Denis Jacob Machado

Acanthobothrium Blanchard, 1848:
diversidade filogenética das linhagens
parasitas de arraias de água doce

São Paulo
2012

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Acanthobothrium Blanchard, 1848:
phylogenetic diversity of the lineages
that parasitize freshwater stingrays

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de Biociências da Universidade de
São Paulo, para a obtenção de Título
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Orientador: Fernando Portella de
Luna Marques

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*Com saudades,
a tia Irma e aos amigos distantes.*

What is a master but a master student?

Neil Peart interview to "Rolling Stone", 2012

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Sumário

1	Introdução geral	1
1.1	O papel de <i>Acanthobothrium</i> na Biogeografia Histórica Neotropical	2
1.2	Presente estado do conhecimento taxonômico e filogenético de <i>Acanthobothrium</i>	4
1.3	Objetivos gerais	5
1.4	Organização da dissertação	6
	Referências	10
2	The forgotten origin of <i>Acanthobothrium</i>	11
	Acknowledgements	14
	References	16
3	Diversification and species boundaries of <i>Acanthobothrium</i>	17
3.1	Introduction	17
3.2	Material and methods	18
3.2.1	Taxonomic representation of marine tetraphyllideans	18
3.2.2	Collection of freshwater stingray specimens	19
3.2.3	Collection of cestode specimens	19
3.2.4	DNA extraction, amplification, and sequencing	19
3.2.5	Phylogenetic analysis	21
3.3	Results	26
3.3.1	Molecular dataset	26
3.3.2	Working hypothesis	26
3.3.3	Measure of support and stability	26
3.4	Discussion	28
3.4.1	On the relationship between <i>Acanthobothrium</i> and <i>Uncibilocularis</i>	28
3.4.2	Freshwater <i>Acanthobothrium</i> and intra-specific variation	31
3.4.3	Conflicting patterns of host specificity	33
3.4.4	Phylogenetic relationships among freshwater <i>Acanthobothrium</i>	35
3.4.5	On the polyphyly of freshwater <i>Acanthobothrium</i>	36
	Acknowledgements	37
	References	45

4	Considerações finais	47
4.1	A origem de <i>Acanthobothrium</i>	47
4.2	<i>Acanthobothrium</i> é monofilético?	47
4.3	Clados negligenciados	48
4.4	Relações filogenéticas entre <i>Acanthobothrium</i> dulcícolas	49
	Referências	52
	Resumo	53
	Abstract	55
	Anexos	57
A.1	Collection of freshwater stingrays	59
A.2	Coleta e fixação do material biológico	62
A.3	Preparação de lâminas de holoenóforos	63
A.4	Original cestode material collected in South America	64
A.5	Protocolo otimizado para extração de DNA genômico com DNAdvance™	71
A.6	Protocolo otimizado para purificação de DNA genômico com AMPure™ kit	74
A.7	Sequence data acquired from GenBank	75
A.8	Sequence data acquired from Fyler (2009)	76
A.9	Strict consensus tree	83
A.10	Cladogram with branch lengths representing the number of transformations	85
A.11	Descrição dos anexos eletrônicos	87

Lista de Tabelas

3.1	Primers used for PCR amplification and cycle sequencing	21
3.2	Tree lengths from the OA combined analysis	26
3.3	Mickevich-Farris Extra Steps Index (MFES) calculations	28
A.1	Potamotrygonid stingray species and localities sampled in this study	59
A.2	Original cestode material collected in South America	64
A.3	GenBank data for Proteocephalidea and <i>Acanthobothrium</i>	75
A.4	Specimens of <i>Acanthobothrium</i> sequenced by Fyler (2009)	76

Lista de Figuras

3.1	Collection sites of potamotrygonid specimens	20
3.2	Flow chart showing the main steps of the combined DO search strategy	23
3.3	Main steps of the Goodman-Bremer calculation	25
3.4	Strict consensus of the six trees of parameter 2:1:1:1	27
3.5	Sensitivity plot	29
3.6	Possible scenarios for the freshwater invasion	38
A.1	Complete strict consensus tree with support and stability measures	83
A.2	Cladogram with branch lengths proportional to the number of transformations.	85

Introdução geral

Acanthobothrium Blanchard, 1848 (Eucestoda: Tetraphyllidea: Onchobothriidae) é um helminto caracterizado por botrídios triloculados que portam um par de ganchos bífidos (Southwell, 1925). Como os demais tetrafilídeos, este helminto atinge a maturidade no interior das válvulas espirais de tubarões e arraias (Campbell & Beveridge, 2002; Zschoche *et al.*, 2011). *Acanthobothrium* foi alvo de diversas revisões (*e.g.*, Linton, 1925; Baer, 1948; Goldstein, 1967; Williams, 1969) motivadas por interesses diversos, entre os quais desponta seu número crescente de espécies. Por exemplo, Goldstein (1967) reportou 44 espécies válidas e, apenas dois anos mais tarde, Williams (1969) reportou 69 espécies válidas. Especula-se que atualmente hajam mais de 200 espécies nominais de *Acanthobothrium* (Campbell & Beveridge, 2002; Ivanov, 2005; Fyler, 2009; Cardoso, 2010; Zschoche *et al.*, 2011). A verificação do status taxonômico destas espécies e do número exato de espécies válidas para este gênero aguarda uma revisão taxonômica que certamente só será alcançada através do esforço conjunto de diversos pesquisadores.

