Scanning for apicomplexan parasites (Suborder Adeleorina) in five Holarctic anuran species

Parasites are ubiquitous, but a poorly known component of biodiversity, with estimates of 0.1 % of species described for some groups (Morrison 2009), while other scientists simply note "we have no credible way of estimating how many parasitic protozoa … exist" (Dobson et al. 2008). Yet parasites are of dual interest for conservation biologists, both for their adverse impact on hosts with parasite-driven declines in wildlife becoming increasingly common (Pedersen & Fenton 2007), but also because, in monophagous parasites, this relationship with the hosts increases their risk of co-extinction. Indeed, models suggest co-extinction may be the most common form of biodiversity loss (Dunn et al. 2009). Given the difficulties in alpha-taxonomy of most groups, and the lack of parasitologists (Šlapeta 2013), molecular analyses, much like the common "DNA barcoding" approach, may be an extremely valuable first assessment for some parasites. Clearly, integrative approaches combining morphological and molecular data would be preferable given time (Will et al. 2005), but molecular examination has many advantages, besides being relatively quick and easy. For a start, sequence data is invaluable for placing parasites in a phylogenetic framework. Furthermore, many times host samples are collected for other purposes, such as host genetic assessments, but these same samples are available for the study of parasites.

Apicomplexan blood parasites are a prime example of a group where this molecular approach can be useful. Assessments of these have in the past focused on groups with strong anthropogenic interests due to health reasons, such as *Plasmodium*, or groups with significant economic impact. Others, such as *Hepatozoon*, despite being the most common blood parasite of reptiles (Telford 2009) gained little attention. Molecular analysis however, using primers specific for a section of the 18S rRNA gene, has greatly clarified phylogeny (e.g., Barta et al. 2012; Harris et al. 2012), identified infections in new host orders (e.g., Pinto et al. 2012), and indicated that predator-prey trophic pathways may be widespread in some cases, such as between lizards and snakes (Tomé et al. 2013). At the same time, other parasites such as Stramenopiles were detected (Maia et al. 2012a). Tests using specific primers can be misleading as some parasites may not be detected (Zehtindjiev et al. 2012). Thus, it is necessary to investigate this aspect in different host groups.

Amphibians as a whole have suffered global declines, and parasites are a key driving factor (Beebee & Griffiths 2005). Although the role of the fungus *Batrachochytrium dendrobatidis* is widely accepted (e.g., Daszak et al. 2003), testing for other parasites is needed. Various infections by *Hepatozoon* species have been identified in amphibians using microscopy (e.g., Stenberg & Bowerman 2010), but also molecular examination of an introduced population of frog, *Pelophylax perezi* (López-Seoane, 1885), from the Azores islands (Harris et al. 2013b). On the other hand, examination of *Bufo calamita* Laurenti, 1768, from the Iberian Peninsula did not detect apicomplexan parasites (Harris et al. 2013a).

Table 1: Species analyzed for parasites, the number tested using alternative source material (tissue or blood), and the number examined on slides under the microscope.

Species	Tissue (toe)	Blood	Slides
Pelobates cultripes (CUVIER, 1829)	45	---	---
Pelophylax saharicus (BOULENGER, 1913)	52	30	26
Hyla meridionalis BOETTGER, 1874	---	11	7
Amietophrynus mauritanicus (SCHLEGEL, 1841)	30	8	10
Bufotes boulengeri (LATASTE, 1879)	4	7	8
Bufo bufo (LINNAEUS, 1758)	5	---	---

The aim of the present study was to scan for apicomplexan parasites a number of amphibians from Europe and North Africa, using samples that had been primarily collected for studies of the host. Because of this, in most cases blood smears to assess the prevalence under the microscope were not available. An established PCR protocol amplifying a region of the 18S rRNA gene, successfully applied to amphibians from the Azores, and reptiles from this region (e.g., MAIA et al. 2012b) was adopted in the present study. It is known that the primers used can also detect organisms other than Apicomplexa (e.g., MAIA et al. 2012a; TOMÉ et al. 2012). Therefore, all successful PCR amplifications were sequenced, since a positive PCR amplification cannot be assumed to indicate the presence of a particular parasite. Results based on molecular and visual methods were compared to assess the efficiency of detection using molecular methods.

