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Phylogenetic relationships and systematic position of the enigmatic *Urotrema* Braun, 1900 (Platyhelminthes: Digenea)

Vasyl V. Tkach[a,*], Stephen E. Greiman[b], Eric E. Pulis[c], Daniel R. Brooks[d,e], Carlos Carrion Bonilla[f,g]

[a] Department of Biology, University of North Dakota, Grand Forks, ND, USA
[b] Department of Biology, Georgia Southern University, Statesboro, GA, USA
[c] Department of Science and Mathematics, Northern State University, Aberdeen, SD, USA
[d] H. W. Manter Laboratory of Parasitology, University of Nebraska State Museum, Lincoln, NE, USA
[e] Evolutionary Systems Research Group, Centre for Ecological Research, Hungarian Academy of Sciences, Tihany, Hungary
[f] Department of Biology and Museum of Southwestern Biology, University of New Mexico, Albuquerque, USA
[g] Zoological Museum, School of Biological Sciences, Universidad Católica del Ecuador, Quito, Ecuador

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ABSTRACT

The systematic position of *Urotrema* Braun, 1900 and the family Urotrematidae Poche, 1926 have always been controversial. Due to its unusual morphological characteristics, lack of knowledge of the life cycle or details of its excretory system, this family was placed within different higher taxonomic groups of digeneans. Despite being one of the most enigmatic digenean families in terms of its phylogenetic affinities, DNA sequence data for Urotrematidae were lacking. Here, we evaluate the phylogenetic relationships of *Urotrema* using newly obtained partial sequences of the 28S rRNA gene from *Urotrema* specimens collected in North, Central and South America including the type species *U. scabridum* Braun, 1900, as well as previously published sequences of digeneans. Our study has demonstrated that *Urotrema* is phylogenetically closest (100% branch support) to members of *Parabascus* Looss, 1907 belonging to the family Pleurogenidae Looss, 1899. Thus, the family Urotrematidae becomes a junior synonym of the Pleurogenidae. *Urotrema* forms a 100% supported clade among the Pleurogenidae, parasitic in warm-blooded vertebrates. However, the phylogenetic relationships and exact systematic position of the remaining 3 genera currently placed in the Urotrematidae remains unclear and requires additional studies as their allocation is mostly based on the terminal posterior position of the genital pore and cirrus-sac. According to our results the genus *Parabascus* appears to be paraphyletic and requires further detailed phylogenetic and morphological analyses.

1. Introduction

The family Urotrematidae Poche, 1926 is a small group of trematodes parasitic in bats, lizards and fishes. They have been differentiated from other digenean groups by the terminal genital pore at the posterior end of the body, cirrus-sac located at the posterior end, pretesticular ovary and vitellarium consisting of mostly lateral fields of numerous small follicles [1]. Life cycles and larval morphology of the Urotrematidae remain completely unknown. The family currently includes 4 genera: *Urotrema* Braun, 1900, *Urotrematulum* Macy, 1933, *Sinineobucephalopsis* Zhang, Pan & Li, 1987 and *Sinogastromyzontrema* Li, Zhang & Li, 1988 [1]. Members of *Urotrema* are known mostly from bats and lizards in the Americas with *U. aelleni* Baer, 1957 from bats in the Ivory Coast being a notable exception [1–3]. Monotypic *Urotrematulum* is a rare trematode known only from North American bats [4,5]. Representatives of *Sinineobucephalopsis* and *Sinogastromyzontrema* are parasites of fishes in China and are a relatively recent addition to the Urotrematidae. Before Bray et al. [3] tentatively transferred these two genera into the Urotrematidae they belonged to a separate family Sinineobucephalidae Zhang, Pan & Li, 1987. However, even before the inclusion of the forms parasitic in fishes, the systematic position of the Urotrematidae has always been unstable and its phylogenetic relationships remained unknown.

In the course of its convoluted taxonomic history the Urotrematidae was included in different higher taxa, usually either in the superfamily Plagiorchioidea Dollfus, 1930 or the Telorchioidea Looss, 1899. As an example of more extreme views, Yamaguti [6] brought up resemblance and possible affinity of this family with the Leucochloridiidae Poche,

* Corresponding author at: Department of Biology, University of North Dakota, Grand Forks, ND 58202, USA.
E-mail addresses: vasyl.tkach@und.edu (V.V. Tkach), sgreiman@georgiasouthern.edu (S.E. Greiman), eric.pulis@northern.edu (E.E. Pulis).

