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Diversity within diversity: Parasite species richness in poison frogs assessed by transcriptomics

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ABSTRACT

Symbionts (e.g., endoparasites and commensals) play an integral role in their host's ecology, yet in many cases their diversity is likely underestimated. Although endoparasites are traditionally characterized using morphology, sequences of conserved genes, and shotgun metagenomics, host transcriptomes constitute an underused resource to identify these organisms' diversity. By isolating non-host transcripts from host transcriptomes, individual host tissues can now simultaneously reveal their endoparasite species richness (i.e., number of different taxa) and provide insights into parasite gene expression. These approaches can be used in host taxa whose endoparasites are mostly unknown, such as those of tropical amphibians. Here, we focus on the poison frogs (Dendrobatidae) as hosts, which are a Neotropical clade known for their bright coloration and defensive alkaloids. These toxins are an effective protection against vertebrate predators (e.g., snakes and birds), bacteria, and skin-biting ectoparasites (e.g., mosquitoes); however, little is known about their deterrence against eukaryotic endoparasites. With *de novo* transcriptomes of dendrobatids, we developed a bioinformatics pipeline for endoparasite identification that uses host annotated RNA-seq data and set of *a priori* parasite taxonomic terms, which are used to mine for specific endoparasites. We found a large community of helminths and protozoans that were mostly restricted to the digestive tract and a few systemic parasites (e.g., *Trypanosoma*). Contrary to our expectations, all dendrobatid frogs regardless of the presence of alkaloid defenses have endoparasites, with their highest species richness located in the frog digestive tract. Some of these organisms (e.g., roundworms) might prove to be generalists, as they were not found to be co-diversifying with their frog hosts. We propose that endoparasites may escape poison frogs' chemical defenses by colonizing tissues with fewer alkaloids than the frog's skin, where most toxins are stored.

1. Introduction

At any given time, many organisms carry several types of symbionts, i.e., organisms that 'live together' in an intimate association, including those that are harmful (i.e., parasites) and those that are not (e.g., commensals and mutualists). Parasites engage in long-term interactions with their hosts (Goater et al., 2013) and play an important role in their immune health, diversification, and population dynamics (Douglas, 2010). However, parasite biodiversity is usually described using morphological identification, amplification of conserved genes (e.g., deep-sequencing of 16S and 18S rDNA) and shotgun metagenomics (Srivathsan et al., 2016). These approaches may overlook complex interactions at the molecular level such as active parasitic infections, which can be revealed by gene expression of host immune responses and by gene expression of the parasites themselves. These limitations are evident in studies of tropical species, whose endoparasite diversity is mostly unexplored. Consequently, host transcriptomes constitute an underutilized resource of symbiont information; for instance, endoparasites can be identified by comparing non-host messenger RNAs (mRNAs) against annotated sequences in public databases. Here, we

Abbreviations: mRNAs, messenger RNAs; RNA-seq, RNA Illumina sequencing; COX1, cytochrome *c* oxidase subunit 1; COX3, cytochrome *c* oxidase subunit 3; HSP, heat shock protein; gGAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MYA, million years ago

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develop a bioinformatics pipeline to characterize a set of seven endoparasite groups (defined *a priori*) that infect many tropical ectotherms and are likely present in poison frogs.

Our knowledge of endoparasites in ectotherms is largely based on their identification from samples found in host vouchers preserved in museums, metagenomic data, and targeted PCRs for specific parasitic taxa (Baker, 1987). These approaches, with the exception of metagenomics, may underestimate the number of parasites unless the entire host environment is surveyed (Poulin and Morand, 2000). This limitation is especially evident in the case of protozoans and larvae, which do not preserve well or are microscopic (i.e., they require molecular identification such as PCR). Thus, detailed ecological characterizations of these organisms are rarely accomplished. In contrast, next-generation (e.g., Illumina) sequencing methods (e.g., RNA-seq and metabarcoding) generate the resolution necessary to characterize broad communities of endoparasites. RNA-seq can also provide a physiological perspective of the infective processes through differential gene expression within the host endoparasitic community.

For amphibians, there are only a few reports of endoparasites, including helminths, protozoans, and trypanosomes (Ferreira et al., 2008). Nevertheless, the underlying host-parasite interactions are largely unknown because they require expensive immunological and epidemiologic studies (Aho, 1990). This lack of knowledge is especially prevalent in frog clades from tropical regions, and only two surveys of poison frogs exist. In one survey, protozoans were observed in fecal samples of *Dendrobates auratus* and *D. pumilio* in both captive-breed and wild-caught populations (Poynton and Whitaker, 1994). This study revealed intestinal protozoa present in a third of the captive colony (i.e., mostly opalinids and coccidian cysts in 26% of 300 fecal samples) and an even higher prevalence of parasites in wild-caught frogs. In this last group, protozoans were represented by symbiont opalinids (*Zelleriella sp.*; present in both species of frogs), ciliates (*Nyctotheroides sp.*; not found in *D. pumilio*), flagellates (retortamonads, trichomonads, diplomonads; found in both species), and a blood parasite, *Trypanosoma sp.* (only in *D. auratus*).

In the second survey, fewer than ten helminth taxa were identified across the ~300 species of poison frogs (Campiao et al., 2014), compared to the 16 species reported in only two species of temperate frogs, *Rana catesbeiana* and *R. clamitans* (Muzzall, 1991). The apparent scarcity of endoparasites in tropical hosts is surprising because these organisms are expected to harbor more parasite species than their temperate counterparts (Chown and Gaston, 2000; Poulin, 1996). Therefore, the known endoparasite diversity and their species richness likely represents a small subset of a vastly unexplored symbiont biodiversity.

In this study, we assessed the richness of endoparasites in poison frogs (Dendrobatidae) using tissue-specific host transcriptomes. Most of these amphibians are diurnal, riparian, and reproduce throughout the year; these life history traits readily expose them to parasitic infestations such as myiasis (Hagman et al., 2005). Interestingly, certain dendrobatids are notable for an anti-predator adaptation known as aposematism (i.e., warning colors and chemical defense) (Santos et al., 2014, 2003). These toxic frogs have skin alkaloids that are distasteful (i.e., repellent) or poisonous to most predators and ectoparasites (e.g., mosquitoes); some even have antibiotic properties (Saporito et al., 2012; Weldon et al., 2006). However, poison frogs are unable to produce these toxins, which must be sequestered from a specialized toxic diet that includes ants and mites (Saporito et al., 2009).

The physiological process of alkaloid sequestration and accumulation in amphibian tissues is unknown (Daly et al., 1997; Santos et al., 2016). The current hypothesis states that poison frogs are able to extract alkaloids from toxic prey items (Daly et al., 1994a; Saporito et al., 2009) and bioaccumulate them in granular secretory glands in their skin (Neuwirth et al., 1979; Saporito et al., 2010). Likewise, new evidence is emerging on the molecular basis of alkaloid resistance of poison frogs, such as point mutations in highly conserved genes (e.g., ion channels and receptors) that encode for the intended molecular targets of these toxins (Tarvin et al., 2017a, 2016; Wang and Wang, 2017). Only recently, these alkaloids have been found in other poison-frog tissues, including muscle, liver and eggs (Grant et al., 2012; Stynoski et al., 2014). These observations suggest a systemic presence of alkaloids coupled with autoresistance in poison frogs, which might make their internal environment inhospitable to most endoparasites and reduce these amphibians' parasite load. However, this hypothesis has never been tested empirically.

The aims of our research are two-fold: (1) assess the species richness (number of taxa) of a set of seven groups of endoparasites, which are most likely found in tropical ectotherms (Goater et al., 2013), i.e., Amoebidae, Aschelminthes, Apicomplexa, Diplomonadida, Kinetoplastida, Opalinida and Platyhelminthes, using a novel bioinformatics and phylogenetic pipeline to mine through large host transcriptome databases; and (2) determine if anti-predator toxins are associated with a decrease in parasite species richness in toxic hosts. We additionally performed an exploratory analysis on the patterns of gene expression of endoparasite genes, and determined their general gene ontology including biological function and cellular location in the endoparasites (See Supplementary Materials Text).

