Twenty-five species of frogs in a liter of water: eDNA survey for exploring tropical frog diversity

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Abstract

Biodiversity is declining at unprecedented rates, with species extinctions being an important component of this process. Currently it is almost impossible to evaluate this loss since biodiversity data is lacking even for emblematic species. Recent advances in eDNA marker gene studies promise to deliver the necessary data, but these approaches have almost never been evaluated in the tropics. Here we evaluate whether 1) eDNA metabarcoding may reliably generate frog faunistic data in species-rich tropical areas, 2) the ecological signal in eDNA data is compatible with data from visual and audio encounter survey (VAES), and 3) eDNA data represent a financially viable solution to biodiversity data acquisition. We applied eDNA metabarcoding to investigate frog species occurrence in five ponds in the Chiquitano dry forest region in Bolivia and compared our data with a simultaneous VAES. We find that taxon lists generated with eDNA and VAES correspond closely, and most deviations are attributable to different species' life histories. The ecological signal in eDNA closely matches the signal from VAES. We find that the cost efficiency of eDNA surveys is mostly influenced by the richness of local fauna and the number of surveyed sites: VAES may be less costly in low-diversity regions, but eDNA quickly becomes more cost-efficient in high-diversity regions, particularly with many sites. The results underline that eDNA is suitable for large-scale biodiversity surveys in high-diversity areas if precautions in data generation and interpretation are taken and this projects spatially extensive standardized biodiversity surveys in the close future.

Keywords

Amphibians, metabarcoding, tropical biodiversity, conservation, community ecology, cost comparison

Introduction

Improvements on most biodiversity loss indicators lag behind the 20 “Aichi Biodiversity Targets” (UNEP 2016) that aim to reduce the decline of biodiversity by 2020 (Tittensor *et al.* 2014). An important component of the biodiversity crisis is the extinction of species: based on current trends in mammals, birds, reptiles and amphibians, it has been projected that the biodiversity crisis may lead to the 6th Mass Extinction over the next three centuries if all threatened species go extinct (Barnosky *et al.* 2011). Current rates of extinction may even be much higher if one considers the extinction that likely occurred during the last decades-centuries, but went unnoticed because the now-extinct species had small ranges, were never described or only described on the eve of their extinct (Pimm *et al.* 2014; Lees & Pimm 2015). It is however difficult to assess which species are endangered and to what extent. First, most taxa remain undescribed: in some highly diverse regions many species will likely go extinct before they are discovered (Costello *et al.* 2013; Lees & Pimm 2015). Second, cryptic genetic diversity is common within morphospecies (Pfenninger & Schwenk 2007; Pauls *et al.* 2013), and global change may impact cryptic diversity more severely than morphospecies (Bálint *et al.* 2011). Third, data on population-level trends is scarce, even for well-known species (Butchart *et al.* 2010). Better population-level biodiversity data is thus urgently needed to 1) understand biodiversity patterns and extinction threats, 2) improve forecasting abilities about future biodiversity, and 3) improve humanity’s responses to the challenges of biodiversity loss. This data is crucial in times when conservation action is increasingly demanded by society (Tittensor *et al.* 2014).

The importance of internationally coordinated, standardized biodiversity data collection is long recognized both in science and in conservation (Henry *et al.* 2008). This is particularly true for the most biodiverse areas. The tropics are generally underrepresented in ecological studies (Clarke *et al.* 2017; Stroud & Feeley 2017). However, encounter-based data collection appears

more difficult here and sufficient funds and expertise are often missing for comprehensive surveys. Indirect species records through environmental DNA are increasingly heralded as an alternative to encounter-based surveys (Thomsen & Willerslev 2015; Pedersen *et al.* 2015). eDNA also allows standardized biodiversity surveys on regional and global scales since community composition data can be obtained by high-throughput-sequencing of standardized, taxonomically informative marker genes (metabarcoding) (Taberlet *et al.* 2012a; Cristescu 2014). eDNA-based metabarcoding may present one of several tools needed to globally coordinate initiatives for ecosystem monitoring and sustainable management (Bush *et al.* 2017; Schmeller *et al.* 2017).

In this study we evaluate whether eDNA metabarcoding is suitable for inventories of frogs, a group with particular high species-diversity in tropical regions. Frogs and other amphibians are particularly hard hit by the biodiversity crisis: more than one-third of the approximately 7500 described species are endangered (Stuart *et al.* 2004; Bishop *et al.* 2012; Whittaker *et al.* 2013). Frogs are also known for being a highly diverse, but incompletely described taxon, especially in the tropics (Ferrão *et al.* 2016; Caminer *et al.* 2017). Many “widespread” morphospecies harbor considerable cryptic genetic diversity and are better considered complexes of closely related species with much smaller ranges (Fouquet *et al.* 2007; Gehara *et al.* 2013, 2014; Ortega-Andrade *et al.* 2015). Efficient actions for amphibian conservation (e.g. the prioritization of areas for conservation, or informing society and stakeholders about conservation needs) are only possible with geographically fine-scale, taxonomically well-resolved faunistic data, but our current understanding of present and future amphibian biodiversity is often based on rare, spatially and temporally scattered observations of phenotypically defined taxa.

