Research Article

The value of including intraspecific measures of biodiversity in environmental impact surveys is highlighted by the Amazonian brilliant-thighed frog (*Allobates femoralis*)

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Abstract
The distribution of many vertebrate species in the Amazon basin is delimited by large rivers, which are frequently regarded as geographic barriers related with speciation and are therefore of high conservation value. Rapid development in the region includes plans to dam one of its thirteen major rivers, the Xingu. Environmental impact assessment is required for large scale development within the Brazilian portion of the Amazon Basin. However, impacts on biodiversity are only considered at the species level, and taxonomic work is far from described for many groups. In particular, anuran diversity is underestimated, in part owing to the scale of the region and, for some taxa, by morphological conservatism. Here we describe genetic and phenotypic variation in the brilliant-thighed frog, *Allobates femoralis*. We show that a unique genetic lineage, with a vocal repertoire distinct from that described for the species throughout its remaining geographic range, is located within the region to be directly and indirectly impacted by damming the Xingu River. Further, genetic variation within the *A. femoralis* group is largely structured in accordance to river systems, despite morphological conservatism. Our data add support for conservation policy to be amended to include intraspecific measures of diversity in order to more effectively conserve current biodiversity and evolutionary processes.

Keywords Amazon, Amphibians, Bioacoustics, Dams, Hydropower, mtDNA

Resumo
Na bacia Amazônica, a distribuição de muitas espécies de vertebrados é delimitada por grandes rios, os quais são freqüentemente considerados barreiras geográficas relacionadas a processos de especiação e, portanto, de grande valor para conservação. O desenvolvimento da região ocorre de forma rápida e envolve projetos de barramento do Rio Xingu, um dos treze maiores rios da bacia. Estudos de impacto ambiental são obrigatórios em grandes projetos de infra-estrutura na Amazona brasileira. Entretanto, impactos sobre a biodiversidade são avaliados apenas até o nível de espécie, apesar de o conhecimento taxonômico ser bastante incompleto para muitos grupos de organismos. Especificamente, a diversidade de anuros é subestimada, em parte devido à escala da região e, para alguns taxa, devido a desafios impostos pela baixa variabilidade morfológica. Neste estudo, nós descrevemos a variação genética e fenotípica na rã-de-coxa-brilhante, *Allobates femoralis*. Nós apontamos que uma linhagem genética única, com um repertório vocal distinto daquele descrito para a espécie ao longo do restante de sua distribuição, é encontrada dentro da região a ser impactada direta e indiretamente pelo barramento do rio Xingu. Além disso, a variabilidade genética em *A. femoralis* é fortemente particionada pelos sistemas fluviais, a despeito da morfologia conservativa do grupo. Os dados fornecidos apóiam que a política de conservação vigente seja atualizada, de forma a reconhecer medidas complementares de diversidade.

Palavras chaves: Amazônia, Anfíbios, Bioacústica, Barragens, Hidrelétricas, ADNmt

Introduction

Conservation of biodiversity in the Amazon basin is hampered by many factors, including the large size of the region, poor taxonomic knowledge, and the rapid pace of environmental change. The Amazon basin covers approximately six million km^2, with predictions that 40–55% of its rainforest will be lost within the next few decades [1-2]. In the Amazon basin, the conservation of river systems and associated riparian and inter-fluvial forested areas is considered high priority by conservation biologists [1,3]. The region contains 13 major rivers sytems: Madeira, Tocantins, Negro, Xingu, Tapajós, Purus, Marañon, Ucayali, Japurá, Juruá, Putumayo, Trombetas and Napo rivers. Their respective watershed areas are modified by expansion of cattle ranching, soy bean cultivation and other agricultural activities [2, 4-5], and more recently, by widespread governmental investment in large infrastructure projects [6-7]. New dams and waterways have been recently listed among the most critical threats to freshwater-dependent ecosystems in the Amazon, their impacts ranging from large-scale alteration of biogeochemical cycles to changes in species composition of nearby biological communities [8]. In particular, the damming of rivers to generate hydroelectric power alters large areas through flooding and disruption of seasonal water flows downstream of reservoirs [7]. The value of conserving watershed ecosystems is underscored by research demonstrating that Amazonian inter-fluvial regions are known to house unique components of biodiversity [9-10].

Many amphibian species have been recorded in some Amazon localities, but as with many tropical regions, taxonomy and patterns of intraspecific diversity are generally poorly known [11]. This problem is compounded by high levels of morphological conservatism within some amphibian taxa [12-14]. Consequently, imperiled species lists, such as the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, may not be directly reliable in assessing the implications of development on amphibians.

Conservation policy of Brazil, which includes the largest portion of the Amazon Basin, requires a succession of environmental studies and the evaluation of the resulting reports by analysts in a licensing institution before allowing any large developmental project and licenses to operate [15]. The first licensing steps involve compiling species inventories for an environmental impact assessment report (EIA) and subsequent environmental monitoring studies. In accordance with current legislation, surveys use species-level taxonomies to assess rarity, extinction risk, and potential value as an economic, scientific or environmental resource [15-17]. There is no explicit objective of conserving evolutionary potential, and therefore, knowledge on intraspecific variation that is currently being accrued for a range of organisms, including amphibians, is not considered when assessing the implications of development.

Globally, the focus of conservation activities in many regions has shifted over the last decade to retaining ecological and evolutionary processes [18-21]. To conserve evolutionary processes, we need to assess the degree to which different parts of a species distribution are differentiated from each other. Descriptions of genetic variation are often used to prioritize parts of a species distribution for protection, and several ways to delimit and prioritize groups of individuals in management and conservation plans have been applied [22]. These may be based not only on their relative genetic differentiation, but also on adaptive significance and ecological exchangeability, and are often referred to as evolutionary significant units (ESU's), or management units (MU's) [20, 23-24]. These approaches and recommendations have resulted in environmental legislation protecting distinct population segments (e.g. U.S. Endangered Species Act, ESA). Policy for conserving evolutionary potential has been successfully used to protect terrestrial vertebrates such as the Florida Panther [25] and the Gray Wolf [26], among others. Acknowledging intraspecific diversity would improve these regulations by providing a means of conserving evolutionary processes and cryptic species prior to formal taxonomic assignments.

