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RESEARCH NOTE

Molecular survey of parasites in introduced *Pelophylax perezi* (Ranidae) water frogs in the Azores

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Abstract

Water frogs, *Pelophylax perezi*, that are introduced in the Azores, were screened for parasites using PCR primers known to amplify Apicomplexa parasites, and using nematode-specific primers. With the former, three different organisms were detected: *Hepatozoon*, a trichodinid protozoan ciliate and a possible Stramenopile. Using the latter set of primers, a single unknown spirurid nematode was also detected. Phylogenetic analyses indicate that *Hepatozoon* detected within amphibian hosts appear to form a clade, although relationships of these parasites do not match the vertebrate intermediate host phylogeny. Regarding the possible Stramenopile, it is unclear whether this organism was actually present on the amphibian or in the water on the surface of the tissue sample. Our findings highlight that many different organisms can be detected with these primers and that they can be used to screen introduced host populations to detect parasites that have been brought with them.

Keywords

Amphibian, hemogregarine, *Hepatozoon*, *Trichodina*, stramenophile, Eimeriidae, nematode, spirurid, Apicomplexa, 18S rRNA

It is known that the introduction of exotic species can drive disease emergence through the extension of parasite ranges and the facilitation of host switching to new naïve hosts. In this fashion the most serious diseases in wild aquatic animals in Europe can be traced to non-native species (Peeler *et al.* 2011). Various examples are known where amphibian introductions are associated with the emergence of new parasites. Myxozoa parasites were apparently introduced with cane toads to Australia, and now infect many endemic frog species (Hartigan *et al.* 2010). Introduction of amphibians has also been implicated in the spread of the fungus *Batrachochytrium dendrobatidis* (Daszak *et al.* 2003). Such introductions can be particularly devastating on islands, where taxa that were previously isolated from many continental competitors and diseases can rapidly decline in numbers following such introductions.

The Azores are a remote archipelago of nine volcanic islands in the North Atlantic Ocean. There are no native non-volant vertebrates, but several have been introduced, such as the lacertid lizard *Teira dugesii* and the newt *Triturus carnifex* (Malkmus 1995). However, the most widespread species is the frog *Pelophylax perezi*, a native of the Iberian Peninsula and South of France, which occurs on all of the islands. Possibly because of the lack of native species with which these introductions might compete, little attention has been paid to their possible influence. To our knowledge, no parasite surveys have been performed on these amphibians, despite the possibility of host switching to freshwater fish that have been introduced for aquaculture.

Identification of parasites has been traditionally conducted based on morphological features. However, molecular techniques are now known to be a powerful tool to identify various parasite groups in reptiles and amphibians, including nematodes (Jones *et al.* 2012, Perera *et al.* 2012) and many different blood parasites (Harris *et al.* 2012). In particular, non-lethal tissue samples, such as tail or toe clips, can be used, which avoids sacrificing the host.

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The aim of this study was to identify parasites from introduced populations of *P. perezi* in the Azores. Parasites were screened using primers already known to amplify various Apicomplexan parasites (Harris *et al.* 2012), and using nematode-specific primers (Floyd *et al.* 2005). All positives were sequenced and when possible compared with sequences retrieved from GenBank in a phylogenetic analysis, both to identify them as precisely as possible, and to shed further light on their evolutionary relationships.

Tissue samples (toe or tail clips) were collected from *Pelophylax perezi* from three different Azorean islands, and stored in 96% ethanol (7 from São Miguel, 6 from São Jorge and