A variação morfológica inter e intraespecífica de *Acanthobothrium* é elevada e constitui um fator complicante para a resolução taxonômica do gênero. De acordo com Fyler (2009) e Zschoche *et al.* (2011), *Acanthobothrium* está representado por formas que variam desde os maiores aos menores membros de Onchobothriidae (*e.g.*, *Acanthobothrium macracanthum* Southwell, 1925, que atinge cerca de 21 cm de comprimento total, e *A. gnomus* Reyda & Caira, 2006, com aproximadamente 1 mm de comprimento total). No gênero estão incluídas desde espécies com apenas três proglótides (*e.g.*, *Acanthobothrium tripartium* Williams, 1969) a espécies com mais de 950 proglótides (*e.g.*, *Acanthobothrium grandiceps* Yamaguti, 1952). Também é grande a diversidade de formas e tamanhos dos ganchos (Cardoso, 2010). Uma vez que raramente são encontrados caracteres diagnósticos para os membros de *Acanthobothrium*, suas espécies são muitas vezes delimitadas por combinações únicas de caracteres amplamente difundidos entre membros do gênero e demais tetrafilídeos (Fyler, 2009). Grande parte destes caracteres são baseados na média ou nos limites de distribuição

de variáveis quantitativas, como comprimento total ou número de testículos (*e.g.*, Brooks & Amato, 1992; Berman & Brooks, 1994; Healy, 2006; Caira *et al.*, 2007), resultando na definição ambígua de muitas espécies nominais (Cardoso, 2010).

Além de seu elevado número de espécies e plasticidade fenotípica, *Acanthobothrium* impressiona por sua distribuição cosmopolita, ampla variedade de hospedeiros e elevada especificidade. Estima-se que aproximadamente 130 espécies de *Acanthobothrium* parasitem membros de cinco das seis ordens de arraias (*i.e.*, Myliobatiformes, Rajiformes, Rhiniformes, Rhinobatiformes e Torpediniformes) e cerca de 80 espécies sejam encontradas em cinco das oito ordens de tubarões (*i.e.*, Carcharhiniformes, Heterodontiformes, Orectolobiformes, Pristiophoriformes e Squatiniformes; veja Fyler, 2009). As espécies marinhas de *Acanthobothrium* apresentam alta especificidade a seus hospedeiros (*sensu* Euzet & Combes, 1980), o que é consistente com o padrão observado para os demais oncobotrídeos, que estão entre os grupos de parasitas com maior especificidade a seus hospedeiros (Caira & Jensen, 2001). Nos rios da América do Sul, espécies de *Acanthobothrium* são encontradas parasitando membros de Potamotrygonidae (Chondrichthyes: Myliobatoidei).

1.1 O papel de *Acanthobothrium* na Biogeografia Histórica Neotropical

Os potamotrigonídeos são endêmicos da região Neotropical e constituem os únicos elasmobrânquios estenoalinos a viverem exclusivamente na água doce. A família compreende mais de 20 espécies válidas e pelo menos 10 novas espécies a serem descritas (Marques, 2000; de Carvalho *et al.*, 2003; Rosa *et al.*, 2008; da Silva & de Carvalho, 2011b,a; de Carvalho & Lovejoy, 2011a; de Carvalho & Ragno, 2011; de Carvalho *et al.*, 2011), distribuídas em quatro gêneros: *Paratrygon* Dumeril, 1865; *Potamotrygon* Garman, 1913; *Plesiotrygon* Rosa, Castello & Thorson, 1987; e *Heliotrygon* de Carvalho & Lovejoy, 2011. Os potamotrigonídeos podem ser encontrados nas principais bacias hidrográficas da América do Sul, incluindo as bacias dos rios Atrato e Magdalena (Colômbia), Orinoco (Venezuela), Lago de Maracaibo (Venezuela), Parnaíba (MA, PI), Uruguai, Paraná e Paraguai, além da bacia Amazônica (Ecuador, Colômbia, Peru, Bolívia e Brasil) e dos rios das Guianas e do Suriname (Thorson, 1970; Thorson *et al.*, 1978, 1983; Rosa, 1985; Nishida, 1990; Lovejoy, 1996; de Carvalho *et al.*, 2003; Rosa *et al.*, 2008).

A convoluta história biogeográfica dos potamotrigonídeos e seu impacto na diversificação de suas linhagens associadas de parasitas fazem deste sistema um interessante modelo para estudos em biogeografia e associação histórica. Dentro deste contexto, Brooks *et al.* (1981) foram pioneiros em explorar o potencial de linhagens de parasitas em oferecerem informações

a respeito da invasão da água doce pelo ancestral dos potamotrigonídeos e a subsequente diversificação das arraias de água doce. Empregando dados morfológicos provenientes de parasitas de potamotrigonídeos (*i.e.*, Tetraphyllidea: Onchobothriidae: *Acanthobothrium* e *Potamotrygonocestus*, Tetraphyllidea: Phyllobothriidae: *Rhinebothrium*, *Rhinebothroides*; e Nematoda: *Eutetrarhynchus* e *Echinocephalus*), Brooks *et al.* (1981) propuseram que os potamotrigonídeos seriam grupo-irmão de “urolofídeos” e teriam colonizado a América do Sul a partir do Oceano Pacífico. Observe que, apesar do uso do termo “urolofídeos”, Brooks *et al.* referem-se a membros de Urotrygonidae (*Urobatis* e *Urotrygon*). De acordo com esta hipótese, os potamotrigonídeos teriam sido confinados no continente a 90 Ma devido à orogênese dos Andes. Esta hipótese ficou conhecida como “hipótese Pacífico/urolofídeos” e passou a ser uma contribuição inovadora para Biogeografia Histórica (Straney, 1982) e um grande exemplo para as salas de aula (Blair, 1994). Desde a sua publicação, porém, esta hipótese tem sido extensivamente questionada com base na inadequação dos métodos e, principalmente, na fragilidade dos dados parasitológicos empregados (*e.g.*, Caira, 1990; Lovejoy, 1997).

Diversos aspectos da análise conduzida por Brooks *et al.* