Apicomplexa primers amplify *Proteromonas* (Stramenopiles, Slopalinida, Proteromonadidae) in tissue and blood samples from lizards

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Abstract

Microscopy has traditionally been the most common method in parasitological studies, but in recent years molecular screening has become increasingly frequent to detect protozoan parasites in a wide range of vertebrate hosts and vectors. During routine molecular screening of apicomplexan parasites in reptiles using the 18S rRNA gene, we have amplified and sequenced *Proteromonas* parasites from three lizard hosts (less than 1% prevalence). We conducted phylogenetic analysis to confirm the taxonomic position and infer their relationships with other stramenopiles. Although our phylogeny is limited due to scarcity of molecular data on these protists, our results confirm they are closely related to *Proteromonas lacertae*. Our findings show that unexpected parasites can be amplified from host samples (blood and tissue) using general procedures to detect hemoparasites, and stress that positive PCR amplifications alone should not be considered as definitive proof of infection by particular parasites. Further validation by sequence confirmation and thorough phylogenetic assessment will not only avoid false positives and biased prevalence estimates but also provide valuable information on the biodiversity and phylogenetic relationships of other parasitic organisms. More generally, our results illustrate the perils of general diagnosis protocols in parasitological studies and the need of cross-validation procedures.

Keywords

Lizard, hemogregarine, 18S rRNA gene, molecular screening, microscopy, hemoparasites

Introduction

Traditionally, microscopy has been the gold-standard in parasitological studies and there has been considerable effort to identify and classify the diversity of protist biota in reptiles, namely intestinal flagellates (e.g., Wood 1935; Wenrich 1947; Janakidevi 1961a, b; Krishnamurthy 1968; Telford 1970; Dollahon and Janovy 1971; Saratchandra and Narasimhamurti 1980a, b; Telford and Bursey 2003) and hemoparasites (e.g., Amo *et al.* 2005, Austin and Perkins 2006, Roca and Galdón 2010). Recently, molecular methods have been increasingly used for the detection of protists in both wild and domestic animals. Compared to more traditional approaches, such as microscopy, molecular methods benefit from good specificity and sensitivity in detecting parasitic infections (e.g., Rubini *et al.* 2005, Merino *et al.* 2009), especially at low parasitemia levels (Moody 2002), they are

straightforward and are relatively low cost and not time consuming, thus allowing the screening of large numbers of samples in a reproducible manner. Moreover, molecular data provides additional and valuable information on the diversity of parasitic organisms and their genetic relationships. However, some studies estimate infection prevalence solely based on PCR screening without sequence confirmation (e.g., Ujvari *et al.* 2004, Vardo *et al.* 2005), which could lead to biased estimates since other related or unrelated organisms may be amplified. In recent years, specific primers for the amplification of Apicomplexa in reptiles have been developed, and have been shown to be very useful, considerably increasing the sensitivity of detection (e.g., Ujvari *et al.* 2004, Harris *et al.* 2011, Maia *et al.* 2011, Tomé *et al.* 2012). In this study we demonstrate that these primers can also amplify other protists, namely *Proteromonas* (Stramenopiles, see Cavalier-Smith and Chao 2006).

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At present, limited molecular data is available for the genus *Proteromonas*; there are currently only data from *P. lacertae* for the mitochondrion genome (Pérez-Brocal *et al.* 2010) and from rRNA genes (Leipe *et al.* 1996, Hoevers and Snowden 2005). Phylogenetic studies have shown that *Proteromonas* and other members of the Slopalinida are related with the genus *Blastocystis*, a group of protozoan parasites of medical and veterinary importance (e.g., Tan 2004, Kostka *et al.* 2007).

Materials and methods

DNA was extracted from blood or tail tissue using standard High Salt methods (Sambrook *et al.* 1989), DNeasy Blood & Tissue kit (Qiagen), or Speedtools Tissue DNA extraction kit (Biotools). Primers used were HepF300 and HepR900 (Ujvari *et al.* 2004) targeting part of the 18S rRNA gene and PCR cycling consisted of 94°C – 30 sec, 60°C – 30 sec, 72°C – 1 min (35 cycles) (see Harris *et al.* 2011 for more details). Negative and positive controls were run with each reaction. PCR products were electrophoresed on 2% agarose gels, stained using sybr safe or gel red and visualized using a UV-transilluminator. The positive PCR products were purified and sent for direct sequencing (Macrogen Inc). All amplifications were sequenced in both directions. Sequence chromatograms were checked manually and assembled using Geneious v. 5.6.4 (Biomatters Ltd.). We then performed a similarity analysis using the Basic Local Alignment Search Tool (BLAST) to find the best match (E-value $\leq 10^{-8}$) for the sequences against published sequences in GenBank (<http://www.ncbi.nlm.nih.gov>). All sequences obtained matched the single *P. lacertae* 16S-

like rRNA gene sequence available (U37108) with 98% similarity. The new sequences have been submitted to GenBank (accession numbers JX276957-JX276959).

To assess phylogenetic relationships, these sequences were aligned with the following sequences retrieved from GenBank: 1) representatives of Proteromonadidae family: *P. lacertae* (U37108), *Karotomorpha* sp. (DQ431242 and DQ431243); 2) representatives of the Opalinidae family: *Protoopalina japonica* (AB175929), *Protoopalina intestinalis* (AY576545), *Opalina ranarum* (AF146089); 3) *Blastocystis* sp. (AY135412) and *Blastocystis pythoni* (AY266472), which were used as outgroups for rooting the phylogenetic trees following Kostka *et al.* (2007). Alignments were performed using the MUSCLE algorithm (Drummond *et al.* 2012) using default parameters implemented in Geneious 5.6.4. The final alignment consisted of 11 sequences of 575 bp. Two different phylogenetic analyses (Maximum Likelihood and Bayesian Inference) were conducted. Maximum Likelihood (ML) analysis included random sequence addition (100 replicate heuristic searches), and support for nodes was estimated using the bootstrap technique (Felsenstein 1985) with 1000 replicates, using PhyML 3.0 (Guindon *et al.* 2010). The AIC criteria carried out in jModeltest 0.1.1 (Posada 2008) were used to choose the model of evolution employed (GTR+I+G). Bayesian analysis was implemented using Mr. Bayes 3.1 (Huelsenbeck and Ronquist 2001) with parameters estimated as part of the analysis. The analysis was run for 5×10^6 generations, saving one tree each 1000 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded. Remaining trees were combined in a 50% majority consensus tree (Fig. 1). Upon collection, slides were air-dried and later fixed with methanol and stained with Giemsa following Telford

Fig. 1. Bayesian Inference tree of the new *Proteromonas* sequences obtained in this study together with sequences retrieved from GenBank. Support for the Bayesian and for ML analysis are given above and below the nodes, respectively

Table I. Comparison between *P. lacertae* sequence retrieved from GenBank (U37108) together with the Apicomplexa specific primers used in this study. Mismatches are indicated in bold

	HEP300F	HEP900R
Primer	GTTTCTGACCTATCAGCTTTTCGACG	GTCAGAGGTGAAATTCTTAGATTG
U37108	GTTTCTG CC CTATCAGCTTTTCGATG	GTCAGAGGTGAAATT CAAG GATT A

(2009). Microscopy was conducted at 400x and 1000x magnification using an Olympus CX41 microscope with an in-built digital camera (SC30).

