



# Convoluted history and confusing morphology: Molecular phylogenetic analysis of dicrocoeliids reveals true systematic position of the Anenterotrematidae Yamaguti, 1958 (Platyhelminthes, Digenea)

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## ABSTRACT

The Dicrocoeliidae is a highly diverse family of digeneans parasitic in amniotic tetrapods. Detailed molecular phylogenetic analysis of dicrocoeliids is lacking and only a few dicrocoeliids from mammals have been included in previous studies. Sequence data were previously absent for the Anenterotrematidae that shares several morphological characteristics with dicrocoeliids. We examined phylogenetic affinities of several newly sequenced (nuclear 28S rDNA) taxa of dicrocoeliids and anenterotrematids collected from small mammals in Ecuador, Panama, Peru, USA and Vietnam. Our analyses demonstrated that the two anenterotrematid genera (*Anenterotrema*, *Apharyngotrema*) belong to the Dicrocoeliidae, placing the Anenterotrematidae into synonymy with the Dicrocoeliidae. Molecular data combined with morphological examination of type and new specimens provided evidence that *Parametadelphis* and *Apharyngotrema* are junior synonyms of *Metadelphis*, with all *Metadelphis* species lacking a digestive system. Phylogenetic analysis demonstrates that reduction of the alimentary tract in *Lutztrema* and its loss in *Anenterotrema* and *Metadelphis* represent at least two independent evolutionary events. Genera *Brachylecithum*, *Brachydistomum*, and *Lyperosomum* proved to be non-monophyletic, each likely representing more than a single genus. Furthermore, phylogenetic analysis did not support monophyly of the two largest subfamilies of the Dicrocoeliidae (Dicrocoeliinae and Leipertrematinae) with the other two subfamilies not included in this study. Therefore, we propose to abandon the current subfamily division of the Dicrocoeliidae. Analysis of host associations indicates multiple host-switching events throughout evolution of dicrocoeliids. Lastly, analysis of dicrocoeliid geographic distribution revealed that nearly all major clades included taxa from more than a single zoogeographic realm with the exception of the clade *Anenterotrema* + *Metadelphis*, found only in the Neotropics.

## 1. Introduction

The Dicrocoeliidae Looss, 1899 is a globally distributed, highly diverse family that includes digeneans parasitic as adults in amniotic tetrapods, predominantly birds. Dicrocoeliids have terrestrial life cycles utilizing terrestrial mollusks and arthropods as intermediate hosts [1,2]. The vast majority of dicrocoeliids parasitize the gallbladder and bile ducts of their definitive hosts, while relatively few taxa inhabit the intestine and, exceptionally, other organs [2]. The family-level taxon for these digeneans was originally established by Looss [3] as the subfamily Dicrocoeliinae Looss, 1899 to incorporate merely four genera. In contrast, the most recent revision of the family by Pojmańska [2] recognizes four subfamilies: Dicrocoeliinae (18 genera),

Leipertrematinae Yamaguti, 1958 (24 genera), Proacetabulorchiinae Odening, 1964 (2 genera), and Prosolecithinae Yamaguti, 1971 (3 genera). Thus, after the recent removal of *Infidum* Travassos, 1916 from the Dicrocoeliidae [4] the family currently contains 46 genera and more than 400 species.

Relatively few dicrocoeliids have been described from mammalian hosts and their relationships with taxa from other host groups, particularly those from birds, are unclear. The situation is aggravated by the fact that the systematics and phylogenetic interrelationships of numerous taxa within the Dicrocoeliidae as a whole are poorly understood due to high levels of morphological homogeneity across genera and insufficient knowledge of the extent of their intraspecific variability [2,5–7]. DNA sequence data provide an obvious complementary source

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of characters for phylogenetic analysis. However, a broad phylogenetic analysis of the family is currently lacking with only a few dicrocoeliid taxa incorporated in past molecular phylogenetic studies; in most cases such studies were focused on a single species or genus and included almost exclusively species parasitic in avian hosts [6–10].