In recent years, the federal government of Brazil has increased its investment in large development projects (a list of current projects is available at the Brazilian Government's *Growth Acceleration Program* website: http://www.pac.gov.br/). Among them, few have received as much public attention as the hydroelectric installations on large rivers in the Amazon basin. The Belo Monte power plant, to be constructed on the Xingu River, has become especially notorious owing to potentially great direct impacts on indigenous communities and on areas of high biodiversity [7]. Contemporary amendments to environmental regulations have been made, specifically in relation to assessing the impact of developing hydroelectric power plants ("Instrução Normativa № 146") [17]. Although these environmental impact statements are an enforced requirement for each project, they still do not consider diversity below the species level.

Here we argue that because major rivers are likely to drive speciation, assessment of riverine locations where development is being planned should ask whether these locations play a substantial role in generating biodiversity. Concordant phylogeographic patterns across taxa can be used to identify the geographic features responsible for genetic divergence, but such information is not available for large areas of the Amazon basin. However, because there are often concordant phylogeographic patterns for similar taxa, a fairly rapid assessment could initially be made using abundant species and proxies for intraspecific genetic divergence [27]. We illustrate this with the brilliant-thighed frog, *Allobates femoralis* (Boulenger 1884) where a diagnosable and geographically restricted lineage had been overlooked during environmental impact assessments for development related to the Belo Monte hydroelectric power plant. *A. femoralis* is a diurnal species found in *terra-firme* primary forest throughout the Amazon basin. It exemplifies the challenges faced when relying on current taxonomy for amphibian conservation. This taxon shows a high degree of morphological conservatism throughout its distribution, yet comprises several evolutionary lineages, some of which have enough exclusive genetic and phenotypic traits to have been recently described as distinct species [12]. Differences in vocal repertoire suggest that further cryptic diversity is still to be uncovered within this group [12, 28].

Here we assess the distribution of genetic variation among *A. femoralis* sampled from five large interfluves using a standard mitochondrial DNA marker for amphibians. The data are used to characterize genetic variation within and between interfluves, thereby contributing to understanding the role of large Amazonian rivers in structuring anuran diversity. We associate haplotype distributions with recordings of vocal repertoire in order to reveal potential impacts of the Belo Monte power plant on the variation overlooked by environmental impact surveys. Finally, we argue that the genetic and phenotypic variation

pattern observed in *A. femoralis* is not likely to be unique among Neotropical anurans, and should be regarded as a cautionary example of why environmental policy in Brazil needs updating.

Methods

From January 2007 to March 2011, we sampled 120 *A. femoralis* from 14 locations in the Brazilian Amazon (Fig. 1, Table 1). Samples covered four locations on the Madeira-Tapajós interfluve, three locations in the Tapajós-Xingu interfluve, and five locations on the Xingu-Tocantins interfluve. Except for the locations of Manicoré and Novo Aripuanã, sampling sites were all located along the available main roads (Rod. Transamazônica, Rod. Santarém-Cuiabá), from the city of Itaituba to Novo-Repartimento. Two additional sampling locations were established on the north bank of the Amazon River, on the interfluves between the rivers Negro and Trombetas and between rivers Trombetas and Jari, these areas being part of the Guyana Shield region. Sampling effort in each location usually took place in more than two sampling sub-sites (*e.g.* Fig. 1c) but, given the scale of the study, all samples were grouped according to sampling location.
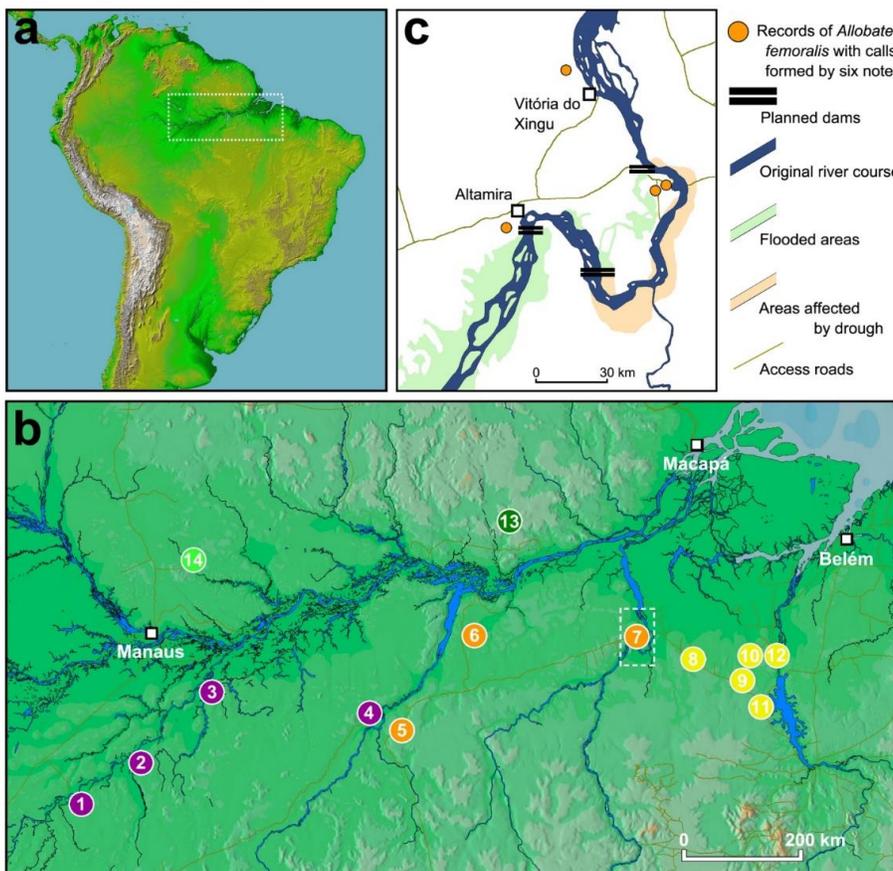

