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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,

Table 1. Reference data on the material studied: *Glypthelmins*- and host species, collecting localities, GenBank accession- and voucher numbers.

Species	Host	Locality	GenBank no.	Vouchers
G. quieta (Stafford, 1900)	*Rana montezumae* Baird	Ciénaga de Lerma, Est. de México, México	AY278049	CNHE 3275–3279; USNPC 93034; HWML 39955
G. quieta (Stafford, 1900)	*Rana* sp.	Coquimatlán, Colima, México	DQ452537	CNHE 4688
G. quieta (Stafford, 1900)	*Rana clamitans* Latreille	Louisiana, USA	DQ452535	
G. quieta (Stafford, 1900)	*Rana catesbeiana* Shaw	Nebraska, USA	DQ452536	
G. californiensis (Cort, 1919)	*Rana montezumae* Baird	Ciénaga de Lerma, Est. de México, México	AY278051	CNHE 3282; HWML 33956, 33957
G. californiensis (Cort, 1919)	*Rana dunni* Zweifel	Lago de Zacapu, Michoacán, México	AY278052	CNHE 3283–3284, 4684–4685; USNPC 93031–93033
G. intestinalis (Lucker, 1931)	*Rana luteiventris* Thompson	Glacier National Park, Montana, USA	AY875673	CNHE 4692
G. facioi Brenes, Madrigal, Arroyo Sancho, Jiménez-Quiroz and Delgado Flores, 1959	*Rana* sp.	Guanacaste, Costa Rica	AY875675	
G. pennsylvaniensis (Cheng, 1961)	*Pseudacris crucifer crucifer* (Wied-Neuwied)	Cabell County, West Virginia, USA	AY875676	CNHE 4693
G. hyloreus Martin, 1969	*Pseudacris triseriata* (Wied-Neuwied)	Nebraska, USA	AY278050	
G. brownorumae Razo-Mendivil, León-Règagnon & Pérez-Ponce de León, 2004	*Rana brownorumae* Hillis	km 50, Carr. 180, Villahermosa-Frontera, Tabasco, México	AY875674	CNHE 4667–4669; USNPC 93039; BM(NH) 2004.3.3.1
G. tuxtlasensis Razo-Mendivil, León-Règagnon & Pérez-Ponce de León, 2004	*Rana vaillanti* Brocchi	Laguna Escondida, Los Tuxtlas, Veracruz	AY278047	CNHE 4670–4672; USNPC 93040; BM(NH) 2004.3.3.2
G. tineri (Babero, 1951)	*Leptodactylus melanonotus* (Hallowell)	San Pedro las Playas, Guerrero, México	AY875677	CNHE 4068–4069
G. poncedeleoni Razo-Mendivil & León-Règagnon, 2001	*Leptodactylus melanonotus* (Hallowell)	San Pedro las Playas, Guerrero, México	AY875678	CNHE 3905–3906
G. hepatica (Lutz, 1928)	*Bufo marinus* (Linnaeus)	San Pedro las Playas, Guerrero, México	AY875679	CNHE 4074

2001a–c, 2002, 2003; Snyder and Tkach 2001; Olson et al. 2003). Allassogonoporidae: *Allassogonoporus amphoraeformis* (Mödlinger, 1930) (AF151924). Brachycoeliidae: *Brachycoelium salamandrae* (Froelich, 1789) (AF151935); *Mesocoelium* sp. (Odhner 1910) (AF433677, AY222277). Cephalogonimidae: *Cephalogonimus retusus* (Walton, 1938) (AY222276). Haematoloechidae: *Haematoloechus abbreviatus* (Bychowsky, 1932) (AF184251); *H. asper* Looss, 1899 (AF151934); *H. breviplexus* Stafford, 1902 (AF387800); *H. complexus* (Seely, 1906) (AF387797); *H. longiplexus* Stafford, 1902 (AF387801); *H. medioplexus* Stafford, 1902 (AF387799); *H. varioplexus* Stafford, 1902 (AF387798). Lecithodendriidae: *Ophiosacculus mehelyi* (Mödlinger, 1930) (AF480167). Macroderidae: *Macrodera longicollis* (Abildgaard, 1788) (AF151913). Macroderoididae: *Macroderoides spiniferus* Pearse, 1924 (AF433674); *M. typicus* (Winfield, 1929) (AF433673). Microphallidae: *Floridatrema heardi* Kinsella and Deblock, 1994 (AY220632); *Maritrema subdolum* Jägerskiöld, 1909 (AF151926); *M. neomi* Tkach, 1998 (AF151927). Ochetosomatiidae: *Ochetosoma kansense* (Crow, 1913) (AF433671); *Dasymetra nicolli* Hall and Allison, 1935 (AF433672). Omphalometridae: *Omphalometra flexuosa* (Rudolphi, 1809) (AF300333). Plagiorchiidae: *Glypthelmins quieta* (Stafford, 1900) (AF433675, AY222278); *G. pennsylvaniensis* (AF433676); *Haplometra cylindracea* (Zeder, 1800) (AF151933); *Lecithopyge rastellus* (Olsson, 1876) (AF151932); *Leptophallus nigrovenosus* (Bellingham, 1844) (AF151914); *Metaleptophallus gracillimus* (Lühe, 1909) (AF151912); *Neoglyphe locellus* (Kossack, 1910) (AF300330); *N. sobolevi* Schaldybin, 1953 (AF300329); *Paralepoderma cloacicola* (Lühe, 1909) (AF151910); *Plagiorchis elegans* (Rudolphi, 1802) (AF151911); *P. koreanus* Ogata, 1938 (AF151930); *P. vespertilionis* (Müeller, 1780) (AF151931); *Rubenstrema opisthovitellinum* (Soltys, 1954) (AF300332). Pleurogenidae: *Parabascus semisquamosus* (Braun, 1900) (AF151923); *Pleurogenes claviger* (Rudolphi, 1819) (AF151925); *Pleurogenoides medians* (Olson, 1876) (AF433670). Prosthogonimidae: *Prosthogonimus ovatus* (Rudolphi, 1803) (AF151928); *Schistogonimus rarus* (Braun, 1901). Telorchiidae: *Telorchis assula* (Dujardin, 1845) (AF151915); *Opisthioglyphe ranae* (Fröhlich, 1791) (AF151929). Troglotrematidae: *Nephrotrema truncatum* (Leuckart, 1842) (AF151936); *Skrjabinophyetus neomidis* Dimitrova and Genov, 1968 (AF184252).

Alignment was performed initially using ClustalX with default parameters (Jeanmougin et al. 1998). Subsequently, the alignment was improved by eye in Bioedit (Hall 1999). The complete alignment has been deposited in EBI, and is available by anonymous FTP from http://ftp.ebi.ac.uk in directory /pub/databases/embl/align or at www.3ebi.ac.uk/Services/align/listali.html under the number ALIGN_001062; sites excluded from analyses are specified. Ambiguous regions in the alignment and uninformative characters were excluded from analysis. All phylogenetic analyses were carried out using PAUP 4.0b10 (Swofford, 2002). An uncorrected distance matrix was obtained for all base pairs of examined sequences. Two species belonging to the suborder Troglotrematata (sensu Tkach et al., 2001a), *Nephrotrema truncatum* and *Skryabinophyetus neomidis*, were used as outgroups, following the results of previous studies of the suborder Plagiorchiata (Tkach et al., 2000a, 2001a, c). Tree searches were conducted with optimality criteria of equally weighted maximum parsimony (MP) and maximum likelihood (ML). In order to determine which model of evolution fit the dataset best, we performed a nested likelihood ratio test (LRT) to compare the fit of the nested general time reversible (GTR) family of nucleotide substitution models (Rodríguez et al. 1990), using Modeltest version 3.06 (Posada and Crandall 1998). The MP and ML trees were generated through 1000 and 100 random taxon addition heuristic searches, respectively, with tree bisection-reconnection (TBR) branch swapping options. Characters were treated as unordered, gaps as missing data. Branch support was estimated using 1000 bootstrap replicates for MP analysis, 100 replicates for ML analysis, with 10 random addition sequences and TBR branch swapping. In addition, Bremer support (Bremer, 1994) was calculated for all internal branches, using Auto-Decay (Eriksson, 1998).

Morphological study

Adult specimens from 15 populations corresponding to 11 species of *Glypthelmins* (see Table 1) collected from frogs and toads were rinsed in saline solution, fixed by sudden immersion in hot 4% formalin, and stored in 70% ethanol or in 4% formalin. For species identification, unflattened worms were stained, dehydrated, cleared in methyl salicylate, and mounted in Canada balsam. Illustrations were made with a drawing tube. Voucher specimens have been deposited at the Colección Nacional de Helmintos (CNHE), Mexico City, the Natural History Museum, London (BMNH), and the United States National Parasite Collection (USNPC), Beltsville, Maryland (see Table 1).

The following additional material of *Glypthelmins* species was used for comparison. *G. quieta*: CNHE 1461, 1562–1563; USNPC 51635, 72268, 84184; Harold W. Manter Laboratory, Lincoln, Nebraska (HWML) 20174–20201. *G. californiensis* (Cort, 1919): CNHE 1181, 1561, 2495; *G. linguatula* (Rudolphi, 1819): Instituto Oswaldo Cruz, Río de Janeiro (CHIOC) 21.823 a–e; CNHE 809; Colección Helmintológica, Departamento de Zoología de Invertebrados, Facultad de Humanidades y Ciencias, Montevideo, Uruguay (CHFHCU) AP11156–AP11157, AP11161;

G. palmipedis (Lutz, 1928): CNHE 1057; HWML 21697; CHIOC 21.974; USNPC 72279; *G. intestinalis* (Lucker, 1931): USNPC 29904 (paratypes); HWML 20835, 23754, 23755; CNHE 4692; *G. intermedius* (Caballero, Bravo-Hollis and Zerecero, 1944): CNHE 741–744, 808, 1166, 1357 (including holotype and paratypes); USNPC 36894; HWML 24901. *G. facioi* Brenes, Jiménez-Quiróz, Arroyo-Sancho and Delgado-Flores, 1959: Colección de Helmintos de Costa Rica, San José (CHCR) 202–22 (lectotype); USNPC 72275, plus specimens from Costa Rica borrowed from Dr. Daniel R. Brooks. *G. pennsylvaniensis* Cheng, 1961: USNPC 59515 (holotype and paratypes); *G. hyloreus* Martin, 1969: USNPC 70464 (paratypes); HWML 20209, 33823, 33824; *G. tineri* (Babero, 1951): USNPC 47089 (paratype); CNHE 4067–4072; *G. robustus* Brooks, 1976: USNPC 73483 (paratypes); *G. poncedeleoni* Razo-Mendivil & León-Règagnon, 2001: CNHE 3733–3738, 3905–3906 (holotype and paratypes); *G. parva* Travassos, 1924: CHIOC 4746 (holotype); CNHE 3391, 4115; *G. rugocaudata* (Yoshida, 1916): USNPC 75618; *G. staffordi* Tubangui, 1928: USNPC 20038; *G. subtropica* Harwood, 1932: USNPC 30878 (holotype); *G. vitellinophilum* Dobbin, 1958: CHIOC 22.098 b–m, 22.099 a–f; USNPC 72278.01–02; *G. incurvatum* Nasir, 1966: USNPC 72277; *G. ramitesticularis* Nasir, 1966: USNPC 60736 (holotype); *G. africana* Dollfus, 1950: HWML 38785; *G. repandum* (Rudolphi, 1819): HWML 21696; *G. shastai* Ingles, 1936: USNPC 8925 (holotype); *G. elegans* Travassos, 1926: CHIOC 21.812 a–c; *G. lenti* (Teixeira de Freitas, 1941): CHIOC 10.763–10.765 (syntype); *G. proximus* Teixeira de Freitas, 1941: CHIOC 10.756–10.761; CHFHCU AP/11176–AP/11184; *G. simulans* Teixeira de Freitas, 1941: CHIOC 10.751–10.752; CHFHCU AP/11162–AP/11168; *G. chaquensis* Mañé-Garzón and Holcman-Spector, 1974: CHFHCU AP/11113–AP/11115 (holotype and paratypes); *G. festina* Cordero, 1944: CHFHCU AP/11155 (holotype).