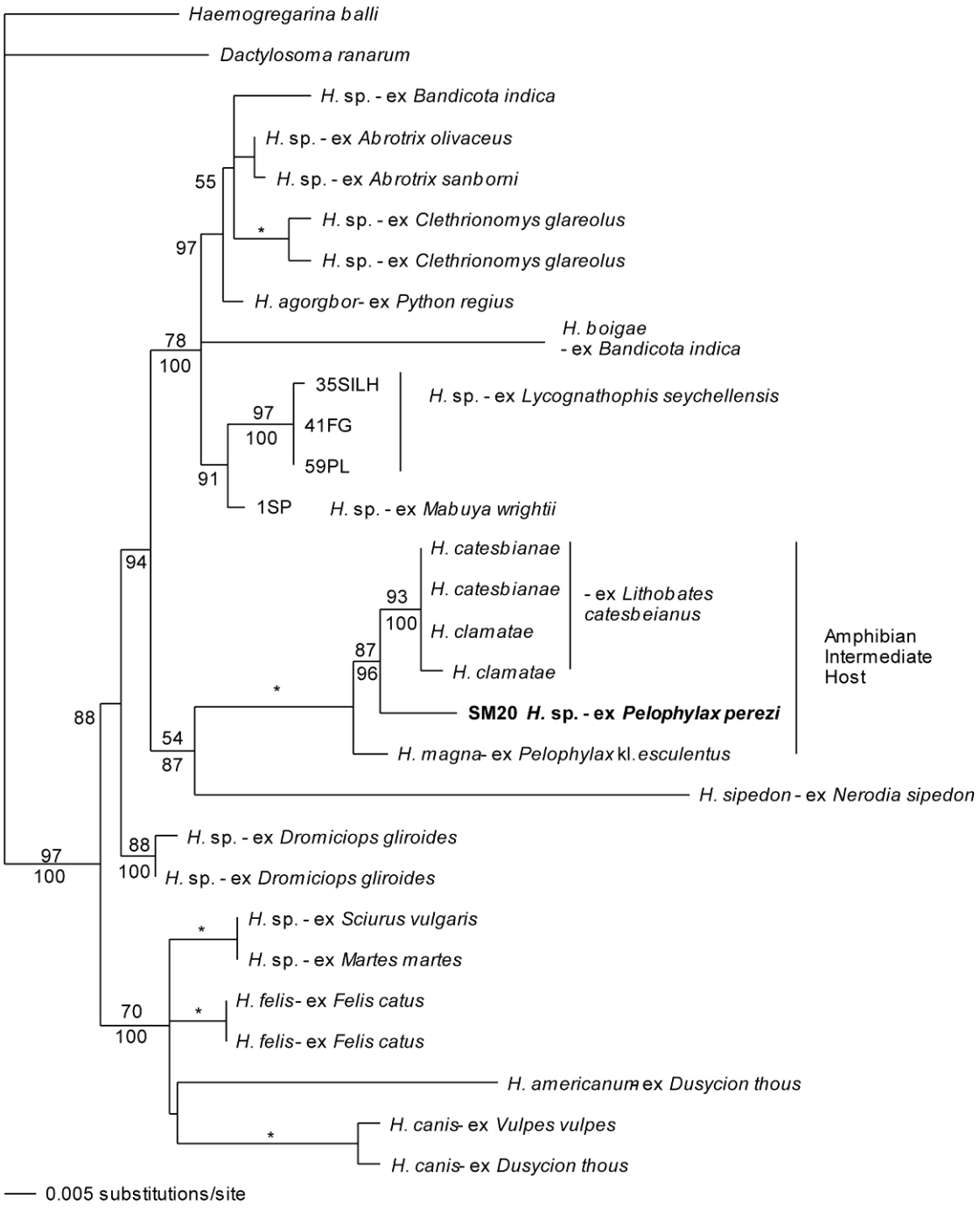

Fig. 1. Tree derived from a Maximum Likelihood (ML) analysis of the *Hepatozoon* sequences. Bootstrap values for ML are given above relevant nodes, and Bayesian Posterior Probability below them. *Indicates 99 or 100% support in both ML and Bayesian analyses. The new haplotype from this study is in bold. For all other codes refer to Harris *et al.* (2012) or Barta *et al.* (2012)

2 from Santa Maria). DNA was extracted using standard High Salt methods (Sambrook *et al.* 1989) or Speedtools Tissue DNA extraction kit (Biotools). Detection of blood parasites was made using PCR reactions with the primers HepF300 and HepR900 (Ujvari *et al.* 2004). These were designed to amplify *Hepatozoon* parasites, but also amplify at least *Eimeria* and *Sarcocystis* species (Harris *et al.* 2012) and stramenopiles (Maia *et al.* 2012). Detection of nematodes was carried out