2. Materials and methods

2.1. Tissue collection and RNA preservation

We collected 1–10 adult males from 15 species of poison frogs (Dendrobatidae) and two outgroup species (i.e., Arthroleptidae and Ranidae). The dendrobatids were collected within their natural distribution in Ecuador, while outgroups were collected in Cameroon (Supplementary Table S1). All animals were euthanized using 10% benzocaine w/w (Orajel® dental gel) by direct application into the mouth cavity. Tissues from brain, liver, intestinal wall and muscle were immediately dissected using forceps in a petri dish with 1 ml of RNAlater® (Life Technologies, CA). We flushed the digestive track tissues (i.e., intestine) with fresh RNAlater® multiple times to remove dietary contents. The tissue samples were submerged in 500 µL of RNAlater solution and incubated for 12–24 h at 0 °C. Then, the tissues were transported for 1–2 weeks at this temperature and subsequently stored long-term at −20 °C.

2.2. RNA extraction and library preparation

We extracted total RNA using the Trizol Reagent (Life Technologies, CA) and precipitated the RNA with a solution of 5 M ammonium acetate, glycogen and ethanol. The RNA pellet was resuspended in 250 µL of ddH2O and stored at −80 °C. The purification of mRNA was performed using the Poly(A) Purist kit (Life Technologies, CA). RNA samples were selected for library preparation if they had an RNA integrity number (RIN) ≥ 6.0, which was determined with a Bioanalyzer 2500 (Agilent Technologies, CA). The library preparation was done using a NEXTflex directional RNA-Seq dUTP-based kit (Bioo Scientific, TX) following the manufacturer's protocol. Briefly, the purified mRNA was fragmented and reverse transcribed using random hexamer primers. After the fragment end-repair, complementary DNA (cDNA) libraries were tagged with indexing barcodes at the sequence fragment ends; separate barcodes were used for each tissue within each species. Then, 10–18 cycles of PCR were used to enrich the barcoded cDNAs followed by quality and quantification analyses of the templates. The resulting cDNA libraries were purified using AMPure XP beads (Beckman Coulter, CA) to a total mean size of 350 bp. During the library preparation, the concentration and purity of the total RNA, mRNA, cDNA and sequencing libraries was determined using Nanodrop and Bioanalyzer 2500 equipment (Agilent Technologies, CA). Before sequencing, the number of reads was targeted to 15 million per library and 10–17 libraries were pooled in each of 5 lanes in a high-output run

mode of the Illumina HiSeq 2100 platform. Libraries were sequenced with ~100 bp paired-end using the Illumina sequencing services at the Genomic Sequencing and Analysis Facility (University of Texas at Austin). However, we make clear that the coverage depth for RNA-seq could not be calculated for our samples as coverage (c) equal to LN/G, where L is the read length, N is the number of reads and G is the haploid genome length as it is applied to genomic DNA (Sims et al., 2014). The main reason for this approach is that we do not know the exact or approximate size/length of the combined transcriptome of the host frog and its parasites. The output of the sequencer comprised of raw sequence data split by unique barcodes in FASTQ format files. For analyses of these data, we developed a bioinformatics pipeline (see Supplementary Fig. S1 schematic and Supplementary Data 1), which includes published software and nine custom R, Perl, and 'sbatch' scripts (see Supplementary Data 3).

2.3. De novo transcriptome assembly and quality assessment

Raw sequences were cleaned of barcodes, primers, over-represented oligomers and low-quality base calls using SnoWhite v 2.0.3 (Dlugosch et al., 2013). We used the following parameters: (1) the quality trimming threshold was set to a minimum of 20 Phred score under which the sequence reads were trimmed at their 3′ end; (2) the TagDust and SeqClean trimming extensions were activated to delete uninformative bases and sequences that matched the Bioo Illumina barcodes with a false discovery rate set to 0.01; (3) the terminal poly A/T repeats trimming parameters were activated with a minimum threshold length of 6 repeats on either sequence read end; (4) the terminal poly A/T trimming was allowed to look inside the cap number of terminal bases beyond 6 bases at the start of the terminal poly A/T with a minimum length of the contained repeats ≥ 10 bases; (5) the internal poly A/T trimming was activated if the repeat was ≥20 bases; (6) the minimum sequence length of cleaned sequence reads to keep was set to 50 bases; and (7) the cleaned sequence reads were written in FASTQ format. After cleaning, custom Perl scripts (Supplementary Fig. S1 and Supplementary Data 1) verified that the final FASTQ files contained only paired sequences.

The cleaned reads were used to reconstruct *de novo* transcriptome assemblies using Trinity v r2013-02-25 (Haas et al., 2013), which uses the de Bruijn graph algorithm to estimate a transcriptome without a reference genome. The parameters for the three Trinity modules (i.e., Inchworm, Chrysalis, and Butterfly) were set to the default values for strand-specific paired data (–SS_lib_type RF) and run on large memory nodes at the Texas Advanced Computing Center at the University of Texas at Austin. The resulting transcriptome assemblies were improved by removing duplicate contigs using a clustering approach at 95% sequence similarity. Finally, we mapped the sequence reads back to the updated transcriptome assemblies using the 'alignReads.pl' utility of Trinity v r2013-02-25 (Haas et al., 2013). This procedure determined the coverage of the assembled transcripts and also excluded all contigs with a mean coverage of < 5 because those might have an increased probability of being misassembled. Finally, we removed all contigs that were ≤200 bp, which we considered incomplete and uninformative.

2.4. Transcriptome annotation and endoparasite sequence selection

We constructed three datasets for the initial transcript identification. These reference libraries included annotated protein sequences at three different levels of taxonomic specificity: *Xenopus laevis*, Amphibia, and the Universal Protein Resource (UniProt) database. Our transcriptomes were annotated against these three reference libraries using the similarity BLASTx tool of BLAST v 2.2.29+ (Camacho et al., 2009). The threshold for the E-value was set to < 1E−20 for all BLASTx searches. The resulting transcripts that did not match either the frog genome or amphibian genes, but did match other eukaryotic organisms were written into a separate dataset for further analyses.

We used a custom Perl script (Supplementary Fig. S1 and Supplementary Data 1) to search for seven specific groups of eukaryotic endoparasites within these datasets. We included the following taxa: Amoebidae (*Acanthamoeba*, *Amoeba, Entamoeba*), Aschelminthes (*Ascaris*, *Brugia*, *Heterakis*, *Trichuris*), Apicomplexa (*Plasmodium*, *Eimeria*, *Cryptosporidium*, *Toxoplasma*), Diplomonadida (*Giardia, Spironucleus*), Kinetoplastida (*Leishmania*, *Trypanosoma*), Opalinida (*Opalina*) and Platyhelminthes (*Diphyllobothrium, Dugesia, Echinococcus, Fasciola, Opisthorchis, Schistosoma, Taenia*). Our selection of the above taxonomic groups aims to maximize the number of hits of unknown endoparasite transcripts with annotated endoparasite sequences (i.e., with defined gene product, genus and species) found in the NCBI public database and those most likely found in tropical ectotherm hosts (Goater et al., 2013). This method increases the possibility of finding an annotated sequence of close phylogenetic relatedness and appropriately identifying our unknown transcripts. We want to emphasize that our script is scalable and can include more taxonomic terms or collections of terms. For example, potential users of our bioinformatics pipeline can include species names rather than genera or phyla.