Fig. 1 (a) Relative location of the study area in northern South America. (b) Geographic position of 14 sampling locations in central Brazilian Amazon from where tissue samples and call recordings of the brilliant-thighed frog (*Allobates femoralis*) were collected. Circles representing sampling locations are colored according to their position in different interfluves (light green = Negro-Trombetas; dark green = Trombetas-Jari; purple = Madeira-Tapajós; orange = Tapajós-Xingu; yellow = Xingu-Tocantins) and numbered according to location names and coordinates listed in Table 1. (c) Depiction of hypothesized effects of Belo Monte power plant in the Altamira and Volta Grande regions of the Xingu River, delimited by a dashed rectangle in (b). Projections of impacted areas were adapted from [54] and do not include impacts caused by deforestation and human population expansion around the urban perimeters of Altamira and Vitória do Xingu. Orange dots in (c) correspond to sampling sub-sites within Altamira location used in this study.

Total genomic DNA was extracted from preserved muscle tissues collected from voucher specimens using a cetyltrimethylammonium bromide protocol (CTAB) [29]. We used primers 16Sar e 16Sbr [30] to amplify a 504 bp fragment of the 16S rRNA gene via polymerase chain reactions (PCR). The 16S rRNA gene has been regarded as one of the standard markers for the study of genetic relationships among anurans because priming sites are largely conserved, and because of its high phylogenetic signal in trees based on genetic distances [11, 31]. Protocols for amplification, purification and sequencing have been described in detail previously [12, 32]. Individual sequence fragments were deposited in National Center for Biotechnology Information's GenBank (accession numbers GU017474–GU017480, JF689976, JF690007–JF690017, KF310912–KF311011).

Table 1 Geographic position of brilliant-thighed frog (*Allobates femoralis*) sampling locations throughout central Brazilian Amazon, number of 16S rDNA sequences obtained from respective tissue samples, and number of call samples recorded at each site in order to determine the number of notes in the advertisement calls of males by inspection of call sonograms.

Locality	Interfluve	Coordinates	Sequence samples	Call samples	Notes/Call
1. Manicoré	Madeira-Tapajós	S 05°49'23", W 61°17'55"	16	21	4
2. Novo Aripuanã	Madeira-Tapajós	S 05°09'01", W 60°20'48"	8	7	4
3. Nova Olinda do Norte	Madeira-Tapajós	S 03°52'28", W 59°02'46"	5	8	4
4. Itaituba	Madeira-Tapajós	S 04°27'44", W 56°17'05"	13	12	4
5. Trairão	Tapajós-Xingu	S 04°40'59", W 56°01'21"	10	13	4
6. Treviso	Tapajós-Xingu	S 03°08'44", W 54°50'13"	9	16	4
7. Altamira	Tapajós-Xingu	S 03°08'24", W 51°44'32"	18	12	6
8. Pacajá km27	Xingu-Tocantins	S 03°34'38", W 51°02'45"	3	4	4
9. Raio de Sol	Xingu-Tocantins	S 03°43'47", W 50°13'03"	10	10	4
10. Transcametá	Xingu-Tocantins	S 03°32'29", W 49°44'27"	2	2	4
11. Novo Repartimento	Xingu-Tocantins	S 04°15'04", W 49°56'42"	3	3	4
12. Tucurui	Xingu-Tocantins	S 03°32'03", W 49°50'45"	7	4	4
13. Serra Azul	Trombetas-Jari	S 01°16'56", W 54°07'47"	8	8	4
14. Balbina	Negro-Trombetas	S 01°55'11", W 59°24'58"	8	9	4

In order to visualize the genealogical relationships among samples, as well as the distribution of haplotypes among localities, we used the 16S rDNA sequence dataset to construct an haplotype network using statistical parsimony, as implemented in TCS 1.21 [33-34], applying a 95% connection limit and considering gaps as a 5th character state. Summary genetic statistics for samples in each sampling locality were calculated in DnaSP v.5.10.1 [35]. Average genetic distances (Kimura 2-parameters) were measured between sampling localities in Mega 5.05 [36-8].

At each sampling location, we recorded advertisement calls of male *A. femoralis* using a Sony WM D6C cassette tape recorder (Sony, Japan) or a Marantz PMD660 digital recorder (DM Professional, USA), and Sennheiser ME66 directional microphones (Sennheiser Electronic, USA). The number of males recorded at each locality is listed in Table 1. Call samples included recordings of unvouchered individuals, thus rendering a total 129 recordings across the study area. In order to evaluate the number of notes that formed advertisement calls recorded for each specimen, we generated and inspected call sonograms in Raven 1.2 [39].

Results

We did not detect any *A. femoralis* 16S rDNA haplotypes shared between interfluves delimited by the Madeira, Tapajós, Xingu and the Amazon Rivers (Fig. 2). Estimates of genetic polymorphism within sampling locations were highly variable, which could be due to differences in sample sizes (Fig. 3). However, a relatively elevated number of haplotypes and segregating sites was observed among sequences from Altamira samples (n = 18, h = 10, S = 19), even when compared to those from localities for which more than 12 sequence samples were available (Manicoré, n = 16, h = 2, S = 1; Itaituba, n = 13, h = 4, S = 3) (Tables S1 and S2).
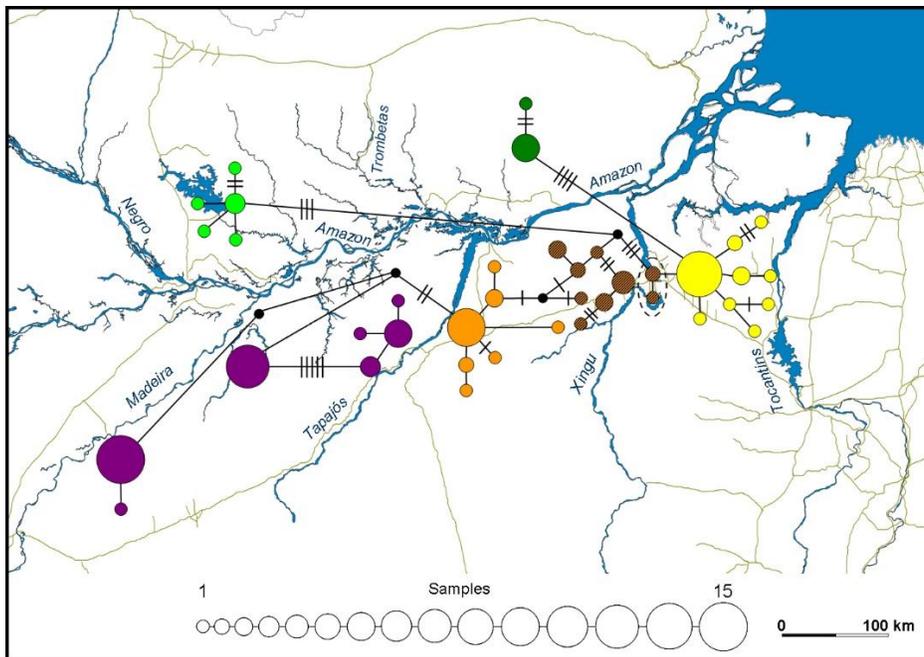

Fig. 2 Haplotype network built from 120 samples of a 504 bp fragment of the mitochondrial 16S rRNA gene of the brilliant-thighed frog (*Allobates femoralis*) using statistical parsimony. Circle diameters are proportional to haplotype frequency. Colors correspond to haplotype distributions according to major interfluves (light green = Negro-Trombetas; dark green = Trombetas-Jari; purple = Madeira-Tapajós; orange = Tapajós-Xingu; brown = Altamira region, in the Tapajós-Xingu interfluve; yellow = Xingu-Tocantins). Brown haplotypes indicate samples collected near the city of Altamira. The dashed line ellipse encloses the approximate locations of sampling sites in the locality of Altamira, all within areas potentially affected by environmental impacts of the Belo Monte power plant.
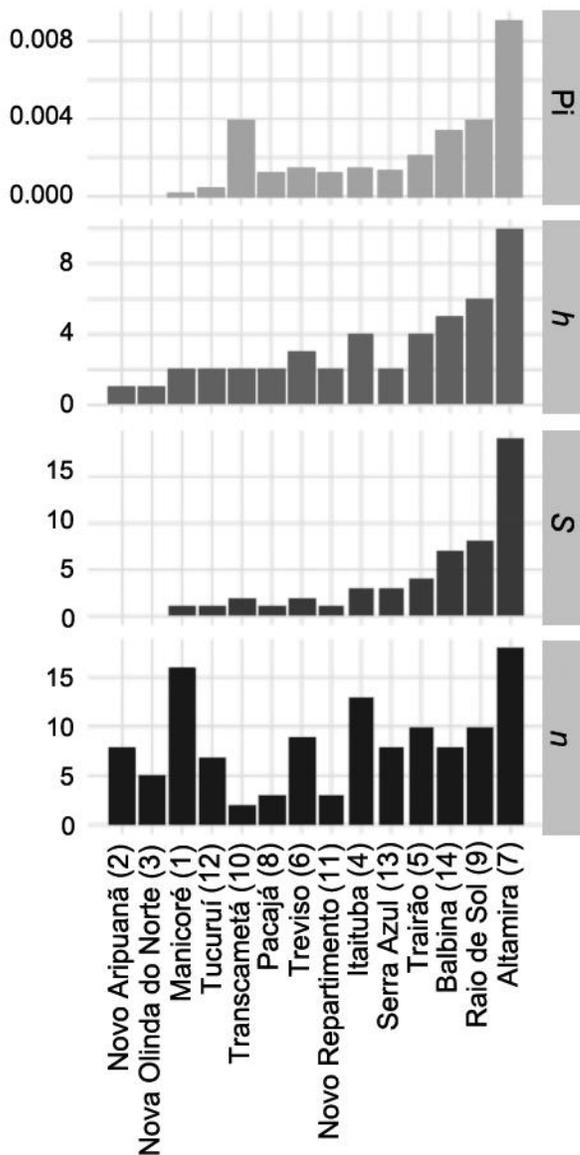
Fig. 3 Summary statistics of genetic polymorphism parameters of the brilliant-thighed frog (*Allobates femoralis*) based on mitochondrial 16S rDNA haplotypes. Samples were collected in 14 locations (as indicated in brackets) throughout central Brazilian Amazon. *n* = sample size; *h* = number of haplotypes; *S* = number of segregating sites; Pi = average pairwise genetic distance between samples in the same locality.

Average genetic distance among samples collected in each locality was 1.5% (Fig. 4). Lowest distances were observed between samples collected in locations within the same interfluve, notably between samples proceeding from Nova Olinda do Norte and Novo Aripuanã (western Madeira-Tapajós interfluve), and between samples from Pacajá, Anapu and Novo Repartimento (across the Xingu-Tocantins interfluve) (Table S3). Highest genetic distances (2.5–2.9%) were observed between samples collected in Itaituba (eastern Madeira-Tapajós interfluve) and locations in other interfluves (Fig. 4). Genetic distances observed between samples from Altamira and other locations were never lower than 1.1%, the lowest values being between Altamira samples and those collected in the western region of the same interfluve (Table S3). Overall, genetic distances between samples collected in different interfluves were appreciably higher than those observed between samples from the same interfluve (Fig. 5).
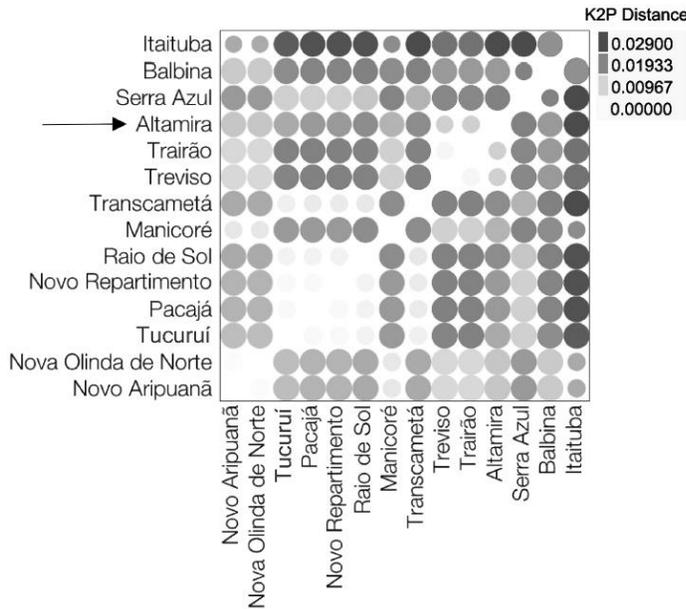
Fig. 4. Average Kimura 2-parameter genetic distances between brilliant-thighed frogs (*Allobates femoralis*) collected in 14 sampling locations along central Brazilian Amazon. Measures were obtained from a 504 bp fragment of the mitochondrial 16S rDNA gene. Arrow indicates the location of Altamira, where *A. femoralis* present a distinct advertisement call phenotype. Refer to Table 1 for the number of sequences available for each locality.

Calls recorded throughout the study area uncovered the existence of a distinct acoustic phenotype of *A. femoralis* restricted to sampling sites in Altamira, on the eastern Tapajós-Xingu interfluve, by the west bank of the Xingu River (Table 1; Fig. 6). At this location, *A. femoralis* males (*n* = 12 recordings) emit advertisement calls formed by six notes, differing from the four-note call pattern prevalent among specimens recorded elsewhere (*n* = 117, Table 1). This call phenotype is apparently distributed in areas indirectly impacted by the construction of the Belo Monte dam. Importantly, individuals with six-note calls did not share haplotypes with those possessing a four-note call structure recorded elsewhere, including individuals from the same interfluve (western Tapajós-Xingu) and individuals sampled immediately across the Xingu River (Figure 2).

Fig. 5 Comparison of genetic distances between pairs of populations of brilliant-thighed frogs (*Allobates femoralis*) occuring in the same or in different interfluves of the major Amazon basin rivers. Diamonds denote mean values (middle horizontal lines) and 95% confidence intervals (extremes). Data were taken in 14 localities. Measures were obtained from a 504 bp fragment of the mitochondrial 16S rDNA gene. Refer to Table 1 for the number sequences available for each locality.

Fig. 6 (a) Live specimen of the brilliant-thighed frog (*Allobates femoralis*) captured near the city of Altamira, Pará State, Brazil. This characteristic color pattern is widespread in the Tapajós-Xingu interfluve. (b) Sonogram of the adve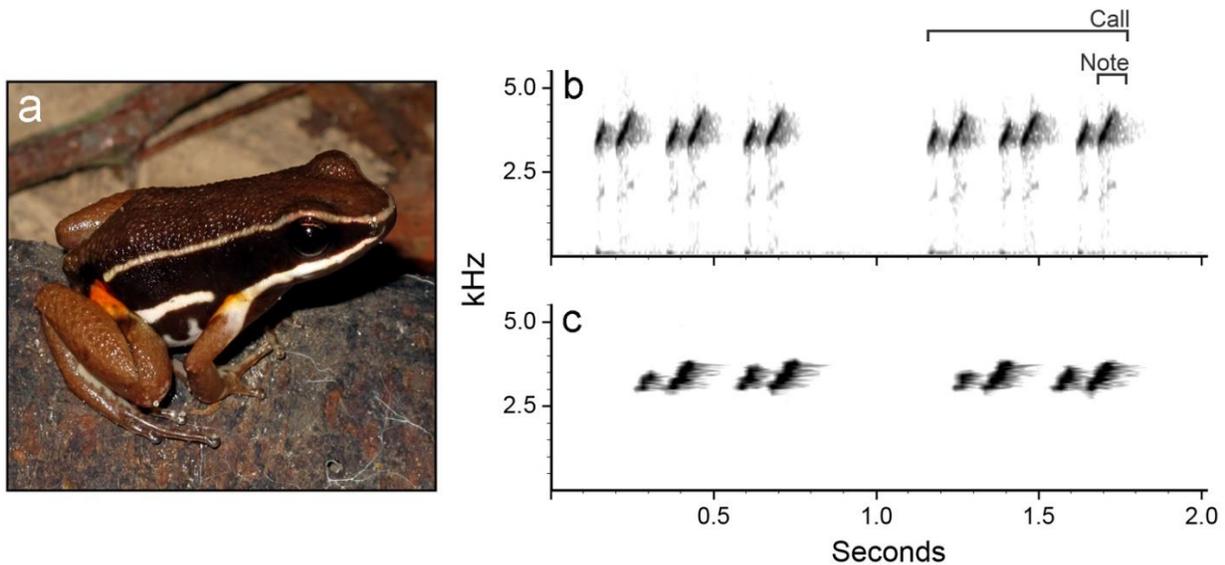rtisement calls of one *A. femoralis* male recorded near Altamira, on the west bank of the Xingu River, in March 2009. All males recorded in this region possess advertisement calls formed by six notes. (c) A typical 'four-note' advertisement call, recorded from a male at Fazenda Treviso, Belterra, Pará State, January 2007. Advertisement calls recorded at all remaining sampling sites covered by this study, except those near Altamira, have a similar, four-note pattern. Recordings were conducted at air temperatures of 28.6 °C (b) and 24.7 °C (c).