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1907 which belongs to a different order of digeneans. More details of the taxonomic history of this family can be found in Bray et al. [3]. We would only like to mention here that all previous attempts of systematic placement of the family using formal phylogenetic analyses were based solely on morphology [7,8]. They were overall inconclusive, with two of these works placing urotrematids in the Telorchioidea using a single morphological synapomorphy of unknown systematic value. Other authors who attempted to reconstruct the phylogeny of digeneans of the suborder Plagiorchiata, based primarily on larval characteristics and/or the organization of the excretory system, were able to only provisionally place the Urotrematidae in the Plagiorchioidea [9–11] due to the lack of sufficient data on the excretory system and the complete lack of knowledge of their life cycles. Olson et al. [12] in their phylogenetic study of the Digenea, that until now remains the most comprehensive molecular phylogenetic analysis of the subclass, considered lack of knowledge of the phylogenetic affinities of the Urotrematidae as one of the crucial omissions in their work and one of the most important questions to be addressed in the future.

In the present study, we evaluate the phylogenetic relationships of *Urotrema*, the type genus of the Urotrematidae, using newly obtained partial sequences of the nuclear large ribosomal subunit DNA of 3 *Urotrema* species from North, Central and South America, including the type species of the genus, *Urotrema scabridum* Braun, 1900. Systematic changes are proposed based on the results of the phylogenetic analysis.

2. Material and methods

2.1. Specimen collecting

Specimens representing 3 species of *Urotrema* were collected between 2004 and 2017 from the intestines of bats and lizards in the USA, Costa Rica, Panama and Ecuador (Table 1) in accordance with the collecting permits issued by governmental authorities in corresponding countries. Ecuadorian specimens were collected and transferred under permit No 064-FAU-2017-DPAP-MA and loaned to University of North Dakota (Vasyl Tkach) under exportation permit No 11-2016-FAU-DPAP-MA. The specimens from Panama were collected and exported under permits issued to Dr. Joseph A. Cook (Museum of Southwestern Biology, University of New Mexico). Upon removal from the host's intestine parasites were rinsed in saline, killed with hot water and immediately fixed in 70% ethanol for enabling both molecular and morphological studies. Morphological vouchers of digeneans were stained with alum carmine, dehydrated in ethanol series, cleared in clove oil and mounted permanently in Damar gum [13]. Specimens were identified using original and subsequent descriptions, and deposited on slides as permanent total mounts in the collection of the Museum of Southwestern Biology (MSB), University of New Mexico (Table 1). Voucher host specimens from Ecuador and Panama were deposited (Table 1) in the MSB and Sección Mastozoología. Museo de Zoología, Escuela de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador (QCAZ-M).

2.2. DNA extraction, amplification and sequencing

Two specimens of *U. scabridum* (one from Panama and one from Ecuador), one specimen of *U. minuta* Macy, 1933 and 2 specimens of *U. shirleyae* Zamparo, Brooks and Tkach, 2005 were used for the molecular study. Specimens of *U. shirleyae* came from the same host individual as the type series used in the original description by Zamparo et al. [14]. Genetic data of the Ecuadorian specimen were obtained under Genetic Resources Access Contract No MAE-DNB-CM-2016-0042 issued by Ministerio del Ambiente del Ecuador to Pontificia Universidad Católica del Ecuador. Genomic DNA was extracted from single digenean specimens following the method described by Tkach & Pawlowski [15] or using a ZR Genomic DNA™ Tissue Micro Prep kit (Zymo Research, Irvine, California, USA) following the manufacturer's protocol. An approximately 1350 base pair (bp) long fragment at the 5′ end of the 28S rRNA gene (*lsrDNA*) was amplified using forward primer dig12 (5′- AAG CAT ATC ACT AAG CGG - 3′) and reverse primer 1500R (5′- GCT ATC CTG AGG GAA ACT TCG - 3′) [16]. PCR reactions were performed in a total volume of 25 µl using OneTaq Master Mix (New England Biolabs, Ipswich, Massachusetts, U.S.A.) following the manufacturer's instructions. Annealing temperature during the cycling was 53 °C and extension time was 1.5 min.