If the annotation for the unknown endoparasite transcript matched any of the seven taxonomic groups, our script copied the corresponding sequence to a new file. Then, the selected transcripts were compared against the NCBI database using tBLASTx tool of the BLAST v 2.2.29+ (Camacho et al., 2009) with an E-value set to 1E−20. This approach further refined the transcript annotation and excluded any amphibian genes and those derived from dietary sources (e.g., flies, ants or mites). Only those transcripts that passed this second filtering procedure were used for the phylogenetic and expression analyses. Because each tissue received a separate barcode (with the exception of brain and muscle, which shared indexes), we determined the compartmentalization of endoparasites within the host based on which tissues contained transcripts of each parasitic species. All of the endoparasite transcript sequences captured under the E-value 1E−20 threshold are provided in Supplementary Data 2; their annotations are provided in Supplementary Table S2.

2.5. Phylogenetic and host-parasite association analyses

The selected transcript sequences were compared against the NCBI GenBank nucleotide repository using BLASTn suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for 'somewhat similar sequences' with an E-value < 1E−20. GenBank sequences and our transcripts were aligned using an iterative approach (i.e., simultaneous alignment and tree estimation) with SATé-II v 2.2.2 (Liu et al., 2009). These alignments were used to estimate the phylogeny and taxonomic classification of each endoparasite. The molecular model, all the nucleotide alignments, and the corresponding accession numbers are provided in Supplementary Data 3. We performed maximum likelihood (ML) and Bayesian estimations of each phylogeny with Garli v 2.0 (Zwickl, 2006) and MrBayes v 3.4 (Huelsenbeck and Ronquist, 2001), respectively. The Bayesian approaches were performed using default settings for all priors. For the Markov Chain Monte Carlo (MCMC), six independent runs were included, each one with two chains of 75 million generations and a sampling rate of 1500 generations. The convergence was determined after 100,000 trees were discarded as burn-in. The node support was calculated with 400 nonparametric bootstrap searches for the ML approach and with posterior probabilities for the Bayesian estimation. Both methods gave similar tree topologies; thus, only ML phylogenies are provided.

We tested for an association between endoparasite species richness and chemical defense in poison frogs using the gut transcriptome dataset, which had the largest number of genes recovered from non-amphibian transcripts across all tissues surveyed (Table 1). We first standardized the total number of unique genes (i.e., those with one UniProt ID) per symbiont group for each frog species with gut transcriptome assembly by its total coverage (i.e., number of paired reads used for this

Table 1
Summary of gene and transcript statistics of the endoparasites found in dendrobatid frogs.

Host antipredator strategy	Tissue	Host species	Total host individuals	Mean reads per tissue	Total reads per tissue	Total reads from symbionts	Total % of reads symbionts	Total of reads symbionts per individual	Identified Genes	Length (bp)	BLAST alignment (bp)	BLAST % identity	BLAST E-value
Aposematic	Brain + muscle	7	48	42,739,743	299,178,204	352	1.176E−06	7.333	32	512 ± 243 (303, 1511)	155 ± 74	83.18 ± 17.00	1.875E−22
	Gut	7	48	39,574,876	277,024,132	13,024	4.701E−05	271.333	263	636 ± 428 (300, 3578)	192 ± 141	71.89 ± 18.75	4.962E−22
	Liver	6	44	39,674,395	238,046,372	4094	1.720E−05	93.045	67	699 ± 507 (302, 2714)	183 ± 117	77.29 ± 19.11	6.266E−24
Cryptic	Brain + muscle	6	35	45,806,896	274,841,376	20	7.277E−08	0.571	2	497 ± 261 (312, 681)	159 ± 96	84.94 ± 14.44	3.500E−26
	Gut	4	27	43,967,269	175,869,076	11,221	6.380E−05	415.593	213	673 ± 437 (301, 3687)	190 ± 132	72.53 ± 16.69	3.831E−24
	Liver	6	42	36,983,151	295,865,208	223	7.537E−07	5.310	18	525 ± 299 (308, 1571)	147 ± 63	87.87 ± 15.35	2.167E−21

assembly reconstruction). Total counts of unique genes were assigned to the corresponding groups: Apicomplexa, Diplomonadida, Platyhelminthes, Kinetoplastida, Aschelminthes and other protozoans (i.e., Amoebidae + Apicomplexa + Opalinea). Then, we evaluated whether the number of genes per group with the dendrobatid species was assigned to defended (i.e., toxic) or non-defended (i.e., non-toxic) categories. Given the non-normal distribution of parasite counts, we used non-parametric unpaired Wilcoxon-Mann-Whitney tests with an alpha level of 0.05. These analyses tested whether parasite species richness was associated with frog host chemical defense (i.e., anti-predator phenotype).

We tested for host-parasite co-diversification by estimating the degree of congruence between endoparasite and poison-frog tree topologies (Desdevises, 2007). For these tests, we used a poison-frog (host) phylogeny from a previous study on dendrobatid diversification (Santos et al., 2014) and two parasitic nematode phylogenies (COX1 and COX3 genes), for which we had the highest number of unique sequences (≥ 7). We used global-fit and individual host-parasite link tests to determine the degree of congruence between host and endoparasite topologies using the 'parafit' function implemented in the ape R-package v 3.1.4 (Paradis et al., 2004).

3. Results

3.1. Overview

A total of 40 *de novo* tissue transcriptomes were obtained from 15 poison frogs and two ranoid species (Fig. 1, Supplementary Tables S1 and S2). These transcripts were categorized by host antipredator strategy (i.e., aposematic/toxic or cryptic/undefended) and tissue type: liver, brain + muscle, and gut (Table 1). For our analyses, we assumed that each endoparasite transcript was evidence of a gene being expressed in the given host tissue. A list of obtained genes, functional annotations, and sequences are provided in Supplementary Table S2. Our bioinformatics pipeline identified a total of 595 endoparasite transcripts in assembled poison frog transcriptomes (i.e., ~0.00001% of all reads which were normalized per individual host; Table 1). Although this number is a conservative estimate of endoparasite transcriptional activity, these transcripts are likely to be accurately identified by our pipeline (i.e., BLAST E-values < 1E−20, identity to reference > 70% and alignment to reference > 150 bp). Given that our goal was to identify transcripts present from seven *a priori* endoparasite groups (see Section 2), we consider our results as a lower bound of the genes expressed by these organisms in the studied poison frogs. We emphasize that our method does not intend to reconstruct transcriptomes or identify all possible endoparasites present and capable of infecting the frog hosts. Consequently, some genes with lower levels of expression or those from metabolically inactive endoparasites might have remained undetected during the sequencing of the host transcriptome (see Supplementary Materials Text). Despite this caveat, we found a large number of eukaryotic endoparasites, most of which have never been reported in poison frogs or any other amphibian.

3.2. Endoparasite richness across host tissues

All seven groups of eukaryotic endoparasites included in our taxonomic input terms – Amoebidae (amoebas), Aschelminthes (parasitic nematodes), Apicomplexa (parasitic coccidia), Diplomonadida (parasitic flagellates), Opalinea (obligate commensals), Kinetoplastida (blood parasites), and Platyhelminthes (flatworms) – were found in poison-frog transcriptomes. The digestive tract harbored the highest species richness of these endoparasites (Fig. 1). Transcripts from all seven groups of endoparasites were identified in this tissue; yet, flatworms, diplomonads, and nematodes were the most prevalent. In contrast, the liver and brain + muscle transcriptomes were found to have mostly kinetoplastid endoparasites. These results suggest that the

