1   Assessing the potential of environmental DNA metabarcoding for monitoring

2   Neotropical mammals: a case study in the Amazon and Atlantic Forest, Brazil

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22   **Abstract**

23   The application of environmental DNA (eDNA) metabarcoding as a biomonitoring

24   tool has greatly increased in the last decade. However, most studies have focused

25   on aquatic macro-organisms in temperate areas (e.g., fishes). We apply eDNA

26   metabarcoding to detect the mammalian community in two high-biodiversity regions

27   of Brazil, the Amazon and Atlantic Forest. We identified critically endangered and

28   endangered mammalian species in the Atlantic Forest and Amazon respectively and

29   found congruence with species identified via camera trapping in the Atlantic Forest.

30   In light of our results, we highlight the potential and challenges of eDNA monitoring

31   for mammals in these high biodiverse areas.

32

33   **Keywords:** Camera traps; critically endangered; eDNA; river

34 **Introduction**

35 A quarter of mammal species are endangered according to the IUCN Red List of

36 Threatened Species (IUCN 2019) and there is clearly a need for more cost-effective

37 and rapid methods for long-term biomonitoring to be applied across different biomes

38 and over large spatial and temporal scales (Sales et al. 2019a). In recent years,

39 environmental DNA (eDNA) metabarcoding (the simultaneous identification via next-

40 generation sequencing of multiple taxa using DNA extracted from environmental

41 samples, e.g., water, soil) has delivered on its initial potential and is now

42 revolutionizing how we monitor biodiversity (Deiner et al. 2017). The majority of

43 eDNA metabarcoding applications have focused on monitoring fish and

44 macroinvertebrates, with mammals being targeted in only 8% of vertebrate studies

45 (Tsuji et al. 2019). However, with the development of universal primers for

46 vertebrates and mammals specifically, there has been a recent surge in studies

47 tailored to detect and/or monitor mammalian communities in terrestrial and

48 freshwater environments (e.g. Ushio et al. 2017, Harper et al. 2019, Sales et al.

49 2019a).

50 Several recent mammal-focused eDNA metabarcoding studies in temperate

51 regions in the northern hemisphere have relied on well-studied systems with

52 accompanying long-term or historical survey data to test the efficiency of this novel

53 biomonitoring tool (e.g., Harper et al. 2019, Sales et al. 2019a). However, mammal

54 conservation can be more challenging in biodiversity-rich countries as long-term

55 monitoring systems are still scarce outside of Europe and North America (Proença et

56 al. 2017) and ecological field studies, usually used to plug this gap, are often

57 hindered due to difficulties of sampling over wide spatial scales. For effective

58 conservation action, adequate knowledge regarding the biodiversity components

59 present in each area is of paramount importance.

60 Environmental DNA from lentic and lotic systems has been found to be

61 effective in not just monitoring aquatic and semi-aquatic mammals, but also

62 terrestrial species (Harper et al. 2019, Sales et al. 2019a). Here, we explore the

63 application of eDNA metabarcoding for Neotropical mammals by verifying its ability

64 to detect aquatic and terrestrial animals from rivers/streams in the highly biodiverse

65 biomes of the Brazilian Amazon and Atlantic Forest. The Amazon is the largest

66 tropical rainforest on Earth, encompassing at least 10% of the world's biodiversity.

67 The Atlantic Forest, which is currently represented by only 11% of its original cover

68 (Ribeiro et al. 2009), is the second most biodiverse biome in South America (WWF

69 2018).

70

71 **Methods**

72 In the Amazon, water samples (500mL each, in three replicates) were obtained from

73 six sites within three main areas (A-C; Fig. 1; Table S1). In the Atlantic Forest, water

74 and sediment samples (500mL of water and 25mL of sediment, in three replicates)

75 were obtained from eight sites located in two valleys of the Caparaó National Park

76 (D-E; Fig. 1; Table S1). Temperature and pH were recorded at each site in the

77 Amazon. Mammal-specific universal primers targeting the mitochondrial 12S rRNA

78 gene were used (Ushio et al. 2017). The workflow was conducted following the

79 protocol described in Sales et al. (2019a) and a more detailed description is included

80 in the Supporting Information.