Positive PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, California, U.S.A.) and cycle-sequenced directly using BrightDye® Terminator Cycle Sequencing Kit (MCLAB, California, USA). PCR primers mentioned above and the additional reverse internal primer ECD2 (5′- CTT GGT CCG TGT TTC AAG ACG GG - 3′) were used for sequencing. After sequencing reactions, DNA was cleaned-up using magnetic beads from McLab (San Francisco, CA) and run on an ABI 3100 DNA sequencer (Applied Bio systems, Foster City, California, U.S.A.). Forward and reverse sequences were visualized, edited and assembled using Sequencher v.5.0.1 (Gene Codes Corp., Ann Arbor, Michigan, U.S.A.), with the resulting sequences deposited in GenBank (Table 1).

2.3. Phylogenetic analysis

A preliminary phylogenetic analysis incorporating representatives of a broad variety of digenean lineages (not shown) as well as a BLAST search of the GenBank database has demonstrated that our sequences of *Urotrema* spp. were closest to multiple representatives of the superfamily Microphalloidea with a high degree of similarity. Therefore, for phylogenetic analysis the newly obtained *Urotrema* sequences were aligned with matching sequences of a broad variety of microphalloidean digeneans available in GenBank, using ClustalW implemented in Mega7 [17]. *Plagiorchis vespertilionis* (Müller, 1780) (GenBank AF151931) was included in the alignment as outgroup based on the results of previously published higher-level phylogenies [12,18]. The GTR + I + G model of nucleotide substitution as determined by jModelTest 2 [19] was implemented to reconstruct a Bayesian inference (BI) phylogeny using Mr. Bayes v.3.2.6 [20]. Markov chain Monte Carlo

Table 1
Urotrema spp. sequences used in the phylogenetic analysis and associated geographic and host information.

Urotrema species	Host species	Geographic origin	Parasite voucher	Host vouchers	GenBank No.
U. scabridum	*Eptesicus innoxius*	Reserva Otongachi, Pichincha, Ecuador; 0°19′14.5”S, 78°57′06.2”W	MSB Para28800	QCAZ-M 17184	MK477545
U. scabridum	*Molossus molossus*	El Llano, Chepo District, Panama Province, Panama; 9° 18′ 39.42”N, 78° 59′ 4.31”W	MSB Para28801	MSB NK263316	MK477546
U. minuta	*Lasiurus seminolus*	Pascagoula wildlife management area, Jackson County, Mississippi, USA; 30° 39′ 14.4”N, 88° 38′ 2.4”W	MSB Para28794	–	MK477547
U. shirleyae	*Anolis oxylophus*	Area de Conservación Guanacaste, Guanacaste Province, Costa Rica; 10°50′15.5”N, 85°37′08.8”W	MSB Para28802	–	MK477548, MK477549

(MCMC) chains were run for 1,000,000 generations at which point the standard deviation of split frequencies stabilized well below 0.01. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees. The trees were visualized in FigTree ver. 1.4 software [21] and annotated in Adobe Illustrator®.

3. Results

The alignment of the *lsrDNA* (n = 36) fragment was 1229 bp long with 48 ambiguously aligned nucleotide positions excluded from the analysis. The topology of the tree resulting from BI analysis (Fig. 1) strongly resembled the results of the recent phylogenetic studies of the Microphalloidea [22,23] with the exception of the addition of *Urotrema* spp. that were missing in previous publications. The phylogenetic tree (Fig. 1) demonstrated very high nodal support for the majority of topologies. Species of *Urotrema* formed a 100% supported clade which in turn formed a 100% group together with 3 species of *Parabascus* Looss, 1907. The clade *Parabascus* + *Urotrema* was a part of a strongly supported larger clade of the Pleurogenidae (Fig. 1).