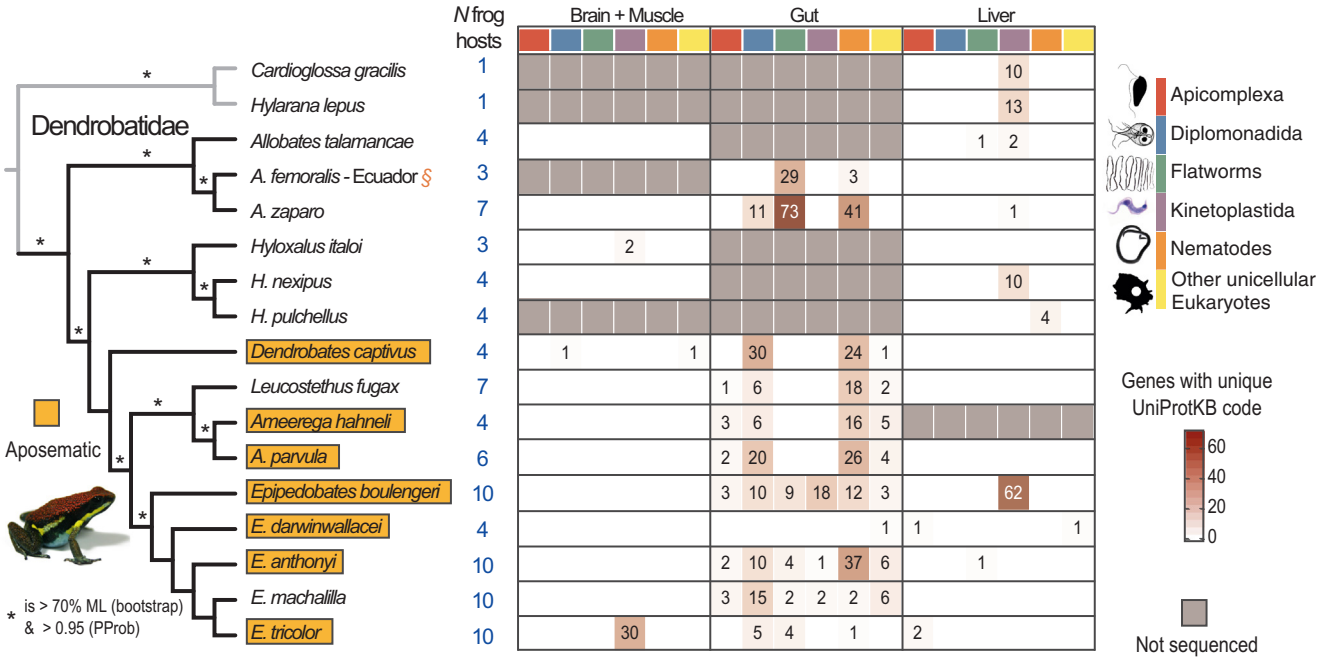

Fig. 1. Total number of unique genes for each host's transcriptome. Dendrobatids are divided based on their antipredator strategy: aposematic (with toxic skin alkaloids; orange boxes) and cryptic (non-toxic; without boxes). Not all tissues were sequenced for each host species (gray boxes) because the initial aim of the RNA-seq was to characterize the transcriptome of specific tissues and not their endoparasites. Significant node support of the phylogeny is indicated (*) for both bootstrap and Bayesian posterior probabilities (PProb). The orange section sign (§) for *Allobates femoralis* indicates that this species might be aposematic, but further evaluation is required (see discussion).

digestive tract, as expected, is where most endoparasites were metabolically active, whereas blood parasites were systemic (i.e., present in all tissues). The annotation of endoparasite transcripts also revealed a diversity of physiological functions (Supplementary Fig. S2 and Supplementary Table S2). However, most captured transcripts derived from highly expressed genes, e.g., those related to housekeeping, oxidative respiration, cell structure, and a few related to pathogenicity (Supplementary Materials Text and Supplementary Fig. S2).

3.3. Endoparasite richness versus frog host toxicity

Our analyses suggest that most dendrobatid species harbor diverse groups of endoparasites. We found diplomonads, kinetoplastids, and parasitic nematodes in both cryptic (i.e., undefended) and aposematic (i.e., toxic) frogs. In general, brain + muscle and liver had a lower quantity of endoparasite reads than the gut. Interestingly, the brain + muscle and liver of aposematic species had 4–16 times more endoparasite reads than the same tissues of cryptic frogs, which suggests that toxicity may influence endoparasite prevalence. The gut tissue had the highest number of parasite-derived transcripts (Fig. 1 and Table 1). However, these differences may be the result of sampling error (e.g., low RNA-seq depth or too few hosts sampled), and higher sequencing depth will be necessary to verify this pattern.

The higher number of endoparasite transcripts in the gut allowed for further exploratory analyses (Fig. 1 and Supplementary Table S2). No group of endoparasites in the gut transcriptomes was over or under represented in aposematic versus cryptic host species (i.e., hosts with or without toxic alkaloids, respectively; 263–213 genes in Table 1). Given the effectiveness of aposematism against vertebrate predators and bacteria (Santos et al., 2016), we tested if the number of unique genes per symbiont group was predicted by the host toxicity (i.e., 0 if host lacks defensive alkaloids versus 1 if otherwise). In this context, we considered that the host chemical defenses are effective against diverse endosymbionts, which yields fewer unique endoparasite genes captured during the host transcriptome sequencing. The results of our test for the association of each of group of symbionts and their host toxicity are as follows (all $N = 11$ and non-parametric unpaired Wilcoxon-Mann-Whitney tests): Apicomplexa ~ host toxicity $W = 16$, $p = 0.924$; Diplomonadida ~ host toxicity $W = 12.5$, $p = 0.714$; Platyhelminthes ~ host toxicity $W = 24$, $p = 0.104$; Kinetoplastida ~ host toxicity $W = 19.5$, $p = 0.353$; Aschelminthes ~ host toxicity $W = 16$, $p = 0.931$; and Amoebidae + Apicomplexa + Opalinea ~ host toxicity $W = 12$, $p = 0.645$. Our results show that the presence of endosymbiont taxa in the gut tissue did not differ between cryptic and aposematic frogs. Consequently, the prediction that chemical defenses in poison frogs might protect them against endoparasites is not supported, at least, for the digestive tract. However, these results do not directly represent endoparasite abundance, which could show a different pattern (see Section 4).

3.4. Phylogenetic diversity of identified endoparasites

We further explored the evolutionary relationships of the seven groups of endoparasites studied in the poison-frog hosts (Fig. 1). We selected and aligned genes with > 6 ortholog sequences from close taxonomic relatives (i.e., genetic distance < 0.2) derived from the NCBI repository (i.e., GenBank). The resulting sequence alignments and selected models of molecular evolution are provided in Supplementary Data 3. We provide a description of the phylogenetic analyses of each endoparasite group.

Protozoans identified in the digestive tract included amoebas, diplomonads, opalinids, and trypanosomes (Supplementary Table S2). Amoebae related to *Acanthamoeba* and *Psalteriomonas* were found in the gut transcriptomes of four lowland poison frogs: *Ameerega parvula*, *Dendrobates captivus*, *Epipedobates darwinwallacei* and *E. machalilla*. Diplomonad protozoans, closely related to the parasitic *Spironucleus* and *Hexamita* genera (Supplementary Fig. S3), were found in the guts of several poison frogs including *Allobates* spp., *Ameerega* spp., *Leucostethus fugax*, *Dendrobates captivus* and *Epipedobates* spp. Transcripts from *Opalina* spp. were found in the gut transcriptomes of *Epipedobates boulengeri* and *Ameerega hahneli*. These commensal heterokonta are often found in the lower intestines of adult amphibians (Goater et al., 2013). A few other rare protists were found in the digestive tract of *Ameerega* and *Epipedobates* species. These were related