Tissue samples (toe clips) were taken from 136 amphibians belonging to six species, from various localities in the Iberian Peninsula, the Balearic Islands and Morocco, and stored in 96 % ethanol (Table 1). The taxonomy of many amphibians in this region is in a state of flux, but here the authors follow BEUKEMA et al. (2013). For a smaller number of specimens, blood drops stored on Whatman paper (56 specimens, 4 species) and blood smears (51 specimens, 4 species) were also available (indicated in Table 1). All samples are part of the DB collection housed at CIBIO, UP. Blood smears were air-dried, fixed with methanol, stained with diluted Giemsa (one part Giemsa solution, nine parts distilled water) for 55 minutes, and examined using an Olympus CX41 microscope with a built-in digital camera (SC30) (Olympus, Hamburg, Germany). Several photomicrographs per slide were taken at 400 fold magnification and stitched using Cell^B software (basic image-acquisition and archiving software, Olympus, Münster, Germany). In case parasites were not detected after ca. 10 minutes of examination, the slides were considered negative. When parasites were identified, even in very low numbers, slides were scored as positive. For some examples, intensity of infection was estimated based on numbers of parasites per 3,000 red blood cells.

DNA was extracted using standard high salt methods (SAMBROOK et al. 1989). Detection of blood parasites was made using PCR reactions with the primers HepF300 and HepR900 (UJVARI et al. 2004), which were designed to amplify *Hepatozoon* parasites. Conditions of the PCR are detailed in HARRIS et al. (2011). A subset of 47 samples was also tested with the HEMO1 and HEMO2 primers (PERKINS & KELLER 2001). Although these are known to be less efficient at detecting *Hepatozoon* relative to the Hep primers (MAIA et al. 2012b), they were used and evaluated in case they gave results in the present amphibian study. Negative and positive controls were run with each reaction. PCR products were analyzed by electrophoresis in 2 % agarose and visualized by Gel Red staining and UV transillumination. The positive PCR products obtained were purified and sequenced by a commercial sequencing facility (Macrogen Europe, The Netherlands). Positive PCRs were compared against the public database GenBank, using a BLAST similarity search.

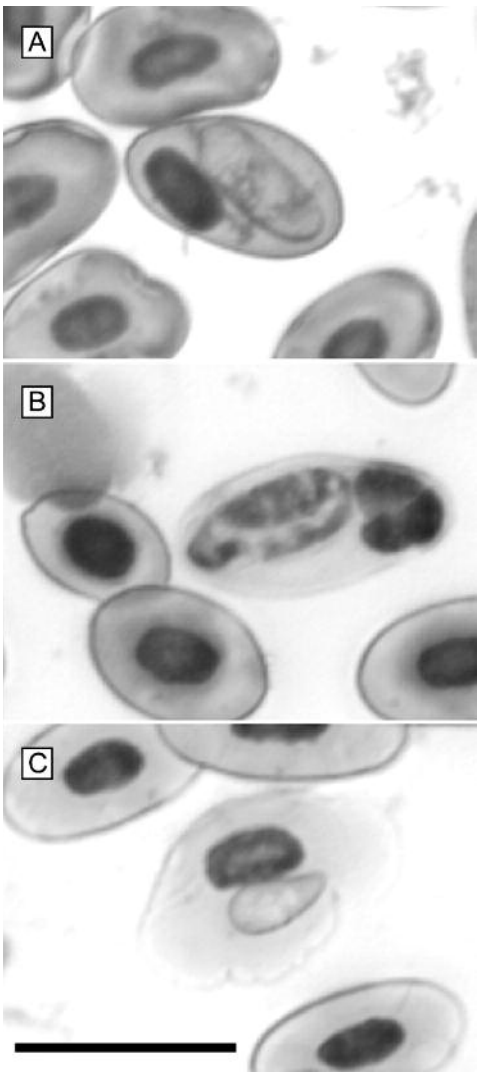

Figure 1:. Samples of red blood cells showing hemogregarine infections in A - *Amietophrynus mauritanicus* (SCHLEGEL, 1841), and B - *Pelophylax saharicus* (BOULENGER, 1913), both of which failed to amplify hemogregarine DNA using the screening protocol employed. C - Infection by presumed *Dactylosoma ranarum* in *Pelophylax saharicus*. Length of the scale bar corresponds to 20 μm.