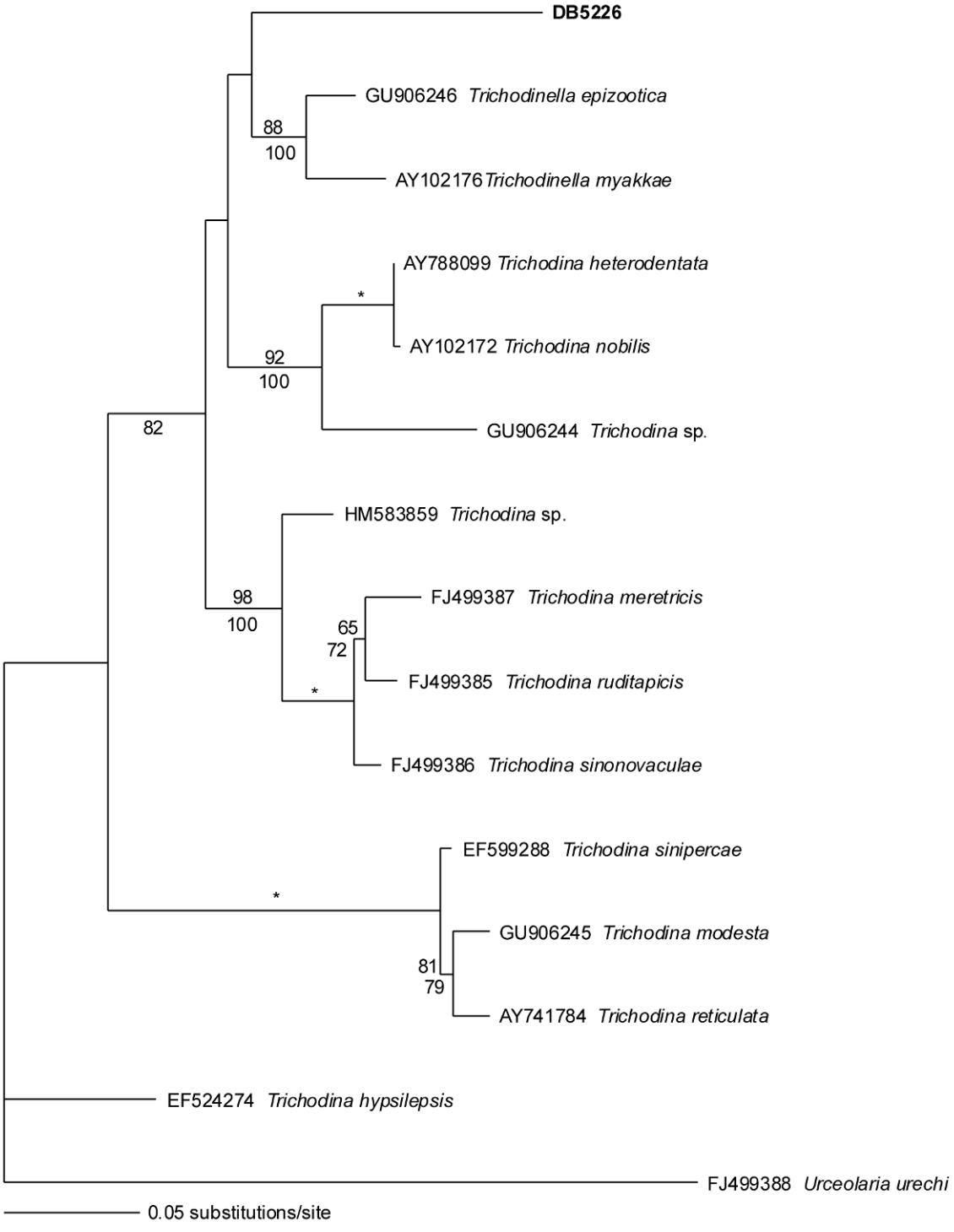

Fig. 2. Tree derived from a Maximum Likelihood (ML) analysis of the *Trichodina* and *Trichodinella* sequences. Bootstrap values for ML are given above relevant nodes, and Bayesian Posterior Probability below them. *Indicates 99 or 100% support in both ML and Bayesian analyses. The new haplotypes from this study is in bold

with Nem18SF and Nem18SR (Floyd *et al.* 2005), which are known to amplify nematodes from reptiles and that have been used successfully on host tissue samples (Perera *et al.* 2012). Conditions of the PCRs are detailed in Harris *et al.* (2011) and Perera *et al.* (2012), respectively. Negative and positive controls were run with each reaction. The positive PCR products were sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea). Sequences were deposited in GenBank.