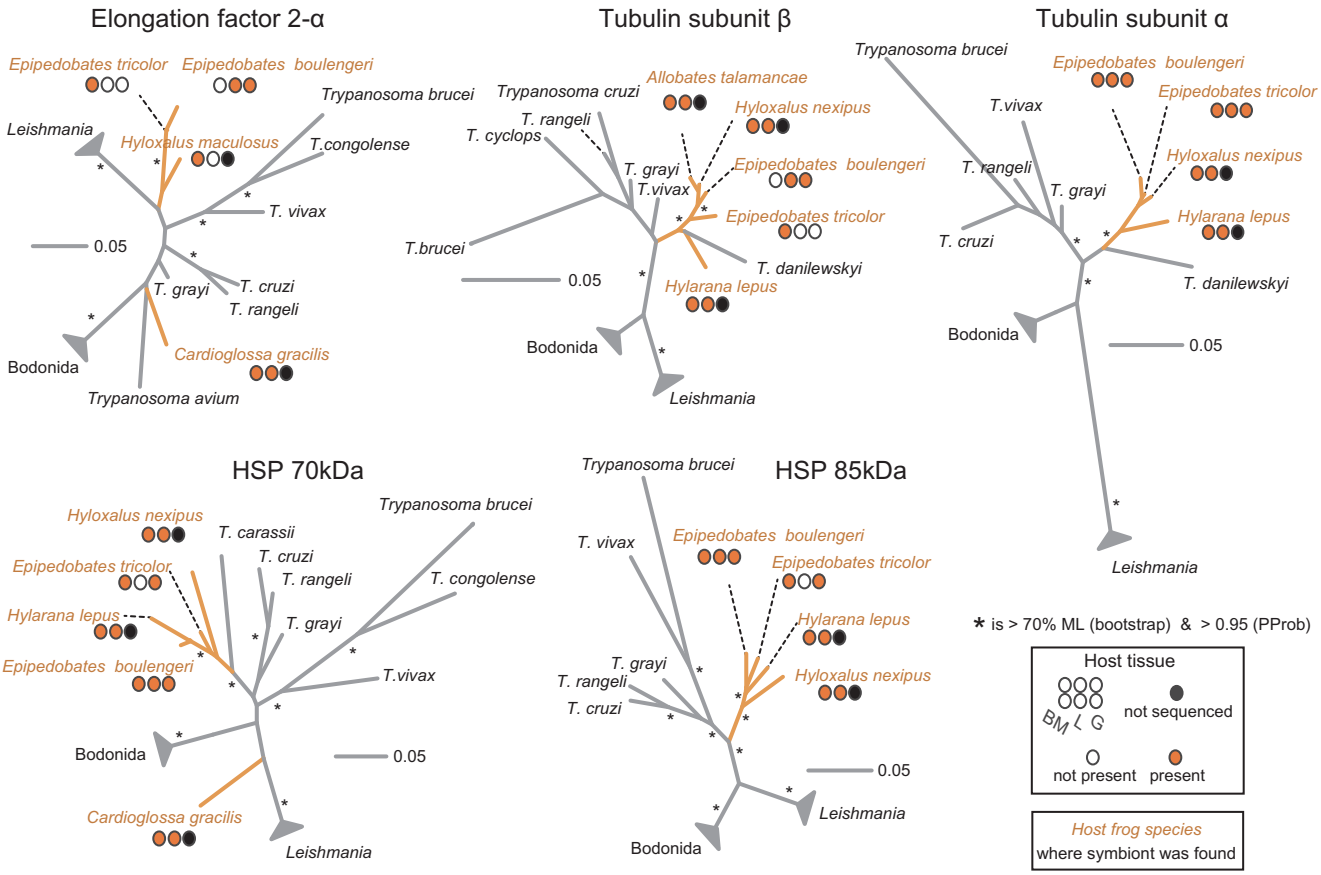

Fig. 2. Species richness of *Trypanosoma* transcripts found in poison frogs based on five genes. Orange branches represent lineages of *Trypanosoma* spp., and the associated tip names indicate the host species where these transcripts were found. Significant node support is indicated (*) for both bootstrap and Bayesian posterior probabilities (PProb). Dot labels indicate the type of host tissue (BM – brain and muscles, L – liver and G – gut or digestive tract).

to parasitic flagellated or ciliated protozoans (e.g., *Oxytricha* and *Tritrichomonas*).

The most diverse genus of unicellular endoparasites was *Trypanosoma*. These blood parasites were found in the liver and brain + muscle tissues of five dendrobatid species (Fig. 2). Phylogenies inferred with tubulin and heat shock protein HSP 70 kDa (Fig. 2B–D) placed all poison-frog kinetoplastids within an aquatic clade of *Trypanosoma*, specifically as the sister lineage to the parasitic *Trypanosoma* lineages found in fish, *T. carassii* and *T. danilewskyi* (Simpson et al., 2006). Similarly, a phylogeny based on the gGAPDH gene sequences (Supplementary Fig. S4) placed the kinetoplastid found in *Epipedobates boulengeri* as the sister group to the fish-platypus *Trypanosoma* lineage, but not within the clade of amphibian-specific parasites that include *T. rotatorium* of pond frogs (*Rana*) and *T. mega* of *Bufo* toads. However, we should emphasize that single gene phylogenies are known to be unreliable for trypanosomatids (Votypka et al., 2015). Consequently, our single-gene data support that the *Trypanosoma* spp. that parasitize poison frogs are evolutionary closer to fish pathogens, but multilocus or whole-genome data will further clarify whether or not these kinetoplastids are phylogenetically closer to known frog-associated *Trypanosoma* species.

The multicellular endoparasites found in poison-frog transcriptomes included trematodes (flatworms), nematodes, thorny-headed worms, and a few fungi. At least two trematode species were found in the digestive tract of six species of poison frogs: *Allobates femoralis*, *A. zaparo*, *Epipedobates anthonyi*, *E. boulengeri*, *E. machalilla*, and *E. tricolor*. We were able to use transcripts of aldolase and paramyosin from these trematodes for phylogenetic analyses because there were > 6 reference species with these genes in the NCBI database (Supplementary Fig. S5). A cestode (tapeworm) transcript found in an *Allobates* frog host was most closely related to sequences of *Hymenolepis diminuta*, a cyclophyllid cestode (i.e., a rat tapeworm). In contrast, the closest taxon to cestode transcripts found in *Epipedobates* hosts is related to *Schmidtea mediterranea*, a free-living planarian. This result suggests either that the *E. boulengeri* trematode is an undescribed parasite or that these transcripts came from an ingested planarian, which has never been reported as a prey item of poison frogs (Santos and Cannatella, 2011). Overall, the few tapeworm transcripts found in poison frogs suggests a lower number of tapeworms compared to other ectotherms (e.g., lizards and fish), which supports the known rarity of these endoparasites across amphibians (Aho, 1990).

Roundworms (i.e., nematodes) were common in the poison frogs studied. Nine of the 15 species of dendrobatids sequenced carried one nematode species, and *Ameerega parvula* carried two (Fig. 3). Most endoparasites were identified from gut tissues, which suggests that these organisms were adults at the time of collection and that the dendrobatids were likely their final hosts. *Contracaecum* is the most common genus of parasitic nematodes across amphibians (Campiao et al., 2014) and, based on our estimated phylogenies (Fig. 3), it is the most likely genus of nematodes found in the studied frogs. However, more molecular information is necessary to rule out other parasitic nematodes (e.g., *Physocephalus*). It is interesting to note that nematodes lack voltage-gated sodium channels (Girard et al., 2007), which are common targets of poison-frog chemical defenses (Santos et al., 2016). Thus, this molecular characteristic may explain the nematode prevalence in our study.