For scanning electron microscopy (SEM), specimens stored in 4% formalin were dehydrated in a gradual alcohol series and critical point dried. The specimens were coated with gold and examined in an Hitachi S2460N SEM.

Results

The fragment of the ribosomal 28S gene varied among the plagiorchiid species, from 1234 bp in *Cephalogonimus retusus* to 1260 bp in *Nephrotrema truncatum*. Among ten species of *Glypthelmins*, sequence length varied from 1247 to 1251 bp. Only 1107 bp could be sequenced for *G. intestinalis*; therefore 127 bp of the 5′ end are missing. Combination of the new sequences generated with those from GenBank produced an alignment of 1287 positions. Thirty-three bp showed position ambiguity and were excluded from the analyses.

For the MP analysis, out of the unambiguously aligned 1254 characters, 746 were constant, 498 variable, and 421 phylogenetically informative. MP analysis produced a single most parsimonious tree ($L = 1587$, $CI = 0.40$, $RI = 0.76$), with some clades showing high bootstrap and Bremer support values (Fig. 1). The 11 species of *Glypthelmins* do not cluster in a single, monophyletic clade. Based on the topology of the tree, *Glypthelmins* as conventionally classified is paraphyletic. *Glypthelmins hepatica* appears as the sister group to members of Ochetosomatidae, whereas *G. tineri* + *G. poncedeleoni* appear closely related to members of Plagiorchiidae. The remaining eight species of *Glypthelmins* constitute an independent, strongly supported group (100% bootstrap, Bremer = 48). The sister group of this clade is represented by members of Haematoloechidae (although with low bootstrap and Bremer values). Hereafter, we refer to those eight species as the *Glypthelmins* clade, since the latter includes the type species of the genus, *G. quieta*. The *Glypthelmins* clade includes two species (*G. californiensis* and *G. intestinalis*) occupying a basal position, and two derived groups. One of the latter comprises *G. brownorumae* as the sister group of *G. tuxtlasensis* + *G. facioi*. The second derived clade consists of *G. hyloreus* + the two haplotypes of *G. pennsylvaniensis* as the sister group to the six haplotypes of *G. quieta*.

The likelihood ratio test indicated that the model best fitting the data set is the Hasegawa-Kishino-Yano (HKY; Hasegawa et al., 1985) model with rate heterogeneity (+G; Yang, 1994), a transition/transversion ratio of 2.3636, and a gamma shape parameter of 2.5890. ML analysis using this model yielded a single best tree with a–ln likelihood of 10,843.85, and with a topology identical to the MP tree.

Sequence variation among the 15 populations belonging to 11 species of *Glypthelmins* ranged from 0.08% to 11.18%. For *G. quieta* and *G. pennsylvaniensis*, the sequences we obtained differed from those reported by Tkach et al. (2001b) in 8 (0.64%), and 2 (0.16%) nucleotides, respectively. However, no differences were found with the sequence reported for *G. quieta* by Olson et al. (2003). This suggests that the differences with the *G. quieta* sequences after Tkach et al. (2001b) might be the product of methodological errors. *Glypthelmins californiensis* from two localities showed one distinct site (0.08%), whereas *G. tineri* and *G. poncedeleoni* showed similar genetic divergence (0.08%).

Light-microscopic (LM) study of several specimens of 28 species included in *Glypthelmins*, and SEM observations on 12 of these species, allowed us to explore the characters traditionally used in the taxonomy of the group. The species *Glypthelmins californiensis*, *G. intestinalis*, *G. facioi*, *G. pennsylvaniensis*, *G. hyloreus*,

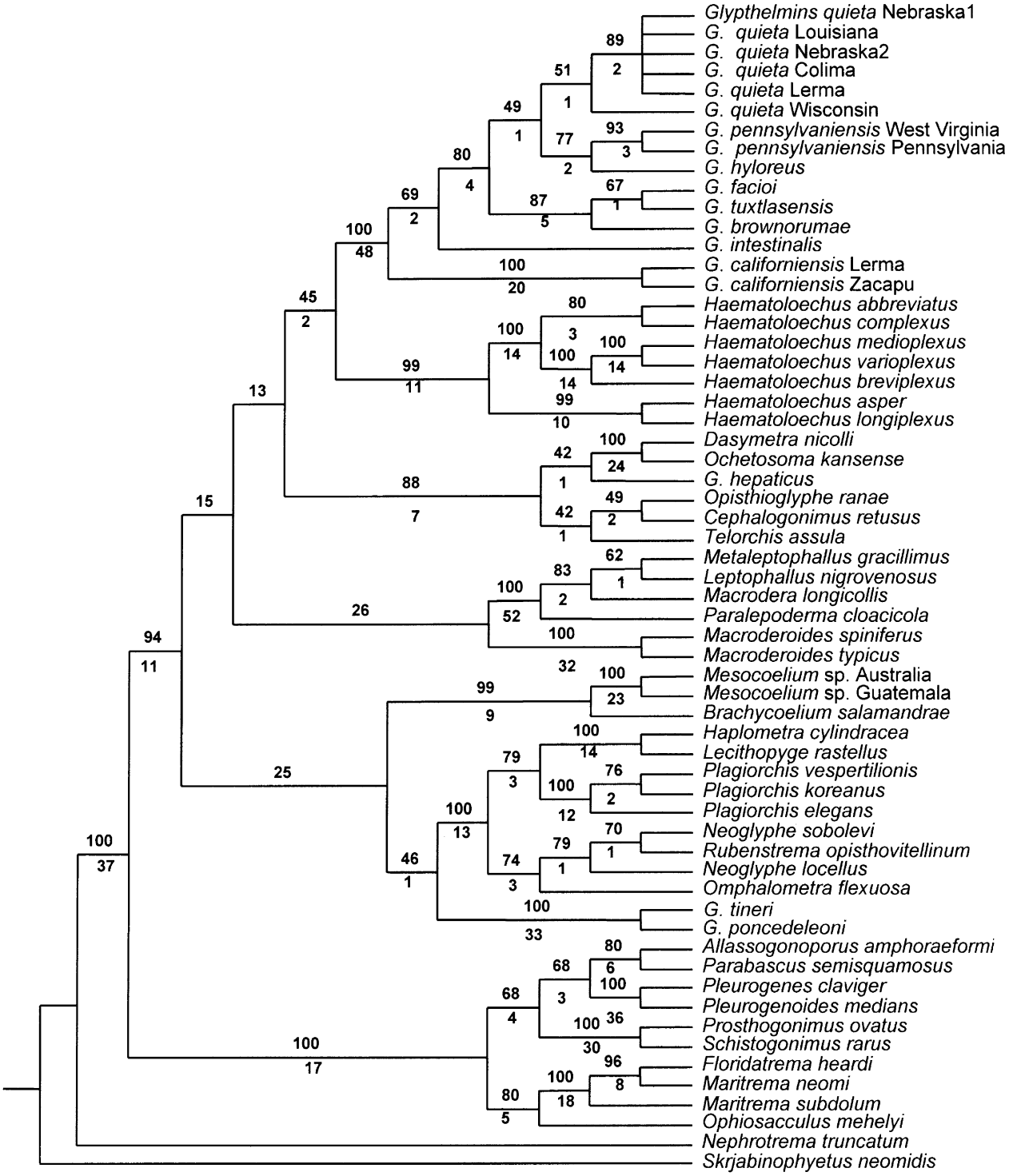
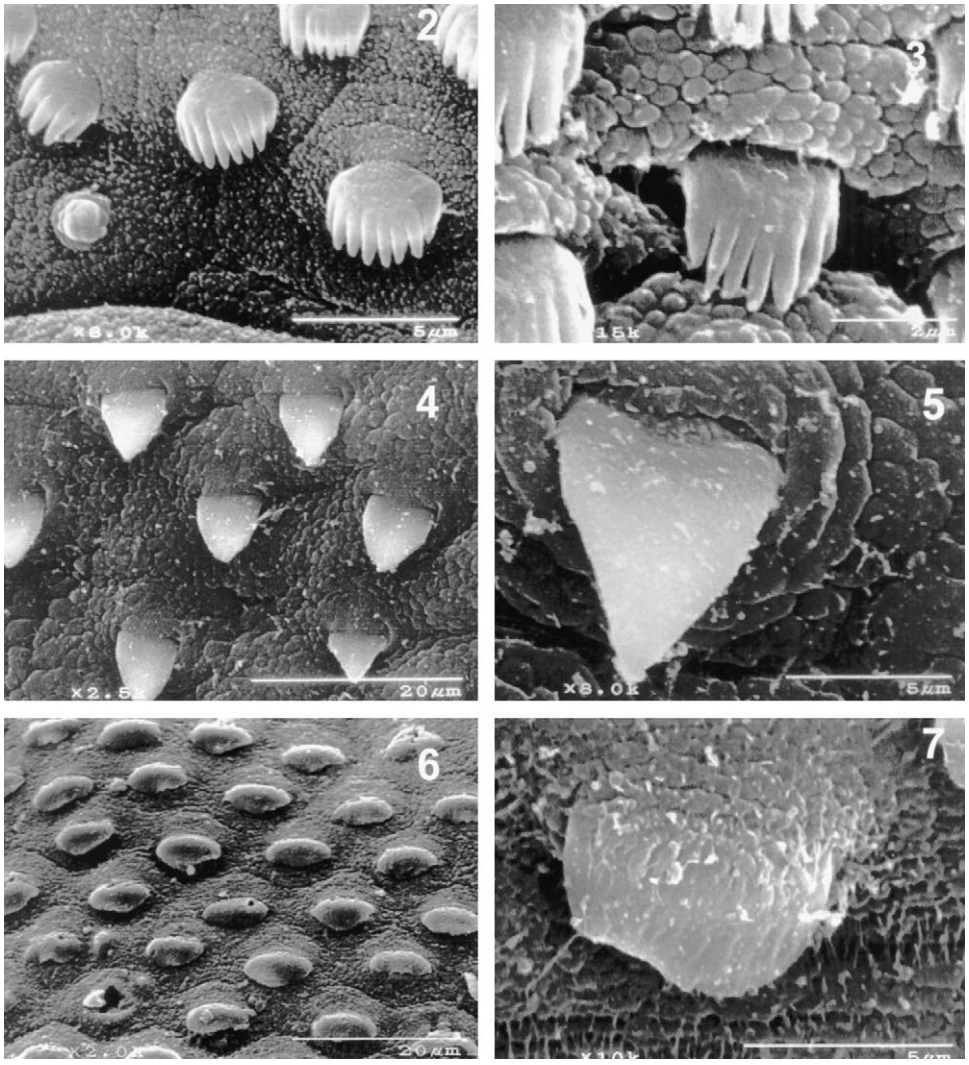


Fig. 1. Single most parsimonious tree from the heuristic MP analysis of 60 sequences of plagiorchiids, including 11 species of *Glypthelmins*.