Discussion

Within the Tapajós-Xingu interfluve, construction of Belo Monte dam will potentially affect habitat occupied by a novel genetic lineage of *A. femoralis*, a lineage with no mtDNA haplotypes in common with other clades. Male *A. femoralis* belonging to this threatened genetic lineage are also characterized by possessing a different call structure. Locally, sampling sites visited in Altamira consist of areas that already have, or are likely to experience environmental change (Fig. 1), including forest fragments surrounding Altamira's urban perimeter (≈ S 03° 14' 36", W 52° 14' 37"), and remnants located in the Volta Grande region (≈ S 03° 07' 39", W 51° 42' 35"). The Volta Grande region is located downstream from the proposed site of the two first dams (Babaquara and Pimental dams) of the Belo Monte power plant, and will be subject to dramatic changes in water level regimes and induced drought [7].

The genetic and vocal differences we have observed suggest that *A. femoralis* within the impact zone of Belo Monte dam are reproductively isolated from other groups of individuals within the same interfluve. Especially given that assortative mating on the basis of call structure has been proposed as a strong evolutionary mechanism, triggering or enhancing reproductive isolation in anurans [43-44]. A comparative approach demonstrating that intraspecific variation in other species or taxa within the impact zone of the Belo Monte Dam would provide evidence that conserving this location is important to maintain evolutionary processes. However, by relying on *Instrução Normativa Nº 146* [17] as the single and unequivocal guideline to pinpoint species potentially affected by new power plants and their associated

reservoirs, environmental researchers and government decision-makers risk overlooking phenotypic differentiation which might be linked to genetic divergence and speciation. We also show that the genetic structure of *A. femoralis* is strongly partitioned among interfluves. Therefore, our data support the debated hypothesis that large Amazonian rivers partition genetic variation of widespread anuran species [40-42], suggesting that this general biogeographic pattern could be a useful proxy for conservation strategies to conserve genetic variation in amphibians with conserved external morphology.

We argue that amendments to legislation for environmental licensing of development projects in the Brazilian Amazon should include guidelines to assess intraspecific biological information. The practice of noting phenotypic characters, such as call repertoire, coloration and morphometric data, to compare with data recorded for the same species at other locations, will provide a valuable first step in evaluating whether the location where development is planned is one that generates biodiversity. If this pattern of intra-specific variation is consistent among several taxa, then these phenotypic data could be complemented with genetic data to more rigorously evaluate the importance of the location to evolutionary processes. Obtaining genetic data from widely applicable markers (e.g. regions of mtDNA) and commonplace genealogical analytical techniques will be sufficient to point out cases of pronounced intra-specific genetic divergence. Given strong evidence for the association between particular geographic features like rivers, and genetic variation for Amazonian anurans [41, 45-47], adopting surrogate measures of diversity in EIA's will reduce the risk of overlooking threatened components of diversity.

Implications for conservation

Increasingly, conservation managers are placing more importance on measures of intraspecific diversity and the conservation of evolutionary potential [27]. A central issue in preserving intraspecific diversity is the conservation of its inherent evolutionary potential in response to environmental change. Current adaptive variation in genotypes and phenotypes of a species is key to its resilience in the face of potential impacts of habitat alteration, emerging disease, or climate shifts; therefore, the focus of conservation planning has shifted to maintaining ecological processes and locations where such variation occurs [20, 23]. In addition to its evolutionary consequences, the extirpation of local amphibian populations can cause severe impacts in ecosystem functioning and ecosystem services, especially due to their biphasic life stages and their role in nutrient cycling [48-49].

We also point to the potential cost to scientific knowledge of losing particular components of intraspecific diversity. For example, *A. femoralis* has become a model organism for behavioral and communication research in this century. The existence of different call morphotypes has been pivotal to experiments investigating inter-population acoustic recognition [50], natural hybridization [32], acoustic signal evolution [28, 51], and the information carried by distinct components of these signals [52]. The premature loss of one of these call morphotypes will undoubtedly leave an irreversible knowledge-gap that will affect many burgeoning research lines.

Genetic and acoustic differentiation may also correlate with differentiation in other traits of commercial significance. For example, *A. femoralis* deposits its eggs in jelly nests that are placed directly on the leaf litter of the forest floor. The exact chemical composition of the nests is largely unknown, but given the wide geographic distribution of *A. femoralis*, it is reasonable to assume that there is great potential for geographic variation of broad spectrum fungicides and antibiotics associated with its nests. Chemical protection could also be location-specific as a result of adaptation to local pathogens. Consequently, there could be additional societal benefits gained from knowledge of intra-specific variation, such as those gained from bio-prospecting.

Recently, improvements in legislation for environmental licensing in Brazil have been suggested that make use of up-to-date ecological reasoning and techniques [53]. Considering that the Amazon is a significant reservoir of global biodiversity, further amendments to licensing procedures might require the inclusion of genetic, phenotypic and behavioural data. Ideally, the selection of groups of individuals deserving conservation or management priorities should be based on both genetic and phenotypic data [23-24]. Elsewhere the adoption of policies that include the protection of intra-specific evolutionary significant units (ESUs) or management units (MUs) have proved to be effective when applied to endangered vertebrates, for example in the U.S.A. [25,26].