First efforts have been done to test the suitability of eDNA for the survey of tropical frog biodiversity (Lopes *et al.* 2016), but important practical aspects remain unaddressed. First, it is

not clear which fraction of the local species pool is represented by amphibian eDNA in tropical water bodies: existing comparisons of encounter-based surveys and eDNA either do not include non-adult life stages, or they use already compiled fauna lists for the evaluation of eDNA performance without consideration of life history traits or behavioral aspects at the moment of sampling. This may confound biodiversity data collection (Petitot *et al.* 2014). Second, ecological signal in aquatic eDNA data remains largely unevaluated: it is not clear whether it reflects ecological conditions. Most studies to date only evaluate the correspondence between encounter-based and metabarcoded taxon lists (Miya *et al.* 2015; Valentini *et al.* 2016), although the ecological signal itself may be of higher importance for many applications (Ji *et al.* 2013; Elbrecht *et al.* 2017). Third, it is not clear whether, and under what conditions eDNA is financially efficient since comparisons are lacking (Lopes *et al.* 2016), although these comparisons are essential for deciding on data collection strategies.

Here we particularly address whether 1) detectability of tropical amphibians with eDNA is linked to species' life history, and 2) there is sufficient ecological signal in amphibian eDNA data to allow inferences about biodiversity patterns. Finally, we present a framework for cost comparisons between encounter- and eDNA-based biodiversity survey that may be adapted to other systems beyond amphibians. We compare the results of long- and short-term encounter-based field surveys, and an eDNA survey of tropical amphibians in a high-diversity area (45 species of frogs on ca. 30 km^2). This area is well characterized with respect to amphibians, including both larvae and adults (Schulze et al. 2015), and cryptic intraspecific diversity (e.g. Jansen et al. 2011, Jansen *et al.* 2016). The area is located the Chiquitano region of Bolivia, a forest-savanna ecotone between Amazon, Cerrado and Gran Chaco in a transition zone among humid and dry forests that are special in regard to their taxonomic and functional diversity (Castro *et al.* 1999). The region contains the largest intact, old-growth block of seasonally dry tropical forests in South America (Miles *et al.* 2006; Power *et al.* 2016).

Materials and methods

A detailed description of the Materials and methods is provided in the Supplementary materials.

The study area is near the Biological Station “Centro de Investigaciones Ecológicas Chiquitos” on the San Sebastián cattle ranch (S16.3622, W62.00225, 500 m a.s.l.), 24 km south of the town of Concepción, Province of Ñuflo de Chávez, Santa Cruz Department, Bolivia. A description of the area is given by Schulze *et al.* (2009). Intensive long-term assessments have resulted in a list of 45 frog species in the area (e.g. Jansen 2009; Schulze *et al.* 2009, 2015; Jansen *et al.* 2011). We selected five ponds for this study. eDNA samples were obtained in three technical replicates from three sampling points on each pond (Fig. 1). All samples and replicates were filtered with a two-step sequential filtering scheme: first, two glass fiber filters (GFF) with a pore size of 2 µm (filter method A: preserved in CTAB; B: dried), and second, the flow-through was filtered with a nylon filter with (pore size 0.2 µm, method C, dried). Combined visual and audio encounter surveys (VAES, Zimmerman 1994), and a tadpole survey (TS, Schulze *et al.* 2015) were conducted by experienced observers (MJ and AS). Frogs were located at night (between 21:00 and 00:30 hours) during 0.5 - 1 hrs transect walks along the ponds to detect and identify specimens in vegetation, water and on ground around ponds with flashlights and headlamps, and by listening. Tadpoles were searched once daytime and once at night in each pond and the riparian vegetation, and caught with dip nets.

eDNA samples were processed in Germany in a low-DNA laboratory. DNA was extracted from GFF samples (A, B, see above) with a CTAB chloroform extraction method according to Strand *et al.* (2014) and Wittwer et al. (2017). Dried nylon filters (C) were extracted with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following Thomsen *et al.* (2012).

Samples were fully randomized before extraction to prevent batch effects (Bálint *et al*. 2017a). The barcode amplification targeted mitochondrial 16S ribosomal RNA. We designed primers for a 150 bp fragment with the reference database of local species (sequences from 159 specimens, Jansen *et al.* 2011; Schulze *et al.* 2015). The primers were tested on a subset of 12 species (Supplementary Table S3). PCRs were run on 96-well plates in 15-µl reaction volumes with a touchdown protocol, with four PCR technical replicates per sample. We included four negative PCR controls (ultra-sterile water), four extraction blanks, and two positive controls on each plate. The amplification primers contained multiplexing indices. PCR products were purified, pooled in equal volumes, and paired-end-sequenced (2 x 150 bp) on an Illumina NextSeq 500 sequencer.

Bioinformatics were done with OBITools v1.2.0 (Boyer *et al.* 2016). We performed two reference-based taxonomic assignments, first with a custom database of 16S sequences of 159 specimens from the regional amphibian fauna (16S_custom), and second with all 16S sequences found in the EMBL (release 125, 16S_EMBL). Further filtering was done in R (R Core Team 2017) with a script supplied on GitHub: https://github.com/MikiBalint/amphibian-eDNA.

We followed recommendations for the statistical analyses of marker gene community data (Bálint *et al.* 2016). eDNA faunistic differences among the five ponds were visualized with a latent variable model-based ordination in R (‘boral’, Hui *et al.* 2015), and tested with multispecies generalized linear models (‘mvabund’, Wang *et al.* 2012). The surveyed ponds were markedly different in size, vegetation, water depth, etc, thus we considered the pond identity as a good predictor of community composition. We performed a model-based ordination also for the VAES presence/absence data and then used a Procrustean superimposition (Peres-Neto & Jackson 2001) to evaluate how the VAES-based ordination of ponds matches the ordination of centroids of eDNA samples. We compared the efficiency of filter preservation

(CTAB or dried) on the successful detection of species with a site occupancy model (MacKenzie *et al.* 2002; Bailey *et al.* 2014), implemented in the R package 'unmarked' (Fiske *et al.* 2011). We used the single season false-positive occupancy model developed by Miller *et al.* (2011).