G. brownorumae and *G. tuxtlasensis* share the following characteristics with the type species of the genus, *G. quieta*: tegumentary spines developed as serrated scales (Figs. 2 and 3), extending from anterior end to varying levels of posterior region; cirrus sac straight or curved, with a bipartite seminal vesicle; genital pore medial, anterior to ventral sucker (Figs. 8 and 9); ovary sinistral; seminal receptacle subspherical; uterus filling posterior region of caeca completely; metraterm running dorsally to cirrus sac; vitellarium constituted by follicles situated laterally, dorsally or between the caeca; excretory vesicle I-shaped; stem reaching post-testicular



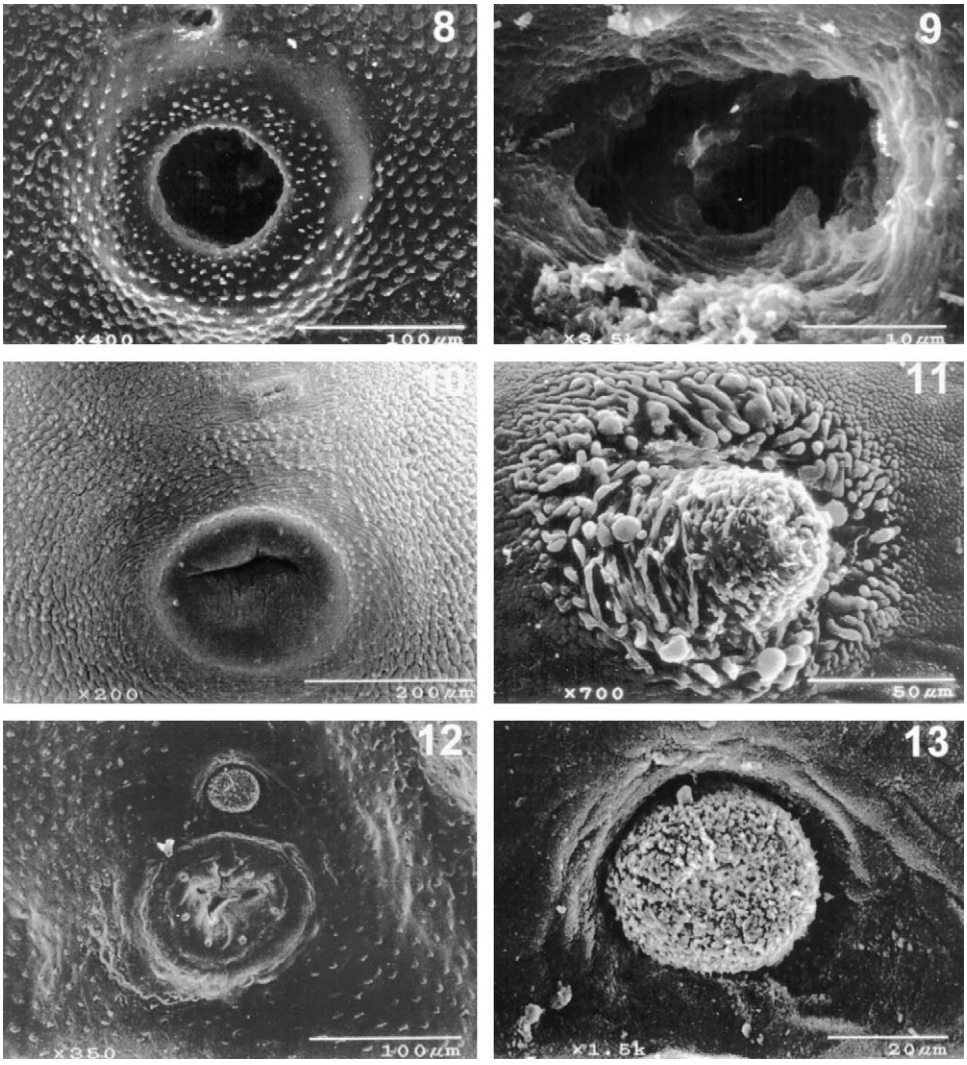
Figs. 2–7. Scanning electron micrographs of spines in *Glypthelmins intestinalis* (2–3), *Choledocystus hepaticus* (4–5), and *Rauschiella tineri* (6–7).

region. On the other hand, *G. hepatica* and *G. elegans* show a tegument covered with triangular spines (Figs. 4 and 5) extending from the region of the oral sucker to the posterior end of the body; sucker-length ratio equal to or higher than 1; cirrus sac globular, with seminal vesicle entire; ovary dextral; seminal receptacle absent; uterus occupying intercaecal, caecal, and extracaecal region; anterior uterine loops extending to ventral sucker region; metraterm muscular, running ventrally to cirrus sac, genital atrium lateral at level of caecal bifurcation, surrounded by a sucker-like structure (Figs. 10 and 11); vitellarium constituted by clusters of acini, lateral fields commence at level of intestinal bifurcation; excretory vesicle Y-shaped; stem extended to pre-testicular region; arms reaching ovary. Finally, the species *G. tineri* and *G. poncedeleoni* possess small, smooth, spine-like scales (Figs. 6 and 7) extending from the anterior end to distinct levels of the posterior region; cirrus sac lunated, with a coiled seminal vesicle; genital pore medial, anterior to ventral sucker; ovary dextral; seminal receptacle spherical; uterus occupying intercaecal and caecal region; uterine loops reaching pretesticular region; metraterm muscular, running ventrally to cirrus sac; genital pore medial (Figs. 12 and 13), anterior to ventral sucker; vitellarium extracaecal, follicles arranged in several rosette-like clusters; excretory vesicle Y-shaped; stem bifurcating between testes; arms reaching ovary (Figs. 14–16).