Initially, analysis was carried out on the toe clips, as these are most readily available from genetic studies of the vertebrate hosts. Only a single positive sample (out of 136) for apicomplexan parasites was found with the Hep primers, and the BLAST comparison revealed 99.8 % similarity with *Dactylosoma ranarum* from a *Pelophylax* synkl. *esculentus* (LINNAEUS, 1758) host from Corsica, France (Accession numbers HQ224957 and HQ224958, BARTA et al. 2012). Only a single nucleotide differed from the GenBank sequences over 613 bp of compared sequence data. Testing of blood drops with both primer sets also failed to detect any positive infections. However, when blood slides were visually examined, various positive samples infected with hemogregarines were identified (Fig. 1). These were found in two host species, *Pelophylax saharicus* (BOULENGER, 1913) and *Amietophrynus mauritanicus* (SCHLEGEL, 1841), and at least for *P. saharicus* prevalence was high (12 in 26 [= 46 %] of individuals screened). With regard to the intensity of infections, samples visually diagnosed positive for low parasitaemia levels of *Dactylosoma* (0.1 % infected red blood cells) were detected by the molecular method, whereas even heavier hemogregarine infections (up to 3 % infected cells in the sample of *A. mauritanicus* - DB15569) were scored as negative in the molecular approach.

Scanning for parasites using conserved primers has the potential to greatly improve knowledge on parasite diversity and distribution. Like all molecular approaches, it can be improved by adoption of an "integrated" approach, as barcoding alone can be misleading (WILL et al. 2005).

For most studies of *Hepatozoon*, the Hep primers (UJVARI et al. 2004) have proven to be efficient at detecting not only divergent *Hepatozoon* lineages (e.g., HARRIS et al. 2012), but also various other parasites (e.g., TOMÉ et al 2013). Most studies indicated that using these primers, identification efficiency was at least as high, or even higher, when compared to the visual assessment of blood smears (e.g., O'DWYER et al. 2013). Yet, in the present study, they failed to amplify DNA of any hemogregarines, which were clearly identified in at least two of the host species examined. One could argue that amphibian toe clips are not ideal sources of material for studies of these parasites. Also, in blood drops stored in

Whatman paper, the authors did not detect any parasites, which suggests that the source of the host sample was not the issue. Rather, the primers used failed to amplify the DNA of these hosts' hemogregarine lineages. This is unexpected given that the primers worked with *Hepatozoon* from *P. perezi* in the Azores Islands, but not *P. perezi* or the related *P. saharicus* in the Iberian Peninsula and North Africa. It is however possible that if several different *Hepatozoon* lineages can be found in the same intermediate host species (e.g., TOMÉ et al. 2013), some common hemogregarines may not be detected by these primers, whereas other lineages which occasionally occur are amplified.

The identity of the hemogregarines identified under the microscope remains unclear. *Hepatozoon* are the most common hemogregarines of reptiles and are common in amphibians, with forty-two species associated with amphibian hosts (SMITH 1996), and since in much of the earlier literature the hosts were not identified below the generic level, previous identifications of *Hepatozoon* from toads and frogs from North Africa may well correspond to *P. saharicus* and *A. mauritanicus*.

The finding of *Dactylosoma* in one sample of *P. saharicus* increases the list of parasites that have now been detected using molecular methods with these primers. The very high similarity with the parasite from GenBank of *P.* synkl. *esculentus* indicates close vicinity to *D. ranarum*, and implies low intermediate host specificity for this parasite. Indeed, *D. ranarum* is thought to occur in amphibians from Africa and North and South America, Asia and Europe (BARTA 1991). Genetic data from hosts from other regions and other genera will be invaluable in assessing this further.

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