For cost comparisons, we considered a typical eDNA survey scenario: samples are collected in the field and later processed in a dedicated laboratory. For the encounter-based survey we considered a scenario with a similar separation of the fieldwork, and species identification: species records (audio or visual) are collected by a field biologist, and later identified by an expert in the office/laboratory. The parameters in our cost model are a good approximation of the variables involved in the present study and involve a learning effect in the efficiency of the taxonomic expert. The sampling and identification costs of VAES are dependent both on the number of sampling sites, and the number of species since each species needs to be recorded (Fig. 4). During the eDNA survey eDNA samples are collected by a field biologist, analysed in a lab and sequenced by an external provider. The sampling and identification costs of eDNA sampling costs of eDNA depend on the number of sites, but not on the number of species (Fig. 4). We kept some cost factors constant: the costs for training the frog taxonomic expert and the VAES observers, the costs for building up the eDNA metabarcoding facilities (clean rooms and equipments to perform DNA manipulations, but not the sequencing), and the databases necessary for the sequence assignment. We assume that travel costs are the same for the two survey types, and that the time necessary to walk between frog observations and eDNA sampling points is the same. All model parameters and calculations are accessible on FigShare (Bálint *et al.* 2017b; https://figshare.com/articles/_/5099842).

Results

The sequencing resulted in 12 742 273 read pairs (deposited in ENA as PRJEB22113**)**, of which 9 479 299 were identified as complete 16S amplicons. These reads were de-replicated into 631 003 unique sequences variants. Only 22 706 of these variants were represented by at least 10 reads and retained for further processing. The sequence cleanup resulted in 14 442 high quality sequence variants, 13 497 coming from the present experiment. Of these, 4 805 sequence variants were taxonomically assigned with the 16S_custom database, and 8 692 with the 16S_EMBL database. The assigned sequence variants represented 8 011 631 sequences, of which 561 sequence variants were kept for further analyses as they were recorded as 'head' in at least one sample (6 965 866 reads). After the removal of potential contaminant reads 5 898 184 sequences were retained. The final sample - sequence variant abundance matrix contained 5 815 014 sequences. These belonged to several groups: frogs (2 158 534), fish (1 692 613), insects (304 059), mammals (14 006), birds (967), and bacteria and other groups (1 063 156).

In the five ponds 31 frog species were detected in total with both methods. The VAES and the eDNA metabarcoding detected 25 species each, and TS detected 4 species; 19 species were detected by both eDNA survey and VAES (Fig. 2a; Supplementary Table S1). Six species were detected only by eDNA, and six species were detected only by VAES. Several of the species detected by VAES but not by eDNA had no aquatic life phase during the time of survey (i.e. no larvae or adults in the water). Of the species that were present in the water (N=20), eDNA detected 19 (Fig. 2b, Supplementary Table S1). With a single sampling eDNA detected more than half of the 45 species known to be present in the area (Fig. 2c, Supplementary Table S2). Of these 45 species only 35 were ever detected at the surveyed ponds, and eDNA detected 23 of those (Fig. 2d, Supplementary Table S2)**.** The eDNA results reported two additional species that were not known during the time of the survey from the area (Fig. 2c,d). We detected 11 species (of the twelve) from the equimolar DNA concentration positive controls,

and 6 in the non-equimolar DNA concentration controls (Supplementary Fig. S2). Read numbers in the equimolar PCR controls were highly variable, but strongly correlated among the controls ($R > 0.7$ for each pair of positive controls, Supplementary Fig. S2A). The read numbers in the not equimolar positive control were strongly linked to the DNA template concentrations of the PCRs (Pearson correlation coefficient $R = 0.97$, Supplementary Fig. S2B). Only *Leptodactylus vastus* was recovered in the field positive control (water from an aquarium with *L. vastus* tadpoles).

Sample preservation / extraction method C (0.2 µm nylon filter, dried in the field, DNA extracted with DNeasy Blood & Tissue Kit) appeared to perform better (p_1=0.327, 95% C.I. = [0.285; 0.372] than methods A (p_1=0.278 [0.242; 0.317]) and B (p_1=0.243 [0.209; 0.281], both 2 µm glass-fiber filters, A - preserved in CTAB and DNA extracted using CTAB protocol, and B - dried in the field, DNA extracted using CTAB protocol, Supplementary Fig. S3). Method A had larger detection probability than method B, but confidence intervals were largely overlapping. The three methods were equivalent with respect to false positive probabilities (Supplementary Fig. S3).

Regarding the ecological signal in the eDNA data, replicate samples of each pond grouped relatively tightly on the latent variable model ordination (Fig. 3a). The pond identity was a statistically significant predictor of frog communities in the five ponds (ANOVA, df = 6, dev = 534.99, $p < 0.01$). This is reflected in the 95% confidence intervals of the group centroids on the ordination which clearly separates all ponds except T1 and T3 (Fig. 3a). The ordination of the eDNA pond centroids closely corresponds with the ordination of observations from the five ponds with VAES (Procrustes permutation test, R = 0.8, p = 0.03, Fig. 3b).

The cost model of VAES and eDNA showed that the starting costs (i.e. with few sampling sites) for VAES are relatively low, but these costs rapidly increase until the taxonomic

expert becomes familiar with the regional frog fauna (Fig. 4). The VAES price is dependent on the species richness: first, the VAES observer needs to record each species on the field, and then the taxonomic expert needs to listen to each recording (Fig. 4). eDNA metabarcoding has a relatively high entry price since consumables and sequencing are costly, regardless of the number of sites. eDNA survey prices are then a linear function of the number of sampling sites, and an increase in the site numbers simply adds to sampling and consumable costs, but does not influence neither the time spent in the laboratory, nor the sequencing costs (Fig. 4).