Other multicellular endoparasites included thorny-headed worms and fungi. The thorny-headed worms (Acanthochephala) were found in the digestive tract of *Leucostethus fugax* and *Epipedobates machalilla*; these worms were closely related to *Southwellina hispida* (a bird

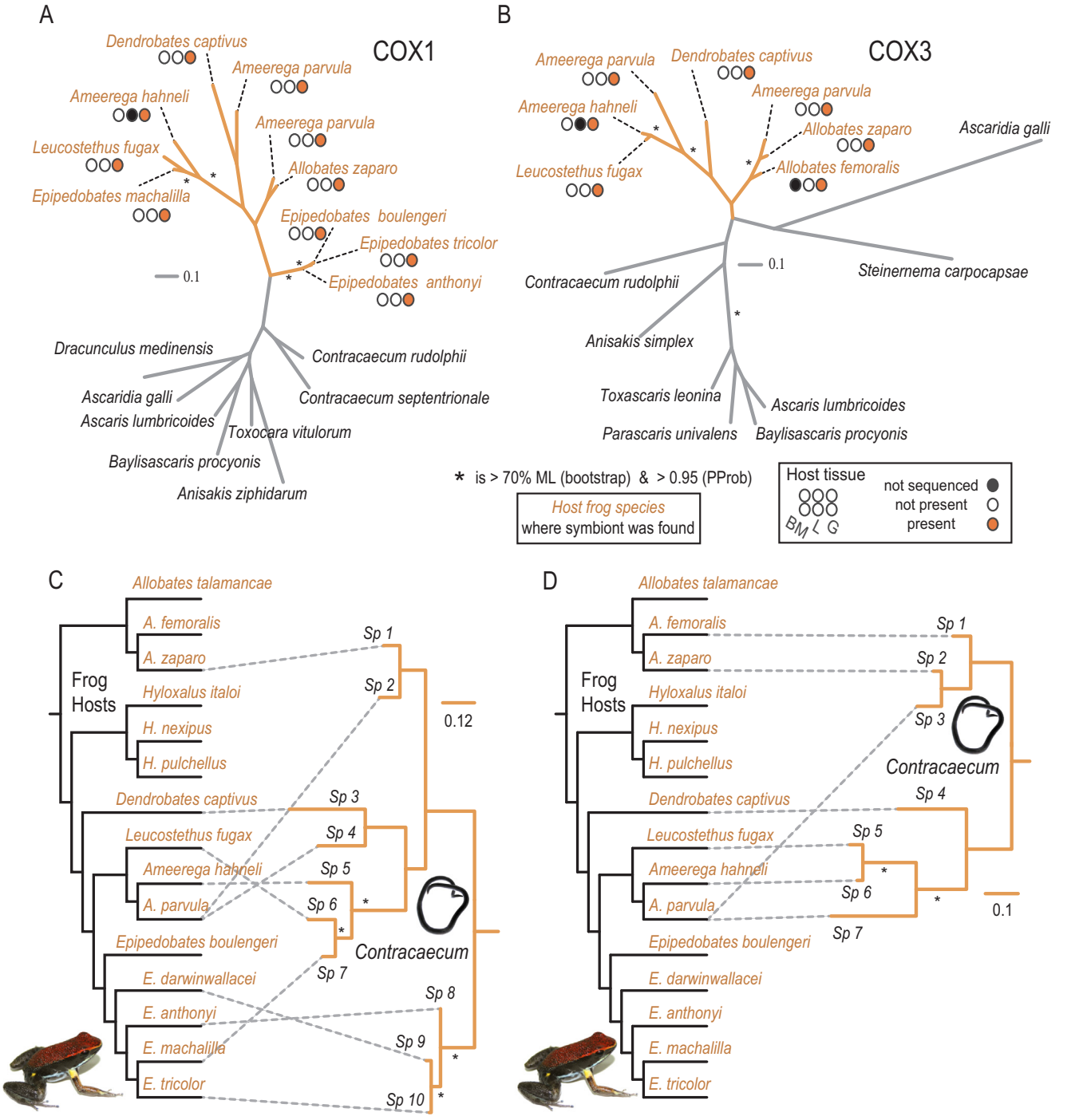

Fig. 3. Species richness of parasitic nematode transcripts found in poison frogs based on sequences of cytochrome *c* oxidase subunits COX1 (A) and COX3 (B). Nematode-host mirror phylogenies are also indicated (C and D) and dashed gray lines indicate the corresponding connections between taxa. The orange branches represent the lineages of nematodes, and the associated tip names indicate the host species where these transcripts were found. Significant node support is indicated (*) for both bootstrap and Bayesian posterior probabilities (PProb). Dot labels indicate the type of host tissue (BM – brain and muscles, L – liver and G – gut or digestive tract).

parasite; data not shown). Of several fungal transcripts, one was the pandemic chytrid fungus (*Batrachochytrium dendrobatidis* or *Bd*; Supplementary Fig. S6) found in *Leucostethus fugax* and *Epipedobates anthonyi*. In these frogs, we found *Bd* transcripts that had a significant similarity to the calmodulin gene of the *B. dendrobatidis* JAM 81 strain (i.e., BLAST E-value < 1E−89, 92.9% identity and a length of 455 bp). This reference transcript was isolated from *Rana muscosa* in the Sierra Nevada Mountains of California (Rachowicz et al., 2006). In our analyses, the calmodulin transcripts were found in the digestive tract of the host frog and not in the skin, where most chytrid infections are detected. However, this result is not unusual because most frogs molt and eat their shed skins, which could be infected with chytrid.

3.5. Host-endoparasite co-speciation

Given the high species richness of parasitic nematodes, we tested for co-speciation between nematodes and their poison-frog hosts. Our tests were based on nematode phylogenies derived from cytochrome *c* oxidase subunits COX1 and COX3 (Fig. 3). We were unable to reject that speciation events were independent between nematodes and their frog

hosts (COX1: ParaFitGlobal = 4496.959, p = 0.386; COX3: ParaFitGlobal = 2830.777, p = 0.131). However, based on our tests of individual host-parasite associations using COX3 topology, only the nematode species found in *Allobates femoralis* and *A. zaparo* (2 of 7 nodes) appear to have co-speciated with both frog hosts ($p < 0.03$). Overall, these results imply that parasitic nematodes rarely co-speciate with their dendrobatid hosts, which suggests that these roundworms are generalists (Aho, 1990).

4. Discussion

Complex habitats and multiple-host systems can increase the species richness of symbiont communities. Characterizing the ecology of such communities requires detailed knowledge of interactions between a host and its symbionts, especially endoparasites. However, most descriptions of these interactions are limited to fortuitous findings of preserved parasites in host specimens. More recent studies target specific endoparasite communities using PCR amplicons or metagenomic analyses, which might only capture the taxon identity and not necessarily the interactions with their host (e.g., active infection or other metabolic processes). Likewise, certain taxa of endoparasites might be difficult to reveal if these organisms are microscopic, do not preserve well in host tissues, or if appropriate probes are unavailable to bind to their DNA (e.g., endoparasite genome unavailable; non-specific primers). Hence, the endoparasite diversity in many taxa including amphibians is likely vastly underestimated, and detailed host-parasite interactions are probably unknown (Goater et al., 2013; Poulin, 1996). Here, we demonstrate that genomic methods and bioinformatic pipelines can provide a first look at the species richness and function of symbionts (e.g., endoparasites and commensals) derived from host transcriptomes.

Our bioinformatics pipeline for endoparasite identification uses host annotated RNA-seq data and set of *a priori* parasite taxonomic terms, which are used to mine a host transcriptome. The identified endoparasite transcripts are compared with reference genes from close taxonomic organisms available in public databases (e.g., GenBank) and further characterized using phylogenetics to place them among their closest relatives. However, some limitations still exist in our approach, such as low sequence depth and the inability to distinguish different endoparasite species from a collection of transcripts of different genes. We propose several remedies in the Supplementary Materials Text to address these limitations. Overall, our pipeline provides a general characterization of endoparasite diversity that, with enough sequencing depth (e.g., more starting reads) and global collection of endoparasite taxonomic terms, will enable the identification of most endoparasites present in any host tissue.

4.1. Endoparasites identified from poison-frog transcriptomes

Most amphibians are exposed to parasites in both aquatic and terrestrial habitats as they transition from larvae to adults. Large metazoan endoparasites (e.g., helminths) can infect tadpoles through their food and water where these parasites live as larvae or cysts. In contrast, protozoans tend to infect adults via hematophagous invertebrate vectors. For example, phylogenetic studies of *Trypanosoma* found in amphibians revealed complex networks of vectors such as leeches, mosquitoes and sand flies (Ferreira et al., 2008). However, little is known about the ecology of these trypanosomes beyond their presence in mostly dipteran vectors and their few amphibian hosts. Gene expression data in combination with sequence barcoding can add to the studies of co-speciation between host and trypanosome physiology (Douglas, 2010; Votypka et al., 2015). Our analyses of gene expression (Supplementary Materials Text) revealed only highly expressed genes (e.g., housekeeping and oxidative respiration). Nonetheless, we were able to identify a remarkable diversity of metabolic functions, which we consider evidence of infective processes of endoparasites in tropical amphibians. For these reasons, our bioinformatics approach provides new insights into the diversity of interactions between poison frogs and their endoparasites using transcriptomic, phylogenetic, and gene expression analyses.