Knowledge of genetic variation among interfluves for co-occurring species will be useful to the design of general conservation management strategies. At present, it is reasonable to suggest that management plans conserve evolutionary potential in geographically widespread anurans should consider, at their outset, protecting groups of individuals restricted to each one of these large interfluves. Further steps should evaluate intra-specific genetic diversity within interfluves in order to identify threatened lineages. For example, in this study, relatively high genetic divergence was detected between samples from Itaituba and those from the remaining locations between rivers Madeira and Tapajós. Itaituba is located on the west bank of the middle course of the Tapajós River, an area severely threatened by the upcoming building of at least two large reservoirs (UHE São Luiz do Tapajós and UHE Jatobá – www.pac.gov.br/energia/geracao-de-energia-eletrica).

Here we have demonstrated that a morphologically conservative frog species, *A. femoralis*, possesses strong genetic partitioning both within and across interfluves. Without formal taxonomic classification, a unique component of diversity within the *A. femoralis* group is threatened by developments related to the Belo Monte power plant. The difficulties and time required to classify biodiversity at any hierarchical level mean that conservation policy that incorporates information on genetically distinctive units or appropriately selected phenotypic traits may safeguard against losses of biodiversity, which would not be recognized by threatened species lists alone. Furthermore, concordant geographic patterns in intraspecific variation can point to locations that are valuable to protect evolutionary processes. The data required to evaluate intraspecific variation are often available, in the process of being generated, or easy to obtain, but not currently made use of in environmental impact statements in the Brazilian Amazon.

Acknowledgements

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Supplementary Information

The value of sub-specific measures of biodiversity in environmental licensing surveys is highlighted by Amazonian frogs. Authors: Pedro Ivo Simões, Adam Stow, Walter Hödl, Adolfo Amézquita, Izeni P. Farias and Albertina P. Lima.

Table S1 Mutation steps and distribution of 39 haplotypes of a 504 bp fragment of the mitochondrial 16S rRNA gene of *Allobates femoralis* sampled in 14 locations across central Brazilian Amazon. Refer to text (Table 1, Fig. 1) for relative position of sampling locations and number of samples collected in each location.

Haplotype	Mutation steps					Location
	10 *	20 *	30 *	40 *	50 *	
Hap_01	CCTTGTCACTAGCCATTGCCCACGTTTCCCGCAATCAAAAAAGCATTCATCC					1
Hap_02	CCTTGTCACTAGCCATTGCCCACGTTTCCCGCAATCAAAAAAGCACTCATCC					1
Hap_03	CCTTGTCACTAGCCATTGCCCACGCATCCCGCAATCAAAAAAGCACTTATCC					2,3
Hap_04	CCTCGTCAATAGCCGTTAACCACGCACCCCGCAATCAAAAGAGCACTCATCC					4
Hap_05	CCTCGTCACTAGCCGTTAACCACGCACCCCGCAATCAAAAGAGCACTCATCC					4
Hap_06	CCTCGTCAATAGCCGTTAACCACGCACCCCGCAATCAAAAGAGCTCTCATCC					4
Hap_07	CCTCGTCAATAGCCGTTAACCTCGCACCCCGCAATCAAAAGAGCACTCATCC					4
Hap_08	CCTTGTCACTAGCCATTGCCCACGCTTCCCGTAATCAAAAAGGCATCTATTC					5
Hap_09	CCTTGTCACTAGCCATTGCCCACGCTTCCCATAATCAAAAAGGCACCTATCC					5
Hap_10	CCTTGTCACTAGCCATTGCCCACGCTTCCCATAATTAAAAAGGCACCTATCC					5
Hap_11	CCTTGTCACTAGCCATTGCCCACGCTTCCCGTAATCAAAAAGGCACCTATCC					5,6
Hap_12	CTTTGTCACTAGCCATTGCGTACACTTTTTGTAATTAAAAAAGCACCTATCC					6
Hap_13	CCTTGTCACTAGCCATTGCCCACGCTTCCCGTGATCAAAAAGGCACCTATCC					6
Hap_14	CCTTGTCACTAGCCATTGCCCACGCTTCCTGTGATCAAAAAGGCACCTATCC					6
Hap_15	CCTTGTCACTAGCCATTGCCCACACTTCTCGTAATTAAAAAAGCACCTATCC					7

Table S1 Continued.