Discussion

The one-time eDNA survey detected 25 species in the studied ponds, including formerly cryptic species that were only recently described by integrative taxonomy. This is on par with the one-time VAES (Fig. 2a), which confirmed the presence of (i) almost all species that were in contact with water during the survey time (Fig. 2b), (ii) all species that had tadpoles in the ponds (Supplementary Table S1), (iii) more than half of all 45 species ever recorded from the area (Fig. 2c), and (iv) about 65% of the 35 species ever recorded in the surveyed 5 ponds (Fig. 2d). The eDNA dataset recorded clear differences among the frog communities in the surveyed ponds, a result also in accordance with the VAES data (Fig. 3). The comparison of VAES and eDNA cost models show that eDNA biodiversity surveys may be a cost-efficient alternative to VAES in species-rich areas, but not necessarily in areas with low species numbers. Recommendations and technical remarks are presented in the Supplementary Materials.

Sensitivity of eDNA in diverse tropical frog communities

The comparison of the three filtration-preservation approaches shows that filters can be feasibly dried in the field and sent to a lab. We could not evaluate whether detection probability differs between larger pore-size GFF and small pore-size nylon filters since DNA was extracted with different methods from these. Currently only few comparative studies exist regarding the preservation of eDNA filtrates on filters: Hinlo *et al.* (2017) showed that the simple refrigeration of filters may be preferred to frozen storage. Here we show that filters can simply be dried in the field: this simplifies transportation since no special precautions are needed, unlike for liquid handling.

During the one-time surveys, six species were detected only with eDNA, but not with VAES (Fig. 2a). Four of these species (*Dermatonotus muelleri*, *Leptodactylus elenae*, *Osteocephalus taurinus*, *Rhinella schneideri*) are quite common in the area. The detection of

these four species by eDNA but not by VAES may result from the low abundance of adults or tadpoles in the ponds, or a lack in acoustic activity. In some cases eDNA detections are likely based on tadpoles only. For example, eDNA detected *L. syphax* from pond T2, although the species was only heard calling from a crevice of a rocky outcrop, its usual habitat (de Sá *et al.* 2014), approximately 30 m away from T2. The remaining two species detected only with eDNA are not known to occur in the area, thus they are candidates for false assignments. Both of these were assigned with sequence data from EMBL (*L. latinasus*: KM091595, *L. laevis*: AY843696). *Lysapsus laevis* would be the first record of this genus in the study area, but the identification of *Lysapsus* populations only using short 16S eDNA sequences is questionable, especially when considering the unclear taxonomy of the group in Bolivia. Nevertheless, all of the four known species of *Lysapsus* (*L. boliviana*, *L. caraya*, *L. laevis*, *L. limellum*) may occur at the site (De la Riva *et al.* 2000; Lavilla *et al.* 2004; Reichle 2004a; b; Angulo 2008; Jansen *et al.* 2011; Frost 2016) since they can easily be overlooked due to their small size and rather inconspicuous advertisement call. *Leptodactylus latinasus* is another species possibly occurring in the area but was so far not recorded (de Sá *et al.* 2014) and we cannot exclude the possibility that the corresponding sequence variant is actually an erroneous variant of one of the six local *Leptodactylus* species.

The species from this study either have no sufficient distributional data for an IUCN evaluation, or they are in the “least concern” category (Supplementary Table S1). Twelve of the 31 species are not assessed by IUCN, because (a) the species were described or removed from synonymy only recently (*Dendropsophus salli*: Jungfer *et al.* 2010; *Scinax madeirae*: Brusquetti *et al.* 2014; *Dendropsophus arndti*: Caminer *et al.* 2017), or (b) the assessment of their taxonomic status is still pending (e.g. various lineages of candidate species *sensu* Jansen *et al.* 2011). Nine species with uncertain taxonomic status were classified as distinct genealogical lineages by Jansen *et al.* (2011). These may represent new species with

considerably smaller distributions. Twelve further species lack information on actual distribution, although only one species is classified as “data deficient” (*Pithecopus azureus*). Nineteen species are classified as “least concern”. These classifications may become obsolete given rapid changes in taxonomy and updates on distributions: cryptic species-level diversity seems to be high in frogs of South America (Fouquet *et al.* 2007; Gehara *et al.* 2013). In summary, very little is known about actual distributions of Neotropical frogs due to sparse sampling and unreliable taxonomy. eDNA might help to complete distribution data of amphibians (including cryptic lineages), and this data is essential for reliable IUCN assessments.

eDNA metabarcoding is strongly facilitated by dedicated sequence databases: we had a database that contained all 45 species that were ever recorded in the area. This database was essential for both initial primer development, and taxonomic assignments: indeed, both eDNA-recorded taxa that were not present on the complete local species list of the present study (Fig. 2, Supplementary Table S2) were identified in the EMBL-based assignment that followed the assignment with the custom local 16S database. The importance of sequence assignment databases is long recognized, with considerable efforts underway to establish them (Ratnasingham & Hebert 2007; Coissac *et al.* 2016).

Six species were detected only with VAES, but not by eDNA (Fig. 2a, Supplementary Table S1). None of these species are strictly bound to ponds in the life stages occurring during our sampling: two hylids, *Dendropsophus arndti* and *D. salli*, usually call from plants on the pond shores (Schulze *et al.* 2009) and have only sporadic contact with water. *Leptodactylus fuscus* and *Pseudopaludicola* sp. also do not enter the water but usually call from nearby muddy grounds or grasslands. We did not detect tadpoles of these species in the ponds: the tadpoles of these species develop during the rainy season which triggers reproduction, but our sampling slightly preceded the rainy season. Some of the other undetected species have terrestrial or quasi-terrestrial life histories: *L. fuscus* deposits eggs within foam nests in underground burrows

at some distance from ponds and these are washed into nearby water bodies by floods that follow heavy rains (Heyer 1978; Lucas *et al.* 2008). If we consider the specific life histories, the one-time eDNA survey only missed a single species (*Sphaenorhynchus lacteus*) that was found calling from the water during the VAES (Fig. 2b, Supplementary Table S1). The hylid *Hypsiboas geographicus* was likely missed as a result of PCR bias: we could never recover it from positive controls when DNA from other species was also present (Supplementary Fig. S2), although single-template PCR reactions worked. Nonetheless, *H. geographicus* was also not detected by VAES, so it is possible that the species was not present during the survey. Regarding the whole local species pool (45 species in 10 years), life history also explains why at least some species (N=10) may not be detectable by eDNA: these species reproduce outside of water (e.g. *Leptodactylus mystacinus*), or are forest dwellers (e.g. *Leptodactylus* cf. *didymus*, Fig. 2d, Supplementary Table S2). In conclusion, with a single sampling eDNA detected about 65% of the detectable species from the area (23 out of 35).

Phenology is long considered important in encounter-based surveys of vertebrates (Wilson & Bart 1985; Møller 2010) and it was recently shown that life history influences insect detections with eDNA metabarcoding in temperate freshwaters (Bista *et al.* 2017). Our sampling was done at the beginning of the rainy season when only few species reproduced in the ponds. Repeating the sampling at during the rainy season may have potentially increased species detections by eDNA. The results show the importance of life history in the metabarcoding-based survey of tropical frogs and emphasize that sampling at specific or even multiple timepoints may be essential for more complete species pools also with eDNA (see Recommendations and technical remarks in the Supplementary Materials).

Ecological signal

There was considerable variation in the species recorded with the spatial replicates of the eDNA samples, and this underlines that eDNA sampling should be replicated for a good

representation of community composition. These samples may be pooled before DNA extraction to optimize costs if site-level variation is not of interest. We recorded similar ecological signal in the two datasets (Fig 3). The eDNA data did not distinguish the communities from ponds T1 and T3, and these communities also grouped closely in the VAES results. We randomized the samples before DNA extraction (Bálint *et al.* 2017a) so the signal is likely of biological origin and not a result of laboratory batch effects. Although the comparison of the ecological signal may be confounded by the different sample sizes (with the eDNA ordination based on many spatial replicates and the VAES ordination on a single observation event per pond), the correspondence of the results is still striking. Similar results were found in insect (Ji *et al.* 2013; Elbrecht *et al.* 2017) and plant communities (Niemeyer *et al.* 2017) where eDNA-based and morphology-based identifications result in similar conclusions about community structure, despite markedly different species lists. Our results provide further evidence that eDNA-based biodiversity surveys are highly sensitive to differences among ecological communities. These inferences are comparable to those derived with encounter-based observations and are informative about processes that underlay community assembly.

Cost efficiency of biodiversity surveys with eDNA metabarcoding

Although central to deciding on a method for biodiversity surveys, cost comparisons are not straightforward since they need to be based on expert knowledge both in VAES and eDNA. Cost comparisons were performed for single species eDNA detection (Huver *et al.* 2015; Davy *et al.* 2015; Smart *et al.* 2016), but we are not aware of frameworks suitable for eDNA metabarcoding. Here the VAES cost estimation is informed by over a decade of field and integrative taxonomic work with tropical frogs (Jansen *et al.* 2007, 2016; Schulze *et al.* 2009; Brusquetti *et al.* 2014), and the eDNA part is informed by multiple years of eDNA biodiversity surveys (Bálint *et al.* 2016, 2017a; Vörös *et al.* 2017). Such comparisons are urgently needed

due to stakeholder demands in eDNA (governmental agencies, conservation NGOs, fisheries, etc.).

We defined two species richness scenarios for the cost comparisons (Fig. 4) since experience shows that the time and resources necessary for VAES depend on the local biodiversity. VAES costs become high in regions with high frog richness since they are a function of both the number of sites and the number of species. Biodiversity surveys with eDNA are not necessarily cheaper in low-richness regions since entry costs for the eDNA work are high: lab consumables and sequencing are costly. However, eDNA survey costs are not dependent on the local biodiversity since metabarcoding can consider thousands of species simultaneously in a sample (Taberlet *et al.* 2012b). Consequently, eDNA costs are function of sample numbers, which influence the collection time spent on the field and consumables. eDNA metabarcoding operations are easily scaled up in a sense that hundreds of samples can be simultaneously processed (Ficetola *et al.* 2015).

Several aspects of our cost models are contentious. One issue is whether the relatively untrained VAES observers, or taxonomic experts perform the fieldwork, since taxonomic experts may identify many of the species immediately on the field. Currently, most surveys of high-diversity areas are directly done by experts interested in the local fauna, but we argue that this will not work for continental - global biodiversity surveys simply because there are not enough taxonomic experts (Buyck 1999; Haas & Häuser 2003). We also did not consider a scenario when VAES surveys are performed with automated recording devices (ARDs), and sounds are automatically identified by algorithms (see Recommendations and technical remarks in the Supplementary Materials). The sound complexity in tropical environments currently prohibits the use of automated sound identifications (Campos-Cerqueira & Aide 2016). It is also difficult to compare fundamental infrastructure and training costs (i.e. the establishment of an eDNA labor VS the training of taxonomic experts). Discussions about cost models are timely since they will

play major roles in devising continental-global biodiversity surveys, much needed today to monitor global biodiversity targets (Tittensor *et al.* 2014).