Previous molecular studies have clarified the systematic position of some taxa included in the Dicrocoeliidae. For instance, the genus *Infidum* has been recently removed from the Dicrocoeliidae based on partial sequences of the nuclear ribosomal 28S gene [4]. Currently, only six of the 46 dicrocoeliid genera have partial 28S rDNA sequences available in GenBank and only three species of mammalian dicrocoeliids representing just two genera have been included in previous phylogenetic analyses [6,7]. No representatives of the somewhat enigmatic family Anenterotrematidae Yamaguti, 1958 (includes only two genera, *Anenterotrema* Stunkard, 1938 and *Apharyngotrema* Marshall et Miller, 1979) have ever been sequenced or included in phylogenetic analyses. It is noteworthy that at least one genus in the family, namely *Apharyngotrema*, is morphologically very similar to typical dicrocoeliids and occurs in the gallbladder.

Herein, we used previously available and newly obtained partial 28S sequences to examine phylogenetic relationships of several species and genera of dicrocoeliids and anenterotrematids collected from shrews, bats and rodents from North, Central and South America, Europe, and Southeast Asia. The results of our study are presented below.

## 2. Material and methods

### 2.1. Morphological data

Specimens used in the present study belonged to the families Dicrocoeliidae and Anenterotrematidae. They were collected from the gallbladder, liver or intestine of several species of bats and one species of rodent in Ecuador, Panama, Peru, United States and Vietnam between 2013 and 2017 (Table 1). Live digeneans removed from host's

organs were briefly rinsed in saline, killed with hot water and fixed in 70% ethanol. Specimens for light microscopy were stained with aqueous alum carmine, dehydrated in an ethanol series of ascending concentrations, cleared in clove oil, mounted permanently in Damar gum and identified using a DIC-equipped Olympus BX40 compound microscope with a digital imaging system. Upon our request, the curator of the collection at the Oswaldo Cruz Institute (Rio de Janeiro, Brazil), Dr. Marcelo Knoff, kindly examined holotype (accession number CHIOC12348) and paratype (12349 to 12354 a-b) specimens of *Metadelphis evandroi* Travassos, 1944 (the type species of *Metadelphis* Travassos, 1944) deposited in the collection. Dr. Knoff also examined and photographed types of *Metadelphis alvarengai* Travassos, 1955 (holotype CHIOC 21137, paratypes CHIOC 21138–40).

### 2.2. Molecular data

DNA was extracted from either partial adult specimens in cases of larger species or complete specimens in cases of very small specimens. In the former case the remainder of the specimen was mounted as the holo-genotype while in the latter case we examined and photographed specimens placed in water under a cover slip prior to DNA extraction. Genomic DNA was extracted according to the protocol described by Tkach and Pawlowski [14]. An approximately 1300 bp long fragment at the 5' end of the 28S rDNA was amplified by polymerase chain reactions (PCR) on a T100™ thermal cycler (Bio-Rad, Hercules, California, USA) using the forward primer LSU5 (5'–TAGGTCGACCCGCTGAAY-TTAAGCA–3') and the reverse primer 1500R (5'–GCTATCCTG AGG GAAACTTCG–3') [15]. PCRs were performed in a total volume of 25 µl using One-Taq quick load PCR mix from New England Biolabs (Ipswich, Massachusetts, USA) according to the manufacturer's protocol and with an annealing temperature of 53 °C.

PCR products were purified using ExoSap PCR clean-up enzymatic kit (Affimetrix, Santa Clara, California, USA) following the manufacturer's protocol. PCR products were cycle-sequenced directly using ABI BigDye™ 3.1 chemistry (Applied Biosystems, Foster City, California,

**Table 1**

List of dicrocoeliid species used in this study including their host species, geographical origin of material, and GenBank accession numbers.