Haplotype	Mutation steps					Location
	10 *	20 *	30 *	40 *	50 *	
Hap_16	CTTTGTCACTAGCCATTGCCCACACTTCTCGTAATCAAAAAAGCACCTATCC					7
Hap_17	CCTTGTCACTAGCCATTACCCACGCTTCTCGTAATTAAAAAAGCACTTATCC					7
Hap_18	CCTTGACACTAGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					7
Hap_19	CCTTGTCACTAGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					7
Hap_20	CCTTGTCCCTAGCCATTGCCCACGCTTCTCGTGATCAAAAAAGCATCTATCC					7
Hap_21	CCTTGTCACTAGCCATTGCCCACGCTTCCCGTAATCAAAAAGGCACCTACCC					7
Hap_22	CCTTGTCACTAGCCATTGCCCACACTTCTCGTAATCAAAAAAGCACCTATCC					7
Hap_23	CTTTGTCACTAGCCATTGCCCACACTTCTCGTAATTAAAAAAGCACCTATCC					7
Hap_24	CCCTGACACTAGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					8
Hap_25	CCCTGTCACTAGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					8,9,11,12
Hap_26	CCCTGTCACTAGCCATTATCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					9
Hap_27	CCCTGTTACTAGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTTC					9
Hap_28	CCCTGTCACTGGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCG					9
Hap_29	CCTTGTCACTAGTCATTATCCATGCTTCTTGCAACCAAAAAAGCACTTCTCC					9
Hap_30	CCCTGTCACTGGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					10
Hap_31	TCTTGTCACTGGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTCC					10
Hap_32	CCCTGTCACTAGCCATTACCCACGCTTCTTGCAACCAAAAAAGCACTTCTTC					11,12
Hap_33	CCCTCTCACAAGCCATGACCCACGCTTCTCGCACTCAAAAAAATACTTCTCC					13
Hap_34	CCCTGTCACTAGCCATGACCCACGCTTCTCGCACTCAAAAAAGTACTTCTCC					13
Hap_35	CCTCGTCACTAACCATTACCCACGCATCTCGTAATCGAAAAAGCACTTATCC					14
Hap_36	CCTTGTCACTAACCATTACCCACGCATCTCGTAATCGAAAAAGCACTTATCC					14
Hap_37	CCTTGTCACTAACCATTACCCACGCATCTCGTAATCGGCGAAGCACTTATCC					14
Hap_38	CCTTGTCACTAACCACTACCCACGCATCTCGTAATCGAAAAAGCACTTATCC					14
Hap_39	CCTTGTCACTAAATATTACCCACGCATCTCGTAATCGAAAAAGCACTTATCC					14

*Relative position of mutation steps along the 16S rDNA fragment: [1] nt_8, [2] nt_16, [3], nt_19, [4] nt_20, [5] nt_33, [6] nt_51, [7] nt_69, [8] nt_81, [9] nt_93, [10] nt_96, [11] nt_110, [12] nt_112, [13] nt_114, [14] nt_156, [15] nt_163, [16] nt_175, [17] nt_196, [18] nt_198, [19] nt_200, [20] nt_208, [21] nt_226, [22] nt_230, [23] nt_236, [24] nt_240, [25] nt_244, [26] nt_251, [27] nt_252, [28] nt_255, [29] nt_256, [30] nt_258, [31] nt_269, [32] nt_270, [33] nt_276, [34] nt_279, [35] nt_282, [36] nt_287, [37] nt_297, [38] nt_308, [39] nt_311, [40] nt_315, [41] nt_324, [42] nt_362, [43] nt_364, [44] nt_366, [45] nt_374, [46] nt_376, [47] nt_384, [48] nt_388, [49] nt_397, [50] nt_409, [51] nt_410, [52] nt_482. Designation of sampling locations: 1) Manicoré; 2) Novo Aripuanã; 3) Nova Olinda do Norte; 4)Itaituba; 5) Trairão; 6) Treviso; 7)Altamira; 8) Pacajá; 9)Raio de Sol; 10) Transcametá; 11)Novo Repartimento; 12) Tucuruí; 13) Serra Azul; 14) Balbina.

Table S2 Summary statistics of genetic polymorphism parameters of *Allobates femoralis* collected in 14 sampling localities across central Brazilian Amazon, based on mitochondrial 16S rDNA haplotypes. Localities correspond to sites presented in Table 1 and Fig. 1 in the text. *n* = sample size; *h* = number of haplotypes; *S* = number of segregating sites; π = average pairwise genetic distance between samples in the same locality.

Sampling locality	*n*	*h*	*S*	π ± 1 s.d.
1. Manicoré	16	2	1	0.0002 ± 0.0002
2. Novo Aripuanã	8	1	0	0.0000 ± 0.0000
3. Nova Olinda do Norte	5	1	0	0.0000 ± 0.0000
4. Itaituba	13	4	3	0.0015 ± 0.0003
5.Trairão	10	4	4	0.0021 ± 0.0007
6. Treviso	9	3	2	0.0015 ± 0.0004
7. Altamira	18	10	19	0.0091 ± 0.0016
8. Pacajá	3	2	1	0.0013 ± 0.0006
9. Raio de Sol	10	6	8	0.0040 ± 0.0011
10. Transcametá	2	2	2	0.0039 ± 0.0019
11. Novo Repartimento	3	2	1	0.0013 ± 0.0006
12. Tucuruí	7	2	1	0.0005 ± 0.0003
13. Serra Azul	8	2	3	0.0014 ± 0.0010
14. Balbina	8	5	7	0.0034 ± 0.0011

Table S3 Average Kimura 2-parameter genetic distances between *Allobates femoralis* collected in 14 sampling localities along central Brazilian Amazon. Measures were obtained from a 504 bp fragment of the mitochondrial 16S rDNA gene. Refer to Online Resource 2 for the number sequences available for each locality.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Manicoré													
2. Novo Aripuanã	0.006												
3. N. Olinda do Norte	0.006	0.000											
4. Itaituba	0.018	0.016	0.016										
5.Trairão	0.011	0.009	0.009	0.025									
6. Treviso	0.011	0.009	0.009	0.025	0.002								
7. Altamira	0.015	0.013	0.013	0.028	0.011	0.011							
8. Pacajá	0.017	0.015	0.015	0.027	0.020	0.020	0.017						
9. Raio de Sol	0.018	0.016	0.016	0.027	0.021	0.021	0.018	0.03					
10. Transcametá	0.018	0.016	0.016	0.028	0.021	0.021	0.018	0.05	0.06				
11. Novo Repartimento	0.017	0.015	0.015	0.027	0.020	0.020	0.017	0.01	0.03	0.05			
12. Tucuruí	0.017	0.014	0.014	0.026	0.020	0.019	0.016	0.01	0.03	0.04	0.01		
13. Serra Azul	0.019	0.017	0.017	0.029	0.022	0.022	0.020	0.11	0.13	0.15	0.11	0.11	
14. Balbina	0.018	0.012	0.012	0.023	0.017	0.017	0.017	0.019	0.020	0.020	0.019	0.018	0.021