81 Additional data regarding species' distribution in the Atlantic Forest were

82 obtained through camera-trap surveys. Both valleys in the Caparaó National Park

83   were surveyed with terrestrial and arboreal camera traps (Bushnell Trophy CamTM,

84   USA; see Supporting Information).

85

86   **Results and Discussion**

87   A total of ~1.3 million mammal reads were obtained after all the bioinformatic filtering

88   steps (Amazon – 833,623 reads; Caparaó – 109,233 reads for water samples and

89   334,593 for sediment samples). Only reads recovered for wild mammals (919,910

90   reads) were retained for downstream analyses.

91   Overall, we detected 28 Molecular Operational Taxonomic Units (MOTUs -

92   Blaxter et al. 2005) from terrestrial and aquatic mammals, representing eight orders

93   and 14 families (Table S2). Considering a threshold of >0.97 minimum identity, only

94   13 MOTUs could be assigned to the species level (Table S2). In the Amazon, six

95   species were recovered, with three currently listed as endangered by the IUCN's

96   Red List (IUCN 2019) in different categories: the Endangered Amazon river dolphin

97   (*Inia geoffrensis*), the Vulnerable giant anteater (*Mymercophaga tridactyla*) and the

98   Vulnerable lowland tapir (*Tapirus terrestris*). Three Least Concern species were also

99   identified: *Thyroptera discifera* and *Rhynchonycteris naso* in the order Chiroptera

100   and the rodent *Toromys rhipidurus*. Only one MOTU was detected for each family

101   (Fig. 1).

102   In Caparaó National Park, nine families were detected using eDNA: five in the

103   west side of the park (D) and nine in the east side (E; Fig. 1 and S1). Of these, only

104   seven could be assigned to the species level (Table S2). Here, camera-trap surveys

105   detected 17 species (and additional unidentified small mammal species),

106   encompassing 12 families (Fig. S2; Table S3). Combining the two non-invasive

107   techniques, 15 families were detected overall (Table 1), six of which by both

108   methods, three exclusively by eDNA metabarcoding and six solely by the camera

109   traps. Although this study was not designed to provide a direct comparison between

110   methods (e.g., Harper et al. 2019, Sales et al. 2019a), it highlights the potential of

111   implementing multiple non-invasive approaches in providing an overview of the

112   mammalian community composition in biodiversity rich areas.

113   More MOTUs were retrieved for the families detected in the Atlantic Forest,

114   suggesting the occurrence of several species of the same family in this area. For

115   example, three MOTUs were recovered in the east side and two from the west side

116   of the Park for both Didelphidae and Cuniculidae. Camera trapping recorded three

117   species of Didelphidae (*Caluromys philander*, *Didelphis* sp., *Philander frenatus*), in

118   accordance with the eDNA data. Only one species from the Cuniculidae (*Cuniculus*

119   *paca*) recorded by camera traps is known to occur in the Caparaó and the existence

120   of three MOTUs for this family might be due to natural intraspecific genetic variability

121   (Fig. 1). Cricetidae had three MOTUs in the west side of the Park: although this

122   family was not identified by camera traps, several species are described for the

123   Atlantic Forest, including endemic and recently described species (Gonçalves &

124   Oliveira 2014). Furthermore, the Critically Endangered primate *Brachyteles*

125   *hypoxanthus* was detected using eDNA, demonstrating its potential to detect

126   arboreal mammals from water samples (Harper et al. 2019).

127   As a similar sampling effort was applied for both areas in this study, there is a

128   need to consider what factors might explain the difference in the number of MOTUs

129   recovered for each biome, particularly if we assume that mammalian alpha diversity

130   should at least be as high in the Amazonian sampling sites as in the Caparaó forest

131   site (see Costa et al. 2000). For example, all the families detected in the Atlantic

132   forest that were not detected in the Amazonian samples are known to occur in Area

133   B of the Amazon (Mendes Pontes et al. 2008). DNA degradation in water is one of

134   the main factors reducing detectability over time and limiting temporal inferences.

135   The sampled black waters in the Amazon have low pH (ranging from 3.85 to 4.27),

136   whereas in the Caparaó the reported values are above 6.5 (Rodrigues 2015). Acidic

137   environments show higher eDNA decay and lower persistence rate due to the

138   increased degradation of DNA via chemical hydrolysis (Seymour et al. 2018).