Conclusions

eDNA seems to be suitable to standardized biodiversity surveys of frogs even in species-rich areas, but it may be overly costly for smaller studies in low-richness regions. Differences between eDNA and traditional surveys seem to result largely from different observational biases, but a consideration of life histories promises to improve correspondence. The eDNA data is certainly suitable to characterize not only community composition, but also the ecological signals of factors that shape communities: this gives an unprecedented opportunity to incorporate eDNA as a standard toolkit for community ecology and macroecology. The quality of the ecological signal in eDNA-based biodiversity data foresees global biodiversity surveys and monitoring that will support both biodiversity research, and informed decisions on sustainable use of biological diversity.

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Data accessibility

Raw sequence data was deposited in ENA as PRJEB22113. Cost model parameters and calculations, and input data for statistical analyses are accessible through FigShare (Bálint *et al.* 2017b) (https://figshare.com/articles/_/5099842). R-based bioinformatic filtering and statistical analyses are accessible through GitHub: https://github.com/MikiBalint/amphibian-eDNA.

Author contributions

M.B., M.J., C.N., C.W. designed research; J.L.A., M.B., M.J., O.M., A.S., C.W., performed research; B.C., T.C., C.N., S.U.P. contributed reagents or analytic tools; M.B., M.J. analysed data; M.B., T.C., M.J., O.M., C.N., S.U.P., wrote the manuscript.

Figures

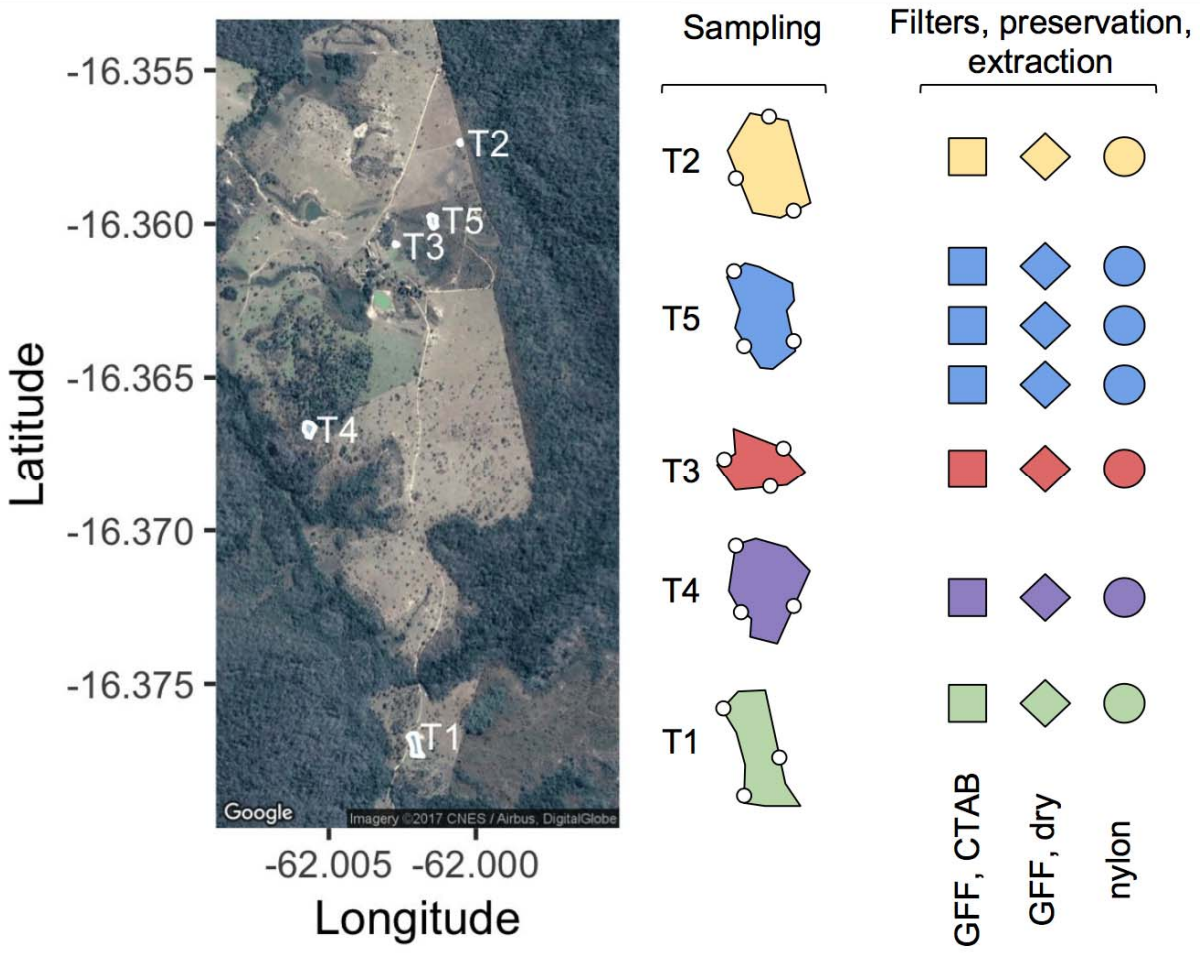

Fig. 1.

Sampled ponds, sampling replication, and filtration - filter preservation strategy. GFF - glass-fiber filter, 2 µm; nylon - nylon filter, 2 µm. Each sampling replicate (three per pond, marked with small circles) were processed with each three filtration strategies. Replicated samples of pond T5 were three times processed with each filtration strategy.

