53	90-100	NA	Bangladesh A, B, C; China D, F; <i>H. trimaculosa</i>					1				1	Yes	
			<i>Cervus</i>	4E-131	99	NA	China F								1	1	No	
			<i>Muntiacus</i>	1E-35	90	NA	China F								1	1	* Yes	
			<i>M. muntjak</i>	6E-71 - 7E-134	93-100	Yes (1 no)	Bangladesh A, B, C; China D, F	5	1					2	1	9	* Yes	
			<i>M. muntjak or rooseveltorum</i>	1E-22 - 1E-47	83-91	NA	China D							2		2	* Yes	
			<i>Rusa or Cervus</i>	1E-47 - 1E-86	87-98	NA	China F; <i>H. hainana</i> ; <i>H. trimaculosa</i>				2	1			1	1	5	Yes
			<i>Rusa timorensis</i>	2E-129 - 1E-130	99-100	Yes and no	China G; <i>H. trimaculosa</i>								1	3	No	
		Suidae	<i>Sus scrofa</i>	3E-30 - 7E-144	77-100	Yes	Bangladesh A, D, E; China D, E, F, G; <i>H. hainana</i> ; <i>H. trimaculosa</i>	3	3	7	8	1	2	4	3	31	Yes	
			<i>S. scrofa or barbatus</i>	7E-26 - 2E-90	94-100	NA	China F; <i>H. hainana</i> ; <i>H. trimaculosa</i>								1	2	5	Yes
		Tragulidae	<i>Tragulus javanicus or kanchil</i>	4E-106 - 8E-120	96	NA	<i>H. trimaculosa</i>				2	1				3	Yes	
Mammalia	Carnivora	Canidae	<i>Canis lupus</i>	9E-103 - 1E-141	97-100	Yes	Bangladesh A, D; China D; <i>H. interrupta</i> ; <i>H. trimaculosa</i>	1		1	2	1		4		9	Yes	
		Herpestidae	<i>Crossarchus obscurus</i>	9E-107	93	Yes	Bangladesh D				1					1	No	
		Felidae	<i>Felis or Pardofelis</i>	1E-130	99	NA	<i>H. trimaculosa</i>					1				1	Yes	
			<i>Felis silvestris</i>	3E-74 - 9E-98	95-100	Yes	<i>H. trimaculosa</i>				1	1				2	Yes	
		Viverridae	<i>Civettictis civetta</i>	1E-40 - 9E-113	87-95	Yes (1 no)	Bangladesh A	5	2							7	No	
			<i>Paguma larvata</i>	2E-58 - 4E-73	92-99	Yes	China F, G								3	3	Yes	
			<i>Paradoxurus hermaphroditus</i>	3E-49 - 7E-134	95-100	Yes	Bangladesh A; China A; <i>H. hainana</i> ; <i>H. interrupta</i> ; <i>H. trimaculosa</i>	1				14	1		3		19	Yes
Mammalia	Chiroptera	Hipposideridae	<i>Hipposideros bicolor</i>	4E-73	93	Yes	<i>H. trimaculosa</i>				1					1	No	
Mammalia	Primates	Hominidae	<i>Homo sapiens</i>	6E-20 - 4E-136	95-100	Yes	Bangladesh A, B, C, D, E; China C, D, F, G; <i>H. hainana</i> ; <i>H. interrupta</i> ; <i>H. trimaculosa</i>	1	5	1	3	13	3	10	7	9	52	Yes
		Cercopithecidae	<i>Macaca mulatta</i>	3E-92 - 1E-141	99-100	Yes	Bangladesh A, B, D	1	2	2						5	Yes	
			<i>M. silenus</i>	3E-36 - 5E-125	86-98	Yes and no	Bangladesh A; <i>H. hainana</i> ; <i>H. trimaculosa</i>	2	2		9	2		2		17	No	
Mammalia	Rodentia	Hystricidae	<i>Hystrix indica</i>	1E-105	93	Yes	China E							1		1	No	
		Muridae	<i>Niviventer confucianus</i>	4E-66 - 3E-112	90-95	Yes and no	China D							3		3	Yes	
			<i>N. excelsior</i>	4E-41	92	Yes	<i>H. trimaculosa</i>					1				1	No	
			<i>Rattus rattus</i>	9E-88	90	Yes	<i>H. interrupta</i>					1				1	Yes	
			<i>R. tanezumi</i>	2E-89 - 3E-132	98-99	Yes (1 no)	Bangladesh A, D	4		1						5	Yes	
		Sciuridae	<i>Callosciurus erythraeus</i>	1E-34 - 8E-114	91-95	Yes	China C							3		3	Yes	
			<i>Dremomys rufigenis</i>	3E-106	93	Yes	China C							1		1	Yes	
			<i>Menetes berdmorei</i>	6E-110	95	Yes	<i>H. interrupta</i>					1				1	Yes	
Mammalia	Scandentia	Tupaiaidae	<i>Tupaia belangeri</i>	8E-114	96	Yes	Bangladesh A			1						1	Yes	

\* IUCN considers this to be *Muntiacus vaginalis* while others consider that to be a subspecies of *M. muntjak*.

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**Fig. 1.** The most likely phylogenetic reconstruction of *Haemadipsa* leeches, with sequences new to this study bolded and underlined. Collection localities are covered by black ovals for the rough range in a given site, with a different colour for each symbol indicating the specific site. The shape of each symbol within these ovals represents the country of a given site, with circles for Bangladesh and triangles for China. These symbols are also displayed next to the clades that had samples from these localities. Bootstrap support values above 50% are displayed as a measure of support.

592x481mm (300 x 300 DPI)