We determined that most poison-frog species have a diverse community of associated parasites. Many of these organisms were identified in the host gut transcriptomes; fewer were present in the liver, brain, and muscle tissues. The amoeboid protozoans that infected poison frogs were related to organisms common in soil and aquatic environments. For example, some transcripts were similar to those of *Acanthamoeba*, an opportunistic pathogen found in humans, yet most species of amoebas found in amphibians are assumed to be commensals (Blumer et al., 2007). Other common protozoans were diplomonads related to *Spironucleus* and *Hexamita*, which are adapted to nearly completely anaerobic environments of the digestive tract of vertebrates (Lloyd and Williams, 2014). We found transcripts of *Spironucleus* spp. related to *Spironucleus salmonicida* in five genera of poison frogs. This diplomonad causes severe systemic infections in salmonid fish (Xu et al., 2014), but we did not find evidence of systemic infection or dermal ulcerations in the studied frogs (JCS field observations).

One interesting group of commensal protozoan found in the gut of poison frogs was *Opalina*. Our results are the second report of these protozoans in dendrobatids, which were previously identified, using microscopy, as the opalinid *Zelleriella sp.*, in samples of the large intestine of *D. auratus* and *D. pumilio* (Poynton and Whitaker, 1994). Opalinids are unique among unicellular symbionts because their sexual reproduction is tied to the amphibian host life cycle (Goater et al., 2013). In adult frogs, opalinids are located in the large intestine where they release micro- and macroflagelated gametes, which are subsequently ingested by tadpoles. These gametes travel to the intestine where they fuse and form a new zygocyst (i.e., an encysted zygote), which is released by the tadpole and consumed by adult frogs. Then, this zygocyst travels to the frog's large intestine and completes the *Opalina* life cycle by developing into a reproductive state. We found opalinid transcripts in the digestive tract, as expected for adult *Opalina*. This result is interesting for poison frogs because their parental care is intensive (Weygoldt, 1980). For example, parents (usually males) take care of the clutches and developing tadpoles by constantly moistening their clutch with urine, which is accumulated in their cloaca (i.e., an extension of the large intestine), a likely habitat for *Opalina*. We propose that the parental care of poison frogs facilitated the colonization and diversification of these *Opalina*.

Our results support that the true diversity of *Trypanosoma* in ectotherms is widely underestimated. Most phylogenetic studies of these kinetoplastids are skewed towards veterinary or medically important strains (Simpson et al., 2006). Consequently, blood parasites might be common in vertebrates, but their prevalence in poison frogs and in other ectotherms is unknown. Our results constitute the first report of *Trypanosoma* in amphibians using RNA-seq approaches. Most blood parasites are highly specialized and produce a debilitating effect on the host's ability to reproduce, although they are not necessarily lethal. For example, trypanosome-infected birds show no significant decline in reproductive performance (Siikamaki et al., 1997). Similarly, we did not observe detrimental effects of *Trypanosoma* infections in the poison frogs obtained for this study; nevertheless, long-term studies are necessary to determine any impact on the fitness of the infected individuals.

These results added two more frog families (i.e., Dendrobatidae and Arthroleptidae) to the reported anuran hosts of *Trypanosoma*, which already includes Bufonidae, Craugastoridae, Hylidae, Leiuperidae, Leptodactylidae and Ranidae (Ferreira et al., 2015, 2008). All of these frog families are species-rich and have tropical distributions. In these regions, the three major groups of vectors of *Trypanosoma* are leeches, sand flies, and mosquitoes (Psychodidade and Culicidae). Amphibians are exposed to leeches as tadpoles and to dipteran vectors (e.g., mosquitoes) as adults. Interestingly, frogs appear to attract many of these

airborne vectors during their mating behavior because dipterans can use frogs' advertisement calls as beacons (de Silva et al., 2014; Meuche et al., 2016). We propose that the main vectors of *Trypanosoma* in poison frogs are sand flies and mosquitoes because poison frogs are essentially terrestrial and diurnal. These dipterans might also use the frogs' mating calls to locate them.

Studies of helminth diversity in amphibians are limited. Only 11 species of these parasites have been identified in poison frogs (Campiao et al., 2014). Of these species, seven are nematodes (four found as adults), three are acanthocephalans (all found as larvae), and one is a cestode (also found as adults). Laboratory experiments have shown that some of these trematodes (e.g., *Ribeiroia ondatrae*) are associated with an increased prevalence of deformities in anurans (Johnson et al., 1999). However, we did not find evidence of deformities or obvious population declines in the poison frogs surveyed for this project (JCS field observations). These results suggest that the nematode discovered might have modest impact on poison frogs, although more studies are necessary to assess long-term population declines.

Most of the nematodes that parasitize amphibians have direct life cycles suited for terrestrial or arboreal hosts (Anderson, 2000). Hence, we can hypothesize that the most common parasitic nematodes of poison frogs are probably monoxenous (single host) and generalists such as *Cosmocerca*. This genus has a global distribution, and its members mostly infect terrestrial amphibians of tropical regions (Campiao et al., 2014). Our results (Fig. 3) support that *Cosmocerca* parasitizes dendrobatid frogs, which includes both aposematic (i.e., *Ameerega* and *Dendrobates*) and cryptic frogs (i.e., *Allobates* and *Leucostethus*). As expected for generalist parasites, we were unable to find any common pattern of co-speciation between *Cosmocerca* and their frog hosts. However, we did find one instance of host-parasite pair association between *Cosmocerca* and *Allobates* frog species. These dendrobatids live in swampy areas, which may be a particularly favorable type of habitat for *Cosmocerca*.

We found evidence of infection by *Batrachochytrium dendrobatidis* (i.e., the chytrid fungus) within the transcriptomes of *Leucostethus fugax* and *Epipedobates anthonyi*. Both of these species are relatively abundant in the lowlands of Ecuador, and the populations where the frogs were collected did not show evidence of decline. However, the chytrid fungus has been suggested to be a causal agent of the catastrophic declines in several amphibian populations across the globe (Lips et al., 2006). Moreover, the transcript obtained from a chytrid in the poison-frog hosts was of calmodulin, a calcium-signaling protein expressed during active chytrid infections (Vieira and Gomes, 2010). Consequently, we cannot rule out that chytridiomycosis is active in both *L. fugax* and *E. anthonyi* host populations.

4.2. Aposematism effectiveness against endoparasites

Poison frogs are well known examples of aposematism (Santos et al., 2014). These amphibians have skin alkaloids that protect them against vertebrate predators, bacteria and arthropods (Santos et al., 2016; Weldon et al., 2006). Therefore, one could predict that these skin alkaloids also protect the frogs against parasitic infections. However, our data do not support this prediction because all tested dendrobatids, chemically defended or not, had endoparasites. This pattern may be due to the fact that dendrobatid defenses involve chemicals that are concentrated primarily in the skin, which may not be effective against endoparasites that originate from ingestion of infected prey items or inoculation by hematophagous vectors. Nevertheless, some evidence suggests that dendrobatids and other alkaloid-defended frogs (e.g., *Melanophryniscus*) accumulate some level of alkaloids in tissues other than skin (e.g., muscle, liver, and oocytes) (Grant et al., 2012; Stynoski et al., 2014), which would ensure that these endoparasites are exposed to some amount of these alkaloids.