Systematic section

Is *Glypthelmins* sensu lato monophyletic?

The results we obtained in this study demonstrate that, considering the traditional classification of the group as proposed by Yamaguti (1958, 1971), *Glypthelmins* sensu lato is paraphyletic. Analyses performed

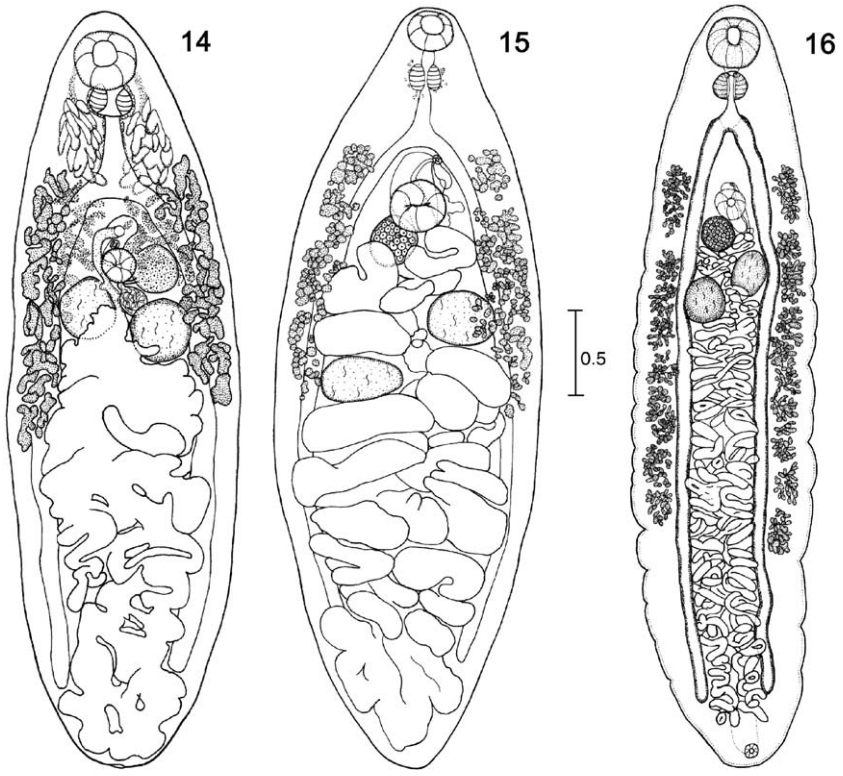


Figs. 8–13. Scanning electron micrographs of genital pores (right column: enlarged details) in *Glypthelmins quieta* (8–9), *Choledocystus hepaticus* (10–11), and *Rauschiella tineri* (12–13).

under criteria of MP and ML are consistent in that conclusion. Both analyses showed that the monophyletic clade including the type species of *Glypthelmins* contains only seven more of the species previously placed in that genus, whereas *G. hepatica*, and *G. tineri* + *G. poncedeleoni* form separate clades elsewhere in the tree. The *Glypthelmins* clade is strongly supported (100% bootstrap, Bremer = 48) and appears as a close relative of Haematoloechidae. These results agree with the recent phylogenetic hypothesis by Olson et al. (2003) for the Digenea. Morphologically, this clade is supported by the metraterm running dorsally to the cirrus sac. In the remaining species of *Glypthelmins* sensu lato, the metraterm runs ventrally to the cirrus sac.

The morphological analysis by Brooks and McLennan (1993) included seven species of the *Glypthelmins* clade as delimited here. They appeared as a monophyletic group, named the ''North American clade'' by those authors, as most included species occur in localities from southern Canada to central Mexico, with only one species, *G. facioi*, occurring in Costa Rica. Brooks and McLennan (1993) mentioned that *Glypthelmins* sensu lato could be paraphyletic, as they found no morphological synapomorphy for the group. Molecular evidence presented here is consistent with their proposal, and shows that our *Glypthelmins* clade is equivalent to their ''North American clade'' and thus to *Glypthelmins* sensu stricto. For *G. parva* and *G. shastai* morphological characters suggest placement in *Glypthelmins* sensu stricto. Whether or not this is appropriate will need to be re-evaluated once molecular information becomes available.

O'Grady (1987) proposed inclusion of *Haplometrana intestinalis* Lucker, 1931 in *Glypthelmins*. This has been supported by morphological evidence in Brooks and McLennan (1993), and by molecular evidence in the present study.



Figs. 14–16. Line drawings of general morphology in *Glypthelmins quieta* (14), *Choledocystus hepaticus* (15), and *Rauschiella tineri* (16); note extension of vitellarium, position of ovary, and extension of uterus.

Phylogenetic relationships among species of *Glypthelmin*s sensu stricto are not finally resolved. In our cladogram (Fig. 1), *G. californiensis* occupies the basal position. In previous analyses, this species is presented as sister to *G. quieta*. Compared to the phylogenetic relationships of species in the ''North American clade'' of Brooks and McLennan (1993) we found substantial differences. Additional information is needed to clarify the relationships among the members of this clade.

Based on the evidence obtained in the present study, we redefine the genus *Glypthelmins* to include at least eight species.

Glypthelmins Stafford, 1905

Type species: *Glypthelmins quieta* (Stafford, 1900).