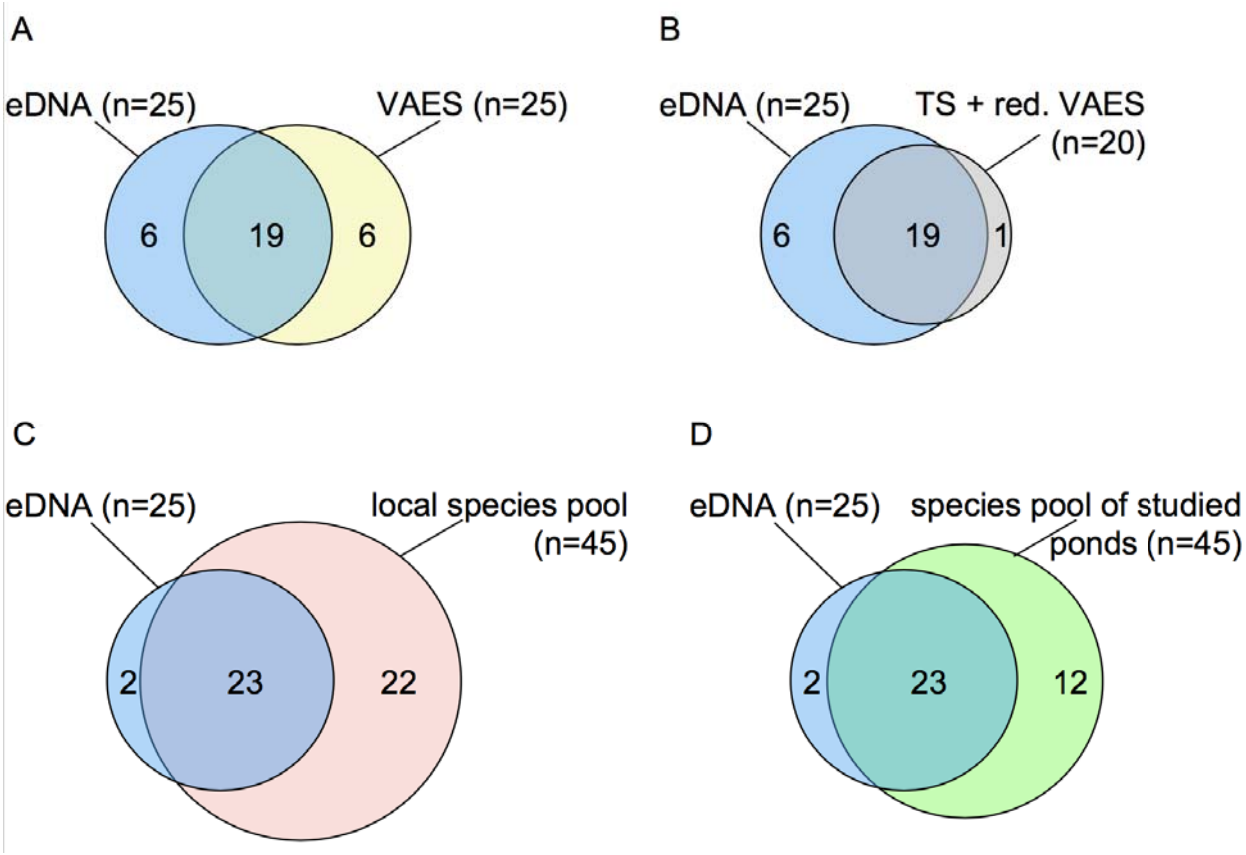

Fig. 2.

Comparison of species lists generated by eDNA (blue, n = 25) and A) visual and audio encounter surveys (VAES, yellow, n = 25), B) tadpole survey (TS) plus reduced VAES - only species with high eDNA detectability in ponds (see text for details; grey, n=20), C) complete local species pool (red, n = 45), and D) species pool of studied ponds based on long-term monitoring (green, n = 35).

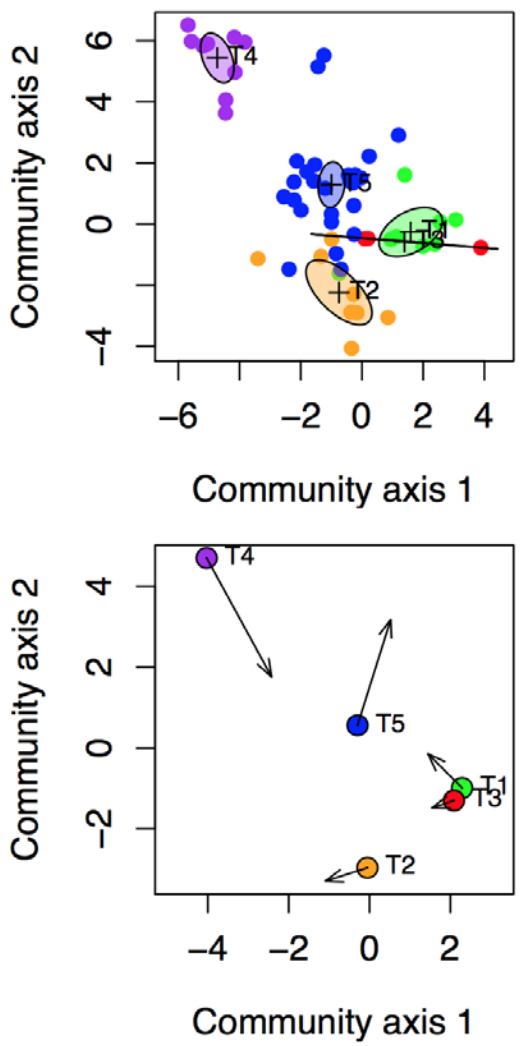

Fig. 3.

