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Brachycephalus pernix (Anura: Brachycephalidae), a new host of *Ophiotaenia* (Eucestoda: Proteocephalidea)

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Abstract. This study reports on a novel record of parasitism of the montane frog *Brachycephalus pernix* (Anura: Brachycephalidae) by the tapeworm *Ophiotaenia* sp. (Eucestoda: Proteocephalidea). Moreover, this is the second record of *Ophiotaenia* parasitizing an anuran in Brazil. The strong tendency for *Brachycephalus* to display isolated microendemic distributions provides an excellent model system to investigate host-parasite coevolution in terrestrial proteocephalidean tapeworms.

Key words: Eucestoda, new record, phylogeny, geography, Terrarana, Brazil.

Brachycephalus is a fascinating frog genus endemic to the Brazilian Atlantic Rainforest, known for its minute size and high level of endemism, particularly in cloud forests (Pie et al., 2013). Several *Brachycephalus* species display conspicuous (aposematic) coloration patterns associated with the presence of tetrodotoxin and its analogues, which are highly potent neurotoxins, particularly concentrated in their integument (Pires et al., 2005). Species of *Brachycephalus* undergo direct development (i.e. they lack a free-living, aquatic tadpole), completing their entire life-cycle on land (Pombal, 1999).

As a part of an ongoing research project on the diversity and distribution of *Brachycephalus* in southern Brazil, we detected the presence of endoparasites in one individual of *B. pernix* collected on November 17, 2012 at Anhangava (25°23'19"S, 49°00'15"W; 1,375 m a.s.l.), Serra da Baitaca, municipality of Quatro Barras, eastern state of Paraná. Five endoparasites were found within the abdominal cavity. Given that the endoparasites were initially preserved in absolute ethanol and therefore were not suitable for traditional parasitological characterization, we extracted genomic DNA from one entire specimen using the PureLink™ Genomic DNA kit (Invitrogen™, USA), according to manufacturer's instructions, and amplified two small regions of 18S and 28S ribosomal genes using the primers 18S5F/18S847R (Wiegmann et al., 2000) and 28S3665F/28S4068R (Belshaw and Quicke, 1997) respectively. For both genes, thermocycling conditions were 3 min at 95°C, followed by 35 cycles of 94°C for 45 s, 47°C for 1 min and 70°C for 1 min, and final extension at 72°C for 5 min. Each reaction was done in 25-µL with 2 units of AmpliTaq DNA polymerase (Invitrogen™, USA), 1X PCR buffer, 1.5mM of $MgCl_2$, 0.5mM of dNTPs and 1.0 µM of each primer.

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Table 1. Genbank accession numbers of neotropical proteocephalid sequences used in the present study.

Species	18S	28S
Ageneiella brevifilis	AY551102	AJ388600
Amphoteromorphus parkamoo	AY551103	AJ388595
Amphoteromorphus piraeeba	AY551104	AJ388624
Choanoscolex abscisus	AY551105	AJ388630
Endorchis piraeeba	AY551107	AJ388603
Ephedrocephalus microcephalus	AY551108	AJ388605
Gibsoniela meursaulti	AY551109	AJ388631
Goezeella siluri	AY551110	AJ388612
Megathylacus brooksi	AY551111	AJ388596
Monticellia coryphicephala	-	AJ238832
Myzophorus pirarara(=Scholzia emarginata)	AY551112	-
Nomimoscolex admonticellia	AY551113	AJ388628
Nomimoscolex dorad	AY551114	AJ388613
Nomimoscolex lenha	AY551115	AJ388611
Nomimoscolex lopesi	AY551116	AJ388618
Nomimoscolex piraeeba	AF286988	AJ388608
Nomimoscolex sudobim	AY551117	AJ388597
Nomimoscolex suspectus	AY551118	AJ275068
Ophiotaenia gallardi	AY551119	AJ388615
Ophiotaenia grandis	AY551128	AJ388632
Ophiotaenia ophiodex	AY551120	AJ388620
Peltidocotyle lenha	AY551122	AJ238837
Peltidocotyle rugosa	AF286989	AF286937
Proteocephalus ambloplitis	AY551123	AJ388633
Proteocephalus brooksi	AY551124	-
Proteocephalus chamelensis	AF267294	AJ275234
Proteocephalus hemioliopteri	AY551129	AJ388622
Proteocephalus singularis	AY551133	-
Pseudocrepidobothrium eirasi	AY551106	AJ388623
Pseudocrepidobothrium ludovici	-	AJ238833
Rudolphiella lobosa	AY551134	-
Rudolphiella szidati	AY551135	AJ388617
Spatulifer maringaensis	AY551136	AJ388634
Thaumasioscolex didelphidis	AF267295	AJ275065
Zygobothrium megacephalum	AF286991	AJ388621

PCR products were electrophoresed on 1.5% agarose gels with E-Gel®1 kb Plus DNA Ladder (Invitrogen™, USA), added to Kasvi Safer Dye fluorescent reagent and visualized under UV light. Positive PCR products were purified using PEG 8000. Samples were sequenced in both directions. The sequencing reaction protocol was performed in 10 µL: 0.5 µL ABI Prism® BigDye™ v3.1 (Applied Biosystems Inc., Foster City, CA), 1.0 µL 5X buffer and 1 µL each (3.2 pmol) primer. The ultra-pure water and template to give 40–50 ng of DNA in each reaction was composed in the remainder of the mixture. The cycle sequencing reaction protocol contained an initial denaturation step of 96°C for 1 min, followed by 35 cycles of 10 s at 96°C denaturation, 15 s at annealing 50°C and 4 min at 60°C. The final DNA precipitation was performed with isopropanol and run on an ABI 3500 sequencer. The sequences were deposited to the GenBank database under the Accession numbers KJ507658 and KJ507659.

Identity of the obtained sequences was detected using the BLAST algorithm (www.ncbi.nlm.nih.gov), which indicated its close relation to the proteocephalid tapeworms. In order to determine a more precise phylogenetic position, we obtained all proteocephalid tapeworm sequences of both 18S and 28S genes for the neotropical clade identified by Hypša et al. (2005) (Table 1). Each gene was aligned separately using ClustalX (Thompson et al., 1994), ambiguously aligned regions were deleted and both fragments were concatenated using Staden Package (Staden et al., 2003), resulting in a final alignment of 1,447 bp. The phylogenetic position of the unknown parasite was inferred based on maximum parsimony using PAUP* Version 4.0b10 (Swofford, 2000) with 100 random sequence additions. Bootstrap support was calculated for 1,000 replicates (other analyses based on maximum likelihood and Bayesian inference provided nearly identical results but are not shown for the sake of brevity.)

The resulting phylogeny indicates that our sample belongs to the terrestrial clade of proteocephalidean tapeworms identified by Hypša et al. (2005). In particular, there is support for its placement in the genus *Ophiotaenia* (Figure 1). So far, 26 species of this genus parasitizing amphibians have been described (de Chambrier et al., 2006; Marsella and de Chambrier, 2008; de Chambrier and Gil de Pertierra, 2012). This is the second record of *Ophiotaenia* parasitizing anuran hosts in Brazil. The first, *O. bonariensis*, was recorded in *Leptodactylus ocellatus* and in *L. pentadactylus* by Rego (1973). Of the remaining *Ophiotaenia* species recorded in this country, 12 are parasites of snakes (see Table 2 in Ammann and de Chambrier, 2008) and one of a turtle [*O. lopesi* (Rego, 1967)]. Our record is not unexpected, given that the geographic distribution of the genus includes Central America and Argentina (de Chambrier et al., 2006; Marsella and de Chambrier, 2008).

The presence of *Ophiotaenia* in a direct-developing frog is particularly intriguing with regard to its life history. Members of this genus exhibit a typical proteocephalan life-cycle involving the development of a procercoid larva with a cercomer within the body of a copepod, which is often accidentally swallowed by the definitive host (Scholz and de Chambrier, 2003). This general pattern was recorded in *O. ranae* parasitizing *Rana nigromaculata* through a copepod intermediate

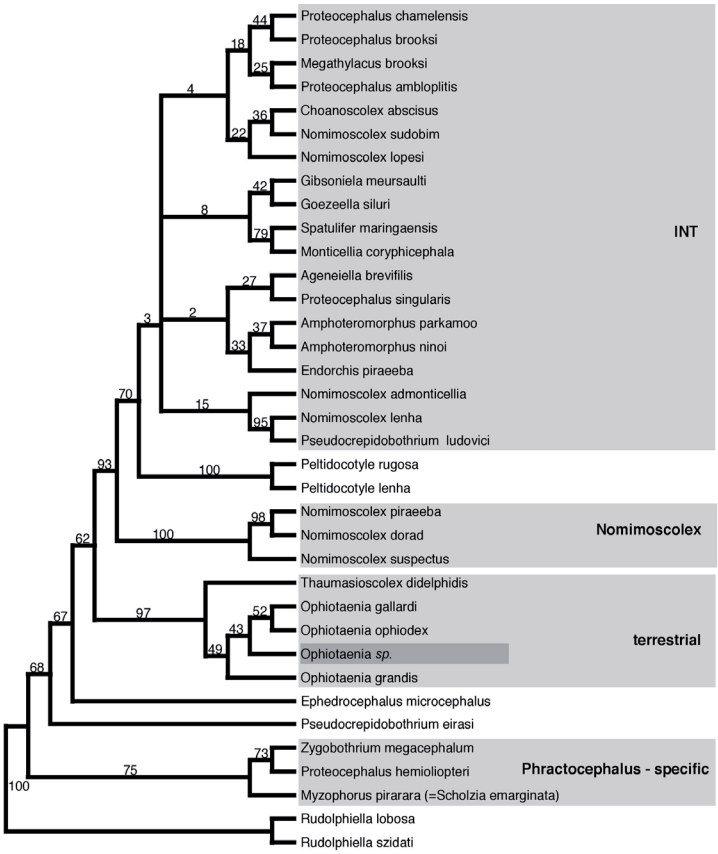

Figure 1. Phylogenetic tree obtained by maximum parsimony (MP) analysis using nuclear 18S and 28S rDNA gene, indicating the placement of the parasite recorded in the frog *Brachycephalus pernix* ("*Ophiotaenia* sp.") within the proteocephalid genus *Ophiotaenia*. The tree is rooted with *Rudolphiella* according to Hypša et al. 2005. Numbers above branches indicate bootstrap values for MP after 1000 replicates. Clade names follow Hypša et al. (2005).

host (*Mesocyclops* sp.) (see Yamaguti, 1943), and in *O. gracilis* parasitizing the bullfrog *Rana catesbiana* through the copepod *Eucyclops agilis* (see Buhler, 1970). Similar results were reported in snakes (e.g. Biserkov et al., 1997). However, this general pattern cannot be the case in *Brachycephalus*, given that all of its species complete their entire life-cycles on land (Pombal, 1999). The only crustaceans in the diet of *Brachycephalus* are isopods (Fontoura et al., 2011; Dorigo et al., 2012), which do not seem to represent suitable intermediate hosts. Finally, the strong tendency of *Brachycephalus* to display isolated microendemic distributions (e.g. Pie et al., 2013) provides an excellent model system to investigate host-parasite coevolution in terrestrial proteocephalidean tapeworms.

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