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Monophyly and systematic position of *Glypthelmins* (Digenea), based on partial lsrDNA sequences and morphological evidence

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Abstract

Species composition and systematic placement within the order Plagiorchiida has been controversial. Species number in *Glypthelmins* Stafford, 1905, a genus of cosmopolitan parasites of anurans, has varied between 19 and 28 species, depending on the taxonomic treatment. The present study performs a phylogenetic analysis using partial lsrDNA sequences to test the monophyly of the genus, and compares new sequences obtained with those published for different plagiorchiids to clarify the systematic position of *Glypthelmins* within the order Plagiorchiida. Maximum parsimony (MP) and maximum likelihood (ML) analyses result in identical tree topology. The single MP tree ($L = 1587$, CI = 0.40, RI = 0.76) includes several clades with high bootstrap and Bremer support values. *Glypthelmins* sensu lato as traditionally classified is paraphyletic. Based on molecular and/or morphological evidence, the taxonomic diagnosis for *Glypthelmins* is emended, only eight species are retained in the genus, and re-establishment of the genera *Choledocystus* Pereira & Cuocolo, 1941 and *Rauschiella* Babero, 1951 is proposed, resulting in the following new combinations: *Choledocystus simulans* (Teixeira de Freitas, 1941) comb. nov., *C. vitellinophilum* (Dobbin, 1958) comb. nov.; *Rauschiella chaquensis* (Mañé-Garzón & Holcman-Spector, 1967) comb. nov., *R. lenti* (Teixeira de Freitas, 1941) comb. nov., *R. linguatula* (Rudolphi, 1819) comb. nov., *R. poncedeleoni* (Razo-Mendivil & León-Règagnon, 2001) comb. nov., *R. robusta* (Brooks, 1976) comb. nov., *R. rugocaudata* (Yoshida, 1916) comb. nov., *R. staffordi* (Tubangui, 1928) comb. nov. In the phylogenetic reconstruction, *Glypthelmins* sensu stricto forms the sister group of *Haematoloechus* Looss, 1899.

Keywords: Anurans; Mexico; United States; Costa Rica; Molecular phylogeny; Ribosomal gene; Parasites

Introduction

The genus *Glypthelmins* was established by Stafford (1905) to include *Distomum quietum* Stafford, 1900, parasitic in anurans from Canada. Monophyly of the group has been a controversial issue, mainly because the original description of the type species, *G. quieta* (Stafford), was incomplete and no characters diagnostic for the genus were specified. Various taxonomic studies have recognized from 19 to 28 species in *Glypthelmins* worldwide, all of them parasitizing amphibians (Yamaguti 1971; Sullivan 1976; Prudhoe and Bray 1982; Brooks and McLennan 1993). Species included in *Glypthelmins* have been combined alternatively with

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one or more of the genus names *Margeana* Cort, 1919, *Haplometrana* Lucker, 1931, *Choledocystus* Pereira and Cuocolo, 1941, *Rauschiella* Babero, 1951, *Reynoldstrema* Cheng, 1959, *Repandum* Byrd and Maples, 1963, and *Hylotrema* Sullivan, 1972. Yamaguti (1971) transferred the species included in *Choledocystus* to *Glypthelmins*, without specifying a justification, and recognized *Rauschiella* as a valid genus. Prudhoe and Bray (1982) supported the validity of the genera *Glypthelmins*, *Choledocystus* and *Rauschiella*, but Brooks and McLennan (1993) suggested that all species should be assigned to *Glypthelmins*.