Taxon	Host	Country	Accession #	Reference
<i>Anenterotrema</i> sp.	<i>Trachops cirrhosus</i>	Ecuador	MH158565	Present study
<i>Anenterotrema</i> sp.	<i>Phyllostomus discolor</i>	Panama	MH158564	Present study
<i>Anenterotrema auritum</i> (Stunkard, 1938)	<i>Glossophaga soricina</i>	Panama	MH158566	Present study
<i>Brachydistomum olsoni</i> (Railliet, 1900)	<i>Apus apus</i>	Czech Republic	KU563712	Heneberg et al., unpublished
<i>Brachydistomum ventricosum</i> (Rudolphi, 1802)	<i>Erethacus rubecula</i>	Czech Republic	KU563713	Heneberg et al., unpublished
<i>Brachylecithum capilliformis</i> (Oshmarin, 1952)	<i>Locustella fluviatilis</i>	Czech Republic	KU212184	[7]
<i>Brachylecithum glareoli</i> (Hildebrand, Okulewicz et Popiołek, 2007)	<i>Myodes glareolus</i>	Poland	KU212203	[7]
<i>Brachylecithum grummi</i> (Odening, 1963)	<i>Attila cinnamomeus</i>	Brazil	KP765768	[6]
<i>Brachylecithum kakea</i> (Bhalerao, 1926)	<i>Acrocephalus arundinaceus</i>	Czech Republic	KU212180	[7]
<i>Brachylecithum laniicola</i> (Layman, 1926)	<i>Lanius collurio</i>	Czech Republic	KU212183	[7]
<i>Brachylecithum lobatum</i> (Railliet, 1900)	<i>Corvus corone</i>	Czech Republic	AY222260	[11]
<i>Brachylecithum</i> sp.	<i>Sylvia atricapilla</i>	Poland	KU563711	Heneberg et al., unpublished
<i>Dicrocoelium dendriticum</i> (Rudolphi, 1819)	<i>Marmota bobak</i>	Ukraine	AF151939	[12]
<i>Dicrocoelium hospes</i> (Looss, 1907)	<i>Bos taurus</i>	–	AY251233	Dittmar, unpublished
<i>Eurytremia pancreaticum</i> (Janson, 1889)	<i>Ovis aries</i>	China	KY490004	[10]
<i>Lutziella microacetabulare</i> (Rohde, 1966)	<i>Hipposideros armiger</i>	Vietnam	MH158562	Present study
<i>Lutztrema attenuatum</i> (Dujardin, 1845)	<i>Turdus philomelos</i>	Czech Republic	KU563714	Heneberg et al., unpublished
<i>Lutztrema monenteron</i> (Price et McIntosh, 1935)	<i>Turdus migratorius</i>	USA	KP765766	[6]
<i>Lutztrema microstomum</i> (Denton et Byrd, 1951)	<i>Cyanocitta cristata</i>	USA	KP765765	[6]
<i>Lutztrema</i> sp.	<i>Acrocephalus arundinaceus</i>	Czech Republic	KT387689	Hildebrand et al., unpublished
<i>Lyperosomum collurionis</i> (Skrjabin et Isaichikov, 1927)	<i>Sylvia atricapilla</i>	Czech Republic	AY222259	[11]
<i>Lyperosomum sarothrurae</i> (Baer, 1959)	<i>Sarothrura pulchra</i>	Uganda	KP765767	[6]
<i>Lyperosomum transcarpathicus</i> (Bychovskaja-Pavlovskaja et Kulakova, 1978)	<i>Sorex minutus</i>	Ukraine	AF151943	[13]
<i>Lyperosomum intermedium</i> (Denton et Kinsella, 1972)	<i>Oryzomys palustris</i>	USA	MH158563	Present study
<i>Metadelphis compactus</i> (Travassos, 1955)	<i>Lonchophylla handleyi</i>	Peru	MH158569	Present study
<i>Metadelphis lenti</i> (Santos et Gibson, 1998)	<i>Lonchophylla robusta</i>	Panama	MH158568	Present study
<i>Metadelphis</i> sp.	<i>Lonchophylla robusta</i>	Panama	MH158567	Present study

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