Additional species included: *Glypthelmins brownorumae*, *G. californiensis*, *G. facioi*, *G. intestinalis*, *G. hyloreus*, *G. pennsylvaniensis*, *G. tuxtlasensis*. Possible further additions: *G. parva*, *G. shastai*.

Emended diagnosis

Body elongate or cylindrical. Tegument covered with small, serrate, scale-like spines extending from anterior end to varying levels of posterior region. Oral sucker subterminal. Prepharynx short. Pharynx globular, strongly muscular, sometimes wider than ventral sucker. Medial glands around pharynx and oesophagus. Conspicuous pharyngeal glands present or absent. Oesophagus bifurcating midway between pharynx and ventral sucker. Caeca extending near end of body. Ventral sucker smaller than oral sucker. Testes intercaecal or sometimes overlapping with caeca, spherical to oval, with smooth edges, usually symmetrical or oblique, occasionally in tandem. Cirrus sac straight or curved, usually behind ventral sucker; containing a straight, bipartite seminal vesicle, followed by an elongate, coiled cirrus. Genital pore medial, anterior to ventral sucker. Ovary spherical to oval, with smooth edges, sinistral and anterior to testes. Seminal receptacle subspherical, situated posterior to ovary or at middle of anterior region of testes. Ootype, Laurer's canal and Mehlis's gland present. Uterus extending posteriorly to end of body, filling posterior region of caeca completely; transversal uterine loops occupying intercecal and caecal regions, sometimes reaching extracecal region. Occasionally, uterine loops occupying pretesticular region. Metraterm muscular, approximately as long as cirrus sac, running dorsally to cirrus sac. Vitellarium conformed by follicles situated laterally, dorsally or between caeca; anterior follicles commencing at distinct levels between level of ovary and pharynx. Posteriorly, vitelline follicles usually extending past posterior margin of testes. Vitelline follicles confluent or not, situated dorsally to caecal bifurcation, cirrus sac or testes. Eggs

operculate and tanned. Excretory vesicle I-shaped, extending to posterior level of testes. Excretory pore terminal. Parasitic in intestines of anurans.

Placement of *Glypthelmins hepatica*, *G. tineri* and *G. poncedeleoni*

The MP and ML analyses revealed that *G. hepatica*, *G. tineri* and *G. poncedeleoni* do not belong to *Glypthelmins* sensu stricto. '*Glypthelmins*' *hepatica* is closely related to members of Ochetosomatidae (*Ochetosoma kansense* and *Dasymetra nicolli*), though with low bootstrap support (<50%); *G. tineri* and *G. poncedeleoni* show close relations to genera that have been assigned to Plagiorchiidae (Fig. 1). High sequence divergence between the members of *Glypthelmins* sensu stricto and *G. hepatica*, *G. tineri* and *G. poncedeleoni* strengthens the proposal that the latter three species do not belong to the genus. The *G. hepatica* sequence differs from those in *Glypthelmins* by 9.8–10.7%, whereas divergence from *Ochetosoma kansense* and *Dasymetra nicolli* ranged from 4.48% to 5.12% only. For *G. tineri* and *G. poncedeleoni*, ranges of sequence difference were 6.1–6.26% from the sister group, but 10.3–11.1% from species of *Glypthelmins* sensu stricto. According to our results, and in contrast with traditional classification, species of *Glypthelmins* sensu lato clearly belong to three independent, morphologically consistent clades. Consequently, we propose the revalidation of two genera to which some species of *Glypthelmins* were allocated in the past: *Choledocystus* Pereira & Cuocolo, 1941, and *Rauschiella* Babero, 1951.

Re-establishment of *Choledocystus*

Glypthelmins hepatica was originally established by Lutz (1928) as *Plagiorchis hepaticus*, found in *Bufo marinus* (L.) from Venezuela, but the name was based on a single figure without description only. Sullivan (1977a) redescribed the species, transferred it to *Choledocystus*, and considered *Choledocystus intermedius* Caballero, Bravo and Cerecero, 1944 as a junior synonym, an opinion that we agree with. The genus *Choledocystus* was established by Pereira and Cuocolo (1941) for *C. eucharis* Pereira and Cuocolo, 1941, the type species. Initially, *Choledocystus* was differentiated from *Glypthelmins* mainly by the absence of a seminal receptacle in the former, and by a combination of traits such as the extension of uterine loops to the ventral sucker region, and the position of the genital pore at the level of the caecal bifurcation (Pereira and Cuocolo 1941). The configuration of uterine loops, the shape of the excretory vesicle, and the possible presence of a small, sucker-like structure surrounding the genital atrium in *Choledocystus* were characters added by Ruiz (1949) to distinguish *Choledocystus* from *Glypthelmins*. Light-microscopical and SEM study of several specimens identified as *G. hepatica* from Mexico, Costa Rica and Venezuela, and of several specimens of *G. elegans* from Panama and Uruguay, allowed us to confirm the characters that were used by Pereira and Cuocolo (1941) and Ruiz (1949) to differentiate *Choledocystus* from *Glypthelmins*. In addition we found that both species show a ratio of oral sucker/ventral sucker length equal to or higher than 1, a dextral ovary, vitellarium constituted by clusters of acini, and a Y-shaped excretory vesicle. These characters are not shared by the eight species here considered as members of *Glypthelmins*.