139   Therefore, the eDNA recovered in the low pH waters of the Amazon might be

140   derived from specimens which have had very recent contact with the water body.

141   Mammal eDNA recovery depends not only on the species presence but also on the

142   direct/indirect contact with the water system (Harper et al. 2019). The junction of the

143   Negro and Amazon Rivers (area C) has an enormous volume of water and possibly

144   much time had elapsed since it flowed under the forest canopy, but the other

145   Amazonian streams (area B; Fig. 1) are similar in size to those in the Atlantic Forest.

146   In the Amazon, all species/MOTUs were detected in a single replicate, except for the

147   lowland tapir (detected in four replicates in three different streams). This species is

148   known to defecate more frequently in water than on land (Tobler et al. 2010) so this

149   may explain its higher rates of eDNA detection. In the Atlantic Forest, several

150   MOTUs/species were recovered from multiple replicates/sites (Fig. S1), suggesting

151   longer persistence of eDNA in this environment.

152   There is a clear limitation in terms of available DNA sequences in public

153   databases (e.g., Genbank) to either match identified MOTUs to species, or to

154   distinguish between closely related species within the same genus. This issue has

155   been highlighted in previous Neotropical eDNA studies for other taxonomic groups

156   (Cilleros et al. 2019, Sales et al. 2019b). A 12S reference database exists for 164

157   Amazonian mammalian species in French Guiana (Kocher et al. 2017) and all

158 Amazonian MOTUs were identified to species level here. However, this was not the

159 case for the Atlantic Forest. This biome hosts more than 300 mammalian species

160 (more than 50% of medium/large species considered at least Vulnerable; Souza et

161 al. 2019). Therefore, for eDNA monitoring to be implemented in this biome, there is a

162 clear need to generate reference DNA barcodes of a large proportion of the

163 mammalian communities present.

164   Here, we demonstrated the potential of applying a cutting-edge non-invasive

165 and cost-effective molecular approach for biodiversity assessment and systematic

166 monitoring scheme of Neotropical mammals, including highly threatened species.

167 This is particularly relevant given the current political climate in Brazil, which is

168 resulting in research funding and environmental crises. However, significant

169 challenges remain to implement this method in the Neotropics, from a better

170 understanding of the ecology of eDNA within these variable environments, to the

171 current lack of appropriate reference sequences for species determination in these

172 biodiversity-rich and anthropogenically-impacted biomes.

173

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180 **Author Contributions**

181 NGS, MDCK, ADM, CB, IC, JPB, WEM and MNFS conceived, and NGS, MDCK,

182 ADM, IC and CB designed the study. NGS, MDCK, AH and CB carried out the eDNA

183   sampling. NGS, MDCK and JCP performed the laboratory work. NGS carried out the

184   bioinformatic analyses. MDCK analysed the camera trap data. NGS, ADM and IC

185   analysed the eDNA data. NGS, ADM and MDCK wrote the paper, with all authors

186   contributing to editing and discussions.

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251 **Table 1.** Number (*n*) of species captured with camera traps and number of Molecular

252 Operational Taxonomic Units (MOTUs) captured with environmental DNA (eDNA)

253 metabarcoding for orders and families within Caparaó National Park, Atlantic Forest.

254 See Tables S2 and S3 for a more extensive breakdown of camera trap and eDNA

255 data, respectively.

Order	Family	Camera (*n* species)	eDNA (*n* MOTUs)
Carnivora	Felidae	1	-
	Mustelidae	1	-
	Procyonidae	2	1
Chiroptera	Phyllostomidae	-	2
Didelphimorphia	Didelphidae	3	3
Pilosa	Myrmecophagidae	1	-
Primates	Atelidae	1	2
	Callithrichidae	1	-
	Cebidae	1	-
Rodentia	Caviidae	1	1
	Cricetidae	-	3
	Cuniculidae	1	3
	Echimyidae	2	1
	Erethizontidae	2	-
	Sciuridae	1	1

256

257 **Figure legend**

258 Figure 1. Sampling areas for environmental DNA (eDNA) in the Amazon (A-C) and

259 Atlantic Forest (D-E) biomes in Brazil. The families recovered from eDNA

260 metabarcoding in each area are represented by stylized drawings and the number of

261 Molecular Operational Taxonomic Units (MOTUs) recovered within each family is

262 indicated.

263

264   Figure 1.