Comparison of ecological signal between visual and audio encounter surveys (VAES) and eDNA metabarcoding surveys. A - Latent variable model ordination of the pond samples according to the read numbers of species. Water samples from each pond were taken at three locations. For ponds T1-T4, three replicate samples at each location were filtered through two GFF (A,B), and one nylon filter (C), resulting in nine samples per pond. For pond T5, each filtration was replicated three times. Not all samples contained reads after the bioinformatic quality filtering and these samples are not shown on the ordination (see e.g. T3 - red). The ellipses represent 95% confidence intervals for the standard errors of the pond centroids

(marked with +). B - Non-metric multidimensional scaling plot of the five ponds (Jaccard distance), according to VAES species presence-absences. The arrows represent the Procrustes rotation of the VAES pond ordination and they target the group centroids of the latent variable model ordination.

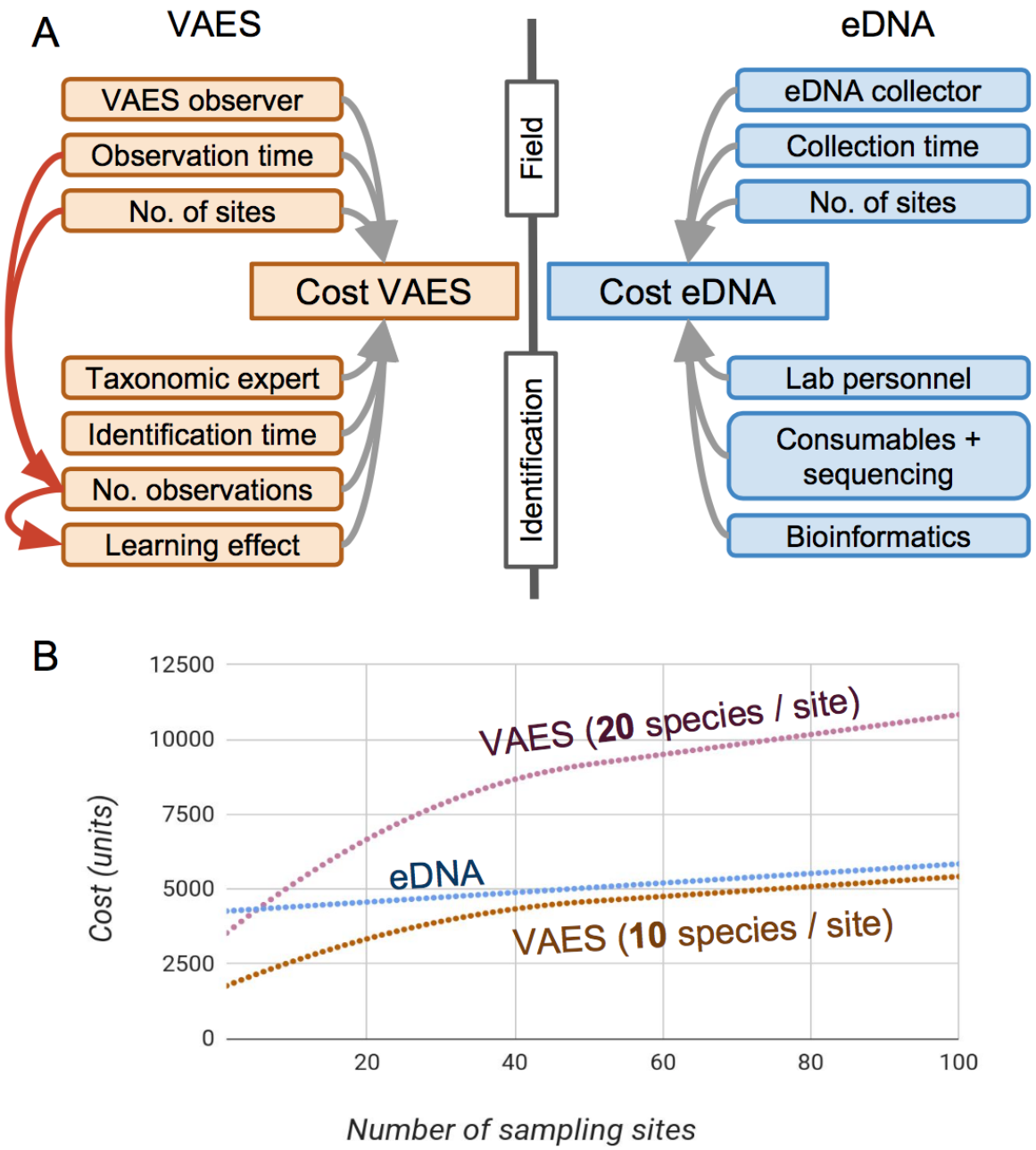

Fig. 4.

Cost comparisons between visual and audio encounter surveys (VAES) and eDNA metabarcoding surveys. A - Schematic overview of the visual and audio encounter survey (VAES) and eDNA cost models. Model parameters and calculations are accessible through FigShare (Balint_et_al_survey_cost_calculations.xlsx, doi: 10.6084/m9.figshare.5099842). B -

Cost comparisons of frog diversity surveys with eDNA, and two VAES scenarios (a low local species richness scenario - 10 species per site, and a medium-high species richness scenario - 20 species per site). eDNA surveys have high initial costs: laboratory and sequencing costs are considerable, regardless of the numbers of sites and species. A consequence is that eDNA surveys are not sensitive to increases in species numbers. This is in contrast with the VAES, where the costs are a direct function of species richness.