Sequenced region of the 28S gene was of equal length in all 3 *Urotrema* species used in our study. They showed no intraspecific sequence variability among 2 samples of *U. shirleyae* from different lizard individuals from Costa Rica or among 2 specimens of *U. scabridum* coming from different bat species in Panama and Ecuador. At the same time, interspecific differences in 28S varied from 0.9% between *U. shirleyae* and *U. scabridum* to 1.7% between *U. shirleyae* and *U. minuta*.

4. Discussion

As mentioned in the Introduction, the systematic and taxonomic history of *Urotrema* and the Urotrematidae was tortuous. Based on morphological characteristics, mainly the position of the testes and posterior, terminal position of the genital atrium and cirrus-sac, different authors included it in the Telorchioidea, Plagiorchioidea and even contemplated their possible close relationship with the Leucochloridiidae [1,3,6]. However, urotrematids have never been previously associated with the Microphalloidea, therefore the results of our phylogenetic study were rather unexpected. The BI analysis firmly placed a 100% supported *Urotrema* clade as a nested group among members of *Parabascus* (Fig. 1), a pleurogenid genus that includes digeneans parasitic mostly in bats and rarely in lizards just like *Urotrema*.

The two genera have significant morphological differences such as tandem, usually posteriorly positioned, testes in *Urotrema* vs. opposite or diagonal testes in *Parabascus*, posterior terminal position of the genital atrium and cirrus-sac in *Urotrema* vs. located in the middle third of the body in *Parabascus*, and vitelline follicles positioned more anteriorly in *Parabascus* than in *Urotrema*. Despite these obvious differences the two genera share common traits, e. g., vitellarium consisting of numerous follicles with a tendency of forming lateral groups or fields, caeca extending posteriorly beyond the level of the testes and sometimes nearly reaching the posterior end of the body and organization of the cirrus-sac.

Unfortunately, the details of the excretory system in *Urotrema* remain unknown. Moreover, to the best of our knowledge, none of the available descriptions of *Urotrema* provided illustrations of the excretory vesicle while it is usually referred to as either V-shaped or Y-shaped with a very short stem [1]. The same description is provided for the excretory vesicle of *Parabascus* [24]. We were unable to study the details of the excretory system from fixed specimens of *Urotrema* because only live, preferably juvenile specimens allow to reveal the protonephridial formula and other aspects of the excretory system organization. Nevertheless, due to the high quality of our specimens we were able to at least partially cover this gap in the knowledge of *Urotrema* anatomy. The excretory vesicle in our specimens of *U. scabridum* and *U. minuta* was essentially intermediate between the two types, and could be called V-shaped with a very short single stem at the base (Fig. 2). Noteworthy, the dextral and sinistral common excretory ducts begin not at the apex of excretory vesicle arms, but from their inner surface at some distance posterior to the apex (Fig. 2). This condition has not been reported in *Parabascus*. Besides, the branches of the excretory vesicle in *Urotrema* are relatively shorter than those in *Parabascus* [25].

Although the terminal position of the genital atrium and cirrus-sac in *Urotrema* is a feature that sets it apart from almost all other members of the Pleurogenidae, this difference is not as dramatic as it seems. Members of the Pleurogenidae demonstrate a wide variability of the position of the genital atrium [24,27]. It varies significantly even among closely related genera (Fig. 1), such as, *Pleurogenes* Looss, 1896 (genital pore antero-lateral, close to the oral sucker), *Pleurogenoides* Travassos, 1921 (genital pore generally antero-lateral, at varying distance from the ventral sucker), *Prosotocus* Looss, 1899 (genital pore lateral at varying distance from oral sucker) and *Brandesia* Stossich, 1899 (genital pore lateral, close to the posterior end of the body). Therefore, a shift of the genital pore to the posterior end seems, to us, entirely feasible. Similarly, albeit less extreme, the position of the genital pore in *Parabascus* (genus most closely related to *Urotrema* according to the molecular phylogeny) also changes quite significantly from various positions in the general ventral sucker area to substantially posterior to the ventral sucker as in *P. joannae* (Zdzitowiecki, 1967) [24,26]. Moreover, as recently demonstrated by Kanarek et al. [28] the genus *Cortrema* Tang, 1951 belongs to the Pleurogenidae. This genus is characterized by a posterior terminal genital atrium while lacking a cirrus-sac. To summarize, the position of the genital atrium among members of the Pleurogenidae varies broadly and its terminal position in *Urotrema* is not a feature that is entirely out of the pattern seen elsewhere in this large digenean family. Therefore, based on the highly convincing results of the phylogenetic analysis as well as morphological considerations, we transfer the genus *Urotrema* into the family Pleurogenidae with the Urotrematidae becoming its junior synonym. The phylogenetic relationships and exact systematic position of the remaining 3 genera currently placed in the Urotrematidae remains unclear. In part, we anticipate that the similarity between two genera from fishes in China, *Sinineobucephalopsis* and *Sinogastromyzontrema*, and the remaining urotrematids will likely prove to be homoplasious and these taxa will need to be removed from the Urotrematidae. However, additional studies including molecular phylogenetic analysis are required to adequately address this question.