SEM observations confirmed the presence of a sucker-like structure around the genital atrium of *Choledocystus hepatica* and *C. elegans* (Figs. 10 and 11). This structure is constituted by several small papillae which under light microscopy give the appearance of a sucker. In contrast, the genital pore of the species of *Glypthelmins* (Figs. 8 and 9) and *Rauschiella* (Figs. 12 and 13) lack such ornamentation. SEM observations showed other traits that should be added to distinguish the genera. *Choledocystus hepatica* and *C. elegans* exhibit a tegument covered with triangular spines extending from the region of the oral sucker to the posterior end of the body. Finally, in *C. hepatica* and *C. elegans* the genital atrium is lateral to the ventral sucker and separated from the latter, whereas in the species of *Glypthelmins* we studied the mid-ventral genital pore is located just anterior to the ventral sucker. Accordingly, we propose that the following species belong to *Choledocystus*: *C. eucharis, C. hepaticus*, *C. elegans*, *C. simulans* and *C. vitellinophilum*; possibly also *G. incurvatum* and *G. ramitesticularis*. Note, however, that this placement based on morphological evidence has been confirmed by molecular data for *C. hepaticus* only.

Re-establishment of *Rauschiella*

Glypthelmins tineri was originally described as *Rauschiella tineri* by Babero (1951), found in the intestine of an unidentified host (''green frog'') from an unspecified locality (''Mexico''). This monotypic genus was differentiated from *Glypthelmins* by a Y-shaped excretory vesicle, uterine coils not extending beyond the posterior margins of the caeca, a coiled seminal vesicle, and a distinctive arrangement of vitellaria (Babero 1951). In addition, Babero transferred *G. repandum* (Rudolphi, 1819) from Brazil, a parasite of *Leptodactylus ocellatus* (L.), to *Rauschiella*. Most authors have treated *Rauschiella* as a valid genus (Yamaguti 1971; Sullivan 1977b; Prudhoe and Bray 1982), but Brooks (1977) and Brooks and McLennan (1993) considered it as a synonym of *Glypthelmins*. Our

results on genetic divergence and the phylogenetic hypothesis support the recognition of *Rauschiella* as a valid genus. In a revision of *Rauschiella*, Sullivan (1977b) included the following species in this genus: *R. tineri, R. repandum, R. palmipedis* (Lutz, 1928) from *Rana palmipes* Spix from Venezuela, *R. sera* (Cordero, 1944) from *Chthonerpeton indistinctum* Reinhardt and Lütken, and *R. proxima* (Teixeira de Freitas, 1941) from *Leptodactylus ocellatus* (L.), the latter two from Uruguay. We examined specimens of *R. tineri, R. repandum, R. sera* and *R. proxima*, as well as of *Glypthelmins chaquensis*, *G. lenti, G. linguatulus*, *G. palmipedis*, *G. poncedeleoni*, *G. robustus*, *G. rugocaudata*, and *G. staffordi* and observed that these species share a coiled seminal vesicle (except *R. proxima*), a Y-shaped excretory vesicle, an intercaecal uterus, a dextral ovary, and a characteristic arrangement of the vitellarium. In addition we examined specimens of *G. tineri, G. poncedeleoni* and *G. linguatula* by SEM and observed that they possess a tegument covered with small, smooth, spine-like scales (Figs. 6 and 7) which clearly differ from the serrated and triangular spines in *Glypthelmins* (Figs. 2 and 3) and *Choledocystus* (Figs. 4 and 5). *Rauschiella* also is characterized by the medial genital pore lacking any papillae-like structures. Consequently, we consider the following species as members of *Rauschiella*: *R. tineri* and *R. poncedeleoni*, as well as *R. chaquensis, R. lenti, R. linguatula, R. palmipedis, R. proxima, R. repandum, R. robusta, R. rugocaudata, R. sera,* and *R. staffordi*; possibly also *G. pseudium* and *G. sanmartini*. Note, however, that this placement based on morphological evidence has been confirmed by molecular data for *R. tineri* and *R. poncedeleoni* only.

Systematic position of *Glypthelmins* within the Plagiorchiida

In the MP and ML analyses *Glypthelmins* sensu stricto resulted as the sister group of the genus *Haematoloechus*, which was represented by seven species in this study. This relationship is weakly supported (<50%, 2), but it is consistent with the phylogenetic hypothesis of Olson et al. (2003), in which *Glypthelmins quieta* forms the sister species of *Haematoloechus longiplexus* + *Skrjabinoeces similis*. Based on morphological grounds and life cycle characters, *Glypthelmins* sensu lato has been placed in three different 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). This fluctuation might have been the result of inadequate definition of the genus in the past, since it included members of at least two other genera.

Molecular data provided in this study show that *Glypthelmins* sensu stricto represents an independent, strongly supported lineage that is not the sister group of any of the aforementioned families. This means that the genus does not belong to Plagiorchiidae, Brachycoelidae or Macroderoididae. Our results partially coincide with those of Tkach et al. (2001a). In these authors' phylogenetic hypothesis based on 28S ribosomal gene sequences *Glypthelmins* formed a separate clade, with no close affinities to any other family. In contrast, although Olson et al. (2003) found *Glypthelmins* to form the sister group of *Haematoloechus* + *Skrjabinoeces*, they placed all these genera in Plagiorchiidae. Our results do not support this interpretation. Tkach et al. (2001a) pointed out that the limited number of taxa included in their analysis prevented definitive conclusions about the systematic position of *Glypthelmins*. However, in their opinion the unusual life cycles of members of this genus suggested that the genus may represent a separate evolutionary lineage within Plagiorchioidea. Our results support this contention. Based on the limited amount of available information, two alternative taxonomic arrangements are conceivable. (1) A new family could be established for the monophyletic *Glypthelmins* sensu stricto. This monotypical new family, however, would constitute a redundant category undesirable in phylogenetic classification. (2) *Glypthelmins* and *Haematoloechus* could be included in a monophyletic Haematoloechiidae. However, it has to be considered that current support for the relationships underlying either solution is very low; further evidence from life cycles and DNA sequences of additional species of *Glypthelmins*, as well as from members of related genera, is needed.

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