Results

During routine molecular screening of Apicomplexa parasites in reptiles, we have amplified and sequenced a portion of the *Proteromonas* 18S rRNA gene in three reptile samples out of around 600 reptile samples analysed, less than 1% prevalence. These samples belong to three different lizard genera: two from the family Lacertidae (tail tissue from *Acanthodactylus erythrurus* from Spain, sample ALC5, and blood drop from *Darevskia armeniaca* from Armenia, sample K18910), and one from the family Gekkonidae (blood drop from *Pristurus carteri* from Oman, sample IBES7122). Of these samples, a blood smear was only available for IBES7122, in which flagellate stages of *Proteromonas* could be identified (Fig. 2). A comparison between U37108 and the Apicomplexa specific primers used in this study (HEP300F and HEP900R) showed only two and four mismatches, respectively (Table I). Our phylogenetic analysis using sequences obtained in this study together with previously published sequences of other related Stramenopiles, confirms these *Proteromonas* 18S rRNA gene sequences to

be closely related to *Proteromonas lacertae* (see Fig. 1). In the conserved regions of the alignment, the three sequences obtained in this study differ from the *P. lacertae* retrieved from GenBank (Leipe *et al.* 1996) by one (K18910), two (ALC5) and four positions (IBES7122). Additionally, there is a short (20 nucleotides) hyper-variable region in which all samples differ considerably in sequence and length, and where is variability within the single host sample for K18910 and IBES7122. The sequence from GenBank also has an ambiguity (A or G) in the same region.

Discussion

In this study, we show that sequences of unexpected parasites can be amplified from host samples (blood and tissue) using general procedures to detect hemoparasites. We have amplified and sequenced a segment of the *Proteromonas* 18S rRNA gene from three lizard host genera using specific primers for the amplification of Apicomplexa. The taxonomic position of these sequences was further confirmed by our phylogenetic analyses. Although our phylogeny is limited due to scarcity of molecular data on these protists, the relationships are congruent with those obtained in previous published works (Kostka *et al.* 2004, 2007; Nishi *et al.* 2005; Hoevers and Snowden 2005). The position of *Karotomorpha* and of opalinids, agrees

Fig. 2. Microscopy picture of the blood smear of sample IBES7122 from *P. carteri* showing flagellate stages of *Proteromonas*. Scale bar = 0.01 mm

indeed with the topology obtained by Kostka *et al.* (2007) that showed the family Proteromonadidae as paraphyletic, with the genus *Karotomorpha* being more closely related to opalinids than to the genus *Proteromonas*.

Interestingly, *Proteromonas* is a genus of obligate anaerobe protists that live as commensals in the colon of lizards. However, in this study we detected *Proteromonas*-like organisms in blood and tail-tissue samples. One hypothesis is that faecal runoff (containing cysts) could be present on the skin of the reptile tissue sampled. Indeed, lizards often defecate when being processed, so there is the possibility that the parasites present in the faeces may be transferred to the blood drops or remain in the skin of the tail tip. Alternatively, cysts or flagellate stages of these organisms may be naturally present in the blood stream (the latter shown in Fig. 2), or occasionally the trophic stages may be able to invade tissues. Other unexpected parasites have also recently been reported from blood samples, including *Eimeria*, which is also typically detected in faecal samples (Harris *et al.* 2012). This clearly warrants further investigation.

Regardless of the source, our study demonstrates the perils of molecular diagnosis protocols in parasitological studies and the need of cross-validation procedures between and within methodologies. First, the primers used were originally designed in a study where 100 pythons were found to have 100% prevalence of presumably *Hepatozoon* spp. based solely on PCR (Ujvari *et al.* 2004). However, these primers are actually less specific and can amplify other distantly related protists (e.g., *Proteromonas*) or even fungi (Tomé *et al.* 2012), as well as various apicomplexans such as *Eimeria* and *Sarcocystis* (Harris *et al.* 2012), which could pose an important bias for infection estimates. Nonetheless, the fact that primers are less specific may also open up new possibilities in providing relevant information on other parasitic organisms, as long as these are confirmed through sequencing and phylogenetic analyses. It therefore should be stressed that it is important not to rely solely on PCR amplifications when screening for infections; PCR products should be sequenced to confirm identification of the parasite detected. This will not only avoid false positives, if other unrelated parasites are amplified, but also has the potential to provide valuable information on the diversity and evolutionary relationships of poorly-known or less common parasites.

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