Our phylogenetic analysis suggests a paraphyletic nature of *Parabascus* with *P. duboisi* forming a branch separate from the clade comprising *P. semisquamosus* and *P. joannae* (Fig. 1). The authors of the present work had in their disposition representatives of these 3 species. Examination of total mounts did not reveal obvious features in adult digeneans that warrant separation of *P. duboisi* into a different genus. It should be noted that at the time of the original description, *P. duboisi* (a European species) was included by Hůrkova [29] into the genus *Limatulum* Travassos, 1921 characterized by short caeca and New World distribution. However, details of the excretory system of either *P. duboisi* or of any member of *Limatulum* are currently unknown. The question of proper systematic position of *Parabascus* species is beyond the scope of the present study and cannot be resolved without additional morphological data and more extensive taxon sampling for DNA sequencing.

In conclusion, our phylogenetic analysis allowed to resolve the long standing and intriguing question regarding the phylogenetic affinities and systematic position of *Urotrema* and the Urotrematidae. Along with other recent publications on the Microphalloidea [22,28,30] it is yet another step toward re-organization and improvement of the systematics of this highly diverse, derived lineage of digeneans.

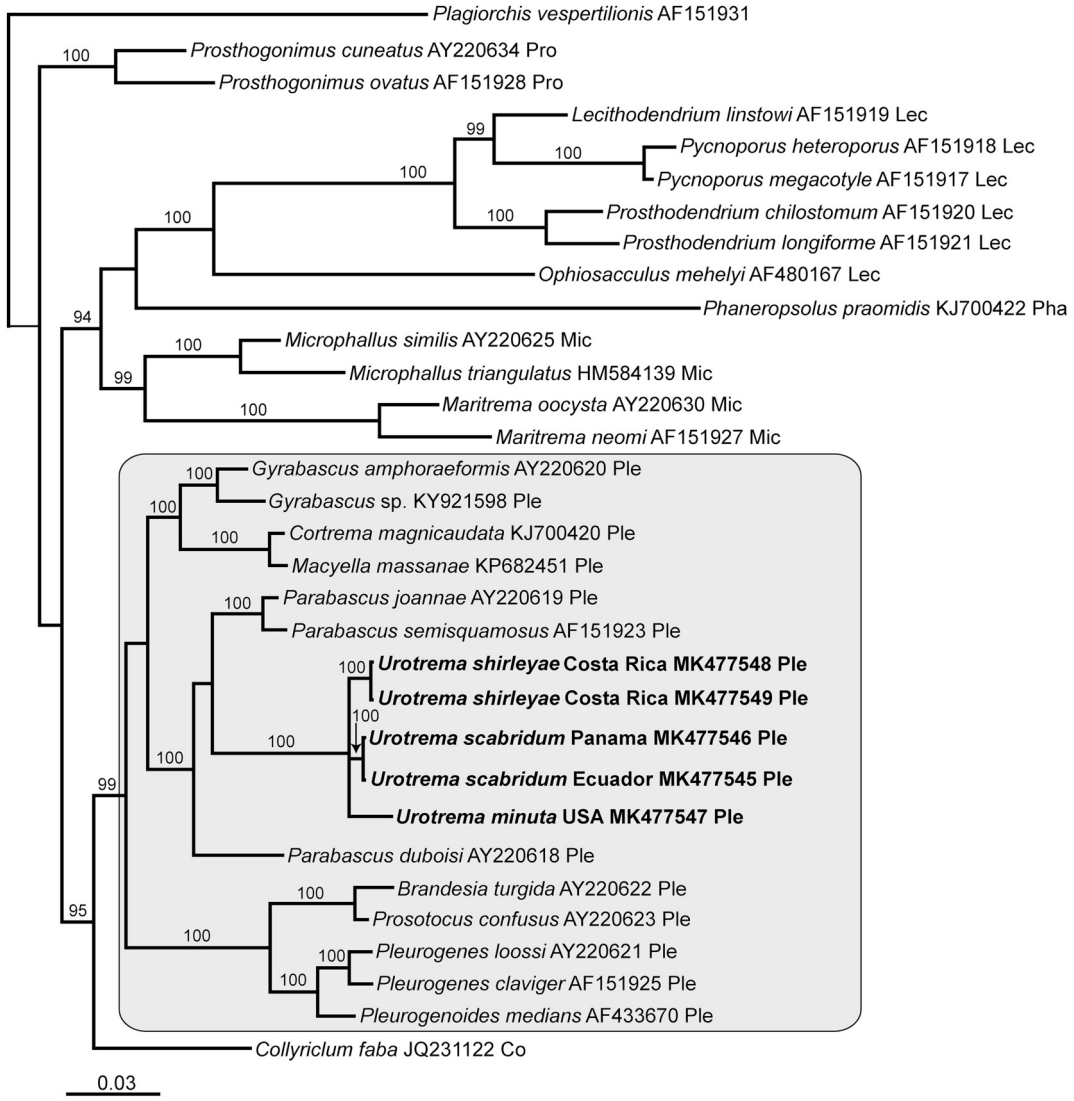

Fig. 1. Phylogenetic relationships among 32 taxa of the Microphalloidea resulting from Bayesian analysis of partial sequences of the 28S rRNA gene. Posterior probabilities greater than 80% are shown above internodes. Shaded rectangle indicates the Pleurogenidae. *Urotrema* species sequenced in the present paper are in bold. Abbreviations: Mic, Microphallidae; Ple, Pleurogenidae; Co, Collyriclidae; Pro, Prosthogonimidae; Pha, Phaneropsolidae; Lec, Lecithodendriidae.

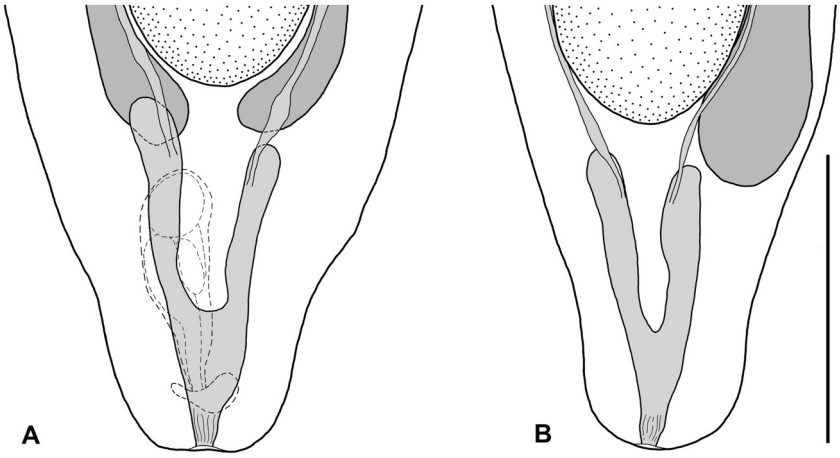

Fig. 2. Posterior end of body showing excretory vesicle of two specimens of *Urotrema scabridum* from *Eptesicus innoxius*. Uterus and details of male reproductive organs not shown for clarity (fig. A shows outlines of genital atrium and cirrus-sac). Fragment of posterior testis and ends of ceca are shown to better present relative length of excretory vesicle arms. Note the antero-lateral branching of common excretory ducts from arms of excretory vesicle at the same position in both specimens. (scale bar: A, B = 500 μm).

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