Our initial hypothesis regarding alkaloid defense against endoparasites may be more complicated if we account the plasticity of the alkaloid sequestration phenotype among individuals and populations of poison frogs (Amezquita et al., 2017; McGugan et al., 2016; Saporito et al., 2006, 2007). Recent lab experiments suggest that some dendrobatid species previously thought to lack chemical defenses (e.g., *Allobates femoralis*) might actually be chemically defended and aposematic. However, whether the methods used in these experiments – injecting toxins into mice and tracking their behavior – is a valid test to evaluate toxicity is unclear (Weldon, 2017).

The case of *A. femoralis* (Fig. 1) is particularly interesting for the following reasons: (1) this taxon has a Pan-Amazonian distribution and likely represents a complex of species that diverged ~8 MYA (Grant et al., 2017; Santos et al., 2014, 2009); (2) previous reports that failed to detect or revealed only trace alkaloids in Ecuadorian *A. femoralis* populations (Daly et al., 1987; Darst and Cummings, 2006; Darst et al., 2006, 2005) contradict those of alkaloid toxicity in mice found recently in the Brazilian *A. femoralis* populations (Amezquita et al., 2017); (3) skin extracts of several species related to the Ecuadorian *A. femoralis* taxon (e.g., *A. zaparo*; its sister species) appear to be non-toxic to lab mice (Darst and Cummings, 2006; Darst et al., 2006) or lack skin alkaloids (e.g., *A. talamancae*; *A. kingsburyi*) (Daly et al., 1994b; Darst et al., 2005; Santos and Cannatella, 2011); and (4) the Ecuadorian *A. femoralis* taxon appears to lack key amino acid substitutions that would make this species immune to alkaloids sequestered from their diet (Tarvin et al., 2017a, 2016). Crucially, the recent report that indicates that the Brazilian *A. femoralis* populations might be toxic to mice (Amezquita et al., 2017) contradicts the evidence of a similar alkaloid toxicity experiment in mice for the Ecuadorian *A. femoralis* populations (Darst et al., 2006). This conundrum is further complicated because the report on the Brazilian *A. femoralis* populations does not provide any identification of the actual frog alkaloids, only its toxicological effects in lab mice. Without direct assays to identify these toxic substances, such as using gas chromatography-mass spectrometry, it is difficult to determine whether changes in mice behavior are associated with alkaloids or other defensive compounds present in the Brazilian *A. femoralis* individuals.

These observations are especially relevant for our study because our transcriptomic data are from individuals of an Ecuadorian *A. femoralis* population, which is purportedly non-toxic and not tested by Amezquita et al. (2017). Considering the available evidence, we can conclude, for the moment, that at least some members of the *A. femoralis* complex (including those used in our study) might be non-toxic and unable to sequester alkaloids. However, we also consider that it is possible that more species of dendrobatids that are currently considered to be non-toxic might actually have skin alkaloids. For example, *E. boulengeri* is a cryptic frog and was previously considered to be non-toxic, yet some of its populations showed substantial amounts of alkaloids (Cipriani-Avila and Rivera, 2009). However, this result was expected given the phylogenetic position of *E. boulengeri* among the alkaloid-bearing *Epipedobates* clade, which has diverged < 5 MYA (Santos et al., 2009; Tarvin et al., 2017b). We suggest that further investigations of aposematism and parasitism in dendrobatids should address the cryptic phylogenetic diversity of the host frogs, account for the plasticity of the alkaloid-sequestration phenotype, and use more sensitive alkaloid profiling techniques (e.g., gas-chromatography) rather than mouse-based assays of toxicity (Weldon, 2017).

Given the knowledge of how poison-frog alkaloids function, are endoparasites even a potential target of alkaloid poisoning? Our current answer is yes. Most common poison-frog alkaloids (e.g., pumiliotoxins) target a subset of ion channel proteins that are present in most animals with a basic nervous system (Santos et al., 2016; Tarvin et al., 2016), including most multicellular endoparasites such as helminths. This intuition is also supported by evidence that certain antihelmintics can effectively target endoparasite ion channels (Greenberg, 2014; Wolstenholme, 2011). Therefore, to our knowledge, it is possible that poison-frog alkaloids can indeed target some multicellular endoparasites, if these have nervous systems with target ion channels. We

propose two hypotheses: (1) poison-frog alkaloids provide a broad-spectrum protection against predators, including some multicellular endoparasites, and (2) an arms race between endoparasite resistance and poison-frog defenses may exist. In either case, toxic alkaloids might not be effective against resistant or immune endoparasites. Consequently, our results constitute the first step towards a general understanding of parasitism in poison frogs and suggest that endoparasites are coevolving with poison frogs.

Chemical defenses in amphibians have been shown to be effective against endosymbionts (Santos et al., 2016; Weldon et al., 2006). However, we did not find that endoparasites were absent or less prevalent in alkaloid-defended dendrobatid hosts. It is possible that we found no such pattern because the number of parasite genes used in our analyses probably does not directly represent the number of individual parasites, in part because most endoparasites vary dramatically in gene-expression patterns. For instance, metabolically active endoparasites might express more genes and in higher abundance compared with quiescent organisms. Moreover, parasites with complex (e.g., multiple duplicated gene families with many isoforms) or larger coding genomes might have a higher number of transcripts. Therefore, gene-transcript abundance alone cannot be assumed to correlate with endoparasite abundance. Given these caveats, we consider that our approach measures only endoparasite richness (i.e., number of taxa) rather than abundance. It is also possible that our categorization of defenses in dendrobatid poison frogs is too broad to identify more specific associations between parasites and particular alkaloids. Moreover, it is plausible that the current state of knowledge regarding the phylogenetic distribution of alkaloid defenses in poison frogs is incomplete as exemplified in the case of the *Allobates femoralis* complex.

Poison frogs are highly social amphibians, and their interactions facilitate the exchange and colonization of parasites (Nunn et al., 2003). These mechanisms might be more efficient in dendrobatids that live densely near streams, congregate to reproduce, and have complex reproductive behavior, including parental care. We hypothesize that tadpoles are infected with helminths in contaminated water habitats where parasites might be present as eggs, while terrestrial adults are infected with blood parasites (e.g., *Trypanosoma*) during courtship because their mating behavior attracts skin-biting vectors (Meuche et al., 2016). A weak association between mating behavior (e.g., advertisement call) and parasite load has been demonstrated in one poison-frog species, *Dendrobates* (*Oophaga*) *pumilio* (Pröhl et al., 2013). Hence, parasite infections may influence mate selection in dendrobatids, but experimental evidence (e.g., females rejecting males with higher parasite loads) is necessary to evaluate this hypothesis.

5. Conclusion

Most studies of amphibian biology do not address endoparasite species richness. Therefore, our knowledge of the extent of co-speciation between frogs and their parasites is limited. However, comparative studies can address the diversity of endoparasites using large-scale host transcriptomic datasets. Here, we developed a bioinformatics pipeline to mine an annotated host transcriptome and characterize the species richness of *a priori* determined groups of endoparasites. We use this pipeline to identify members of seven groups of endoparasites most likely found in tropical ectotherms, such as poison frogs. We propose that endoparasites might escape these frogs' chemical defenses by colonizing tissues with lower concentrations of alkaloids than the skin, where the bulk of toxins are stored. Alternatively, certain endoparasites may be intrinsically less sensitive to these toxins, owing to their distinct nervous systems, or they could have evolved physiological adaptations to resist poison-frog toxins. Future research may reveal how the varying levels of toxic alkaloids influence the already diverse endoparasite community associated with aposematic amphibians.

Fieldwork permissions statement

Collections were done under Ecuadorian permits 003-11 IC-FAU-DNB/MA, 001-13 IC-FAU-DNB/MA and CITES 32/VS.

Ethics statement

All the animal manipulations followed protocols: Harvard University IACUC # 12-10-1 and California Academy of Sciences IACUC # 2014-2).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.03.015.

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