The taxonomic position of *Glypthelmins* within the order Plagiorchiida has been controversial as well. The genus has been placed in the families Plagiorchiidae (Olsen 1937; Skrjabin and Antipin 1958; Yamaguti 1958; Prudhoe and Bray 1982), Brachycoelidae (Caballero 1938; Dollfus 1950; Cheng 1959, 1961), and Macroderoididae (Schell 1962; Odening 1964; Yamaguti 1971). Tkach et al. (2001a) conducted a molecular phylogenetic study of the families Macroderoididae and Ochetosomatidae, based on partial sequences of the nuclear 28S rDNA, and included two species of *Glypthelmins*: *G. quieta* and *G. pennsylvaniensis* Cheng, 1961. This study demonstrated that *Glypthelmins* and *Macroderoides* are not monophyletic; both genera were assigned to the family Macroderoididae. Olson et al. (2003) proposed a more comprehensive phylogenetic hypothesis for the Digenea, based on partial ssrDNA and lsrDNA sequences, in which *G. quieta* was treated as a representative of Plagiorchiidae, constituting a natural group with *Skrjabinoeces* and *Haematoloechus*. Monophyly of *Glypthelmins* was not tested in those papers. For the present study, we obtained partial lsrDNA sequences from 11 species of *Glypthelmins* and compared them with 45 previously published sequences from different plagiorchiidians in order to test the monophyly of *Glypthelmins* and clarify its systematic position within the order Plagiorchiida.

Material and methods

Sampling

Between July 1996 and April 2002, specimens of 11 species of *Glypthelmins* were collected from 13 host species at six localities in Mexico, four localities in the United States and one in Costa Rica (Table 1). Anurans were captured by hand or with seine nets and kept alive prior to parasitological examination. Hosts were sacrificed with an overdose of sodium pentobarbitol and all organs were examined under a stereo microscope.

Digeneans belonging to *Glypthelmins* were initially placed in a 0.65% saline solution; some worms from each host were mounted as semi-permanent slides in saline and assigned to morphospecies in vivo.

Molecular study

For molecular work, specimens were morphologically identified in vivo. Species identifications, hosts and geographical distributions are provided in Table 1. Specimens were washed with saline solution and preserved in 100% ethanol. One or more worms per species were digested individually with proteinase K (25 mg/ml) in 500 µl STE buffer, 75 µl 10% SDS, and incubated for 12–24 h at 55 °C. Genomic DNA was extracted with phenol/chloroform, precipitated with 96% ethanol, and dissolved in 100 µl deionized sterile distilled water (Hillis et al. 1996). Polymerase chain reaction (PCR) was used for amplifying the 5′ end of the lsrDNA gene, including the D1–D3 variable domains. PCRs were performed in a final volume of 25 µl (2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP mixture (200 µM each), 0.8 µl 50 mM $MgCl_2$, 1 µl of each primer (10 pmol), 1 µl template DNA, 0.5 µl Taq DNA polymerase (5 units), and 17.7 µl of sterile distilled water). Amplification and sequencing were performed using forward primer 28Sy (5′ CTA ACC AGG ATT CCC TCA GTA ACG GCG AGT 3′) and reverse primer 28Sz (5′ AGA CTC CTT GGT CCG TGT TTC AAG AC 3′) (Palumbi 1996), and forward primer 28Sl (AAC AGT GCG TGA AAC CGC TC) combined with reverse primer LO (5′-GCT ATC CTG AG(AG) GAA ACT TCG-3′) (Tkach et al. 2000a). With the exception of annealing temperatures, reaction conditions used were the same regardless of primer set employed. An initial denaturation at 95 °C for 5 min was followed by 30–35 cycles at 94 °C for 1 min, primer annealing at 45 °C for 45 s (primers 28Sy and 28Sz) or at 55 °C (primers 28Sl and LO), and extension at 72 °C for 1 min; mixes were held at 72 °C for 10 min to complete elongation, then cooled to 4 °C. PCR products were purified using the Qiaquick™ Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The purified PCR products were sequenced directly on an ABI PRISM 310™ automated DNA sequencer (Applied Biosystems) using the Big Dye Terminator™ chemistry according to manufacturer's, protocols. Chromatogram files were initially checked using the computer program Chromas (version 1.43). Subsequently, sense and anti-sense sequences were assembled using the computer program Bioedit, version 5.0.9 (Hall 1999). New sequences obtained in this study have been submitted to GenBank (Table 1).

Alignment and phylogenetic analyses

Sequences of *Glypthelmins* spp. were compared with the following sequences available from GenBank (respectively generated by Tkach et al. 1999, 2000a, b,