Since the detected parasites were unrelated, separate phylogenetic analyses were performed for each. New sequences generated in this study were aligned with related sequences retrieved from GenBank. Sequences were aligned using ClustalW software implemented in the program BioEdit (Hall 1999). The final datasets contained 29 and 15 taxa with 613 and 481 bp in length, respectively. Maximum Likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to estimate their evolutionary relationships using the software PAUP (Swofford 2002). Support for nodes was estimated using the bootstrap technique, with 1000 replicates. The model of evolution employed was chosen using the AIC criteria carried out in Modeltest 3.06 (Posada and Crandall 1998). Bayesian analysis was implemented using Mr. Bayes v.3.2 (Huelsenbeck and Ronquist 2001). The analysis was run for $1x10^7$ generations, saving one tree every 1000 generations. All trees prior to reaching stationary were discarded as burn-in samples (25%), and remaining trees were combined in a 50% majority consensus tree. Following Barta *et al.* (2012), *Babesiosoma stableri* and *Dactylosoma ranarum* were used as outgroups for rooting the phylogenetic tree for the *Hepatozoon* sequences. Following Zhan *et al.* (2009), *Urceolaria urechi* was defined as an outgroup to root the *Trichodina/Trichodinella* analysis.

One individual from São Miguel island (SM20) was found to be infected with *Hepatozoon* sp. (Fig. 1). When included in a phylogenetic analysis with published *Hepatozoon* sequences, this sample was strongly supported as a member of a clade of other *Hepatozoon* from various amphibians. It was sister taxa to *Hepatozoon caesbianae* and *Hepatozoon* cf *clamatae*, from *Lithobates catesbeianus* and *Lithobates clamitans* from North America, respectively, rather than *Hepatozoon magna* from *Pelophylax esculentus*. Thus, although all currently analysed *Hepatozoon* from amphibians form a clade, relationships within this clade do not match the vertebrate intermediate host phylogeny. Remaining relationships within the *Hepatozoon* are similar to those previously inferred (Barta *et al.* 2012, Harris *et al.* 2012).

Two tadpoles from Santa Maria island (DB5225 and DB5226) were infected with a peritrichous ciliate (Fig. 2), apparently a trichodinid (*Trichodina* or *Trichodinella*) of the Mobilia subclass (Zhan *et al.* 2009). This is a group of parasitic or commensal ciliates that attach to their aquatic hosts using a characteristic adhesive disc. This is, to our knowledge, the first report of a trichodinid from the Azores. A sample from São Miguel island (SM21) also produced a positive PCR, with the closest match on GenBank being a member of a clade of marine Stramenophiles – KKTS.E5, MAST-12 (Kolodziej and Stoeck 2007), from which it differed by 10 mutations or circa 2%. Other slightly more divergent sequences were identified as Eimeriidae environmental sample and uncultured eustigmatophyte clones (Lesaulnier *et al.* 2008). Given that all of these closest matches were environmental samples, it is not clear if this positive was from the actual frog, or was from water that would have been on the frog's skin when it was sampled. It was not therefore analysed further.

With the nematode specific primers, a single positive was identified in an individual from São Miguel island (SM16). Although a BLAST search showed that all closest matches were nematodes of the order Spirurida, none had closer than 84% similarity. With no closely related taxa available a phylogenetic analysis was not performed, but clearly at least one nematode species is infecting *P. perezi* and further research should address if these parasites were introduced to the Azores with the host. Helminth communities have been assessed for *P. perezi* from the South of Spain (Navarro and Lluch 2006), but were generally considered depauperate.

These results are important for several reasons. The first is that they indicate that multiple parasites may have been brought to the Azores with the introduction of *P. perezi*. In an introduced *Podarcis* wall lizard population there is a marked absence of blood parasites, and reduced diversity of nematodes (Burke *et al.* 2007). It will be useful to look at other introduced vertebrate groups to see if any pattern regarding parasite introductions is evident. It is also important to determine how widespread these parasites are in *P. perezi* both by screening introduced and continental populations to confirm the source of the parasites. Secondly they show that various additional organisms can be detected with the Hep primers, not just parasite groups that had previously been identified (Harris *et al.* 2012, for Apicomplexa parasites, and Maia *et al.* 2012, for Stramenopiles), but also trichodinids. Their use in parasite surveys can therefore not only be used to detect various groups in a variety of vertebrate hosts, but the obtained sequences can also give considerable new insights into the evolutionary history of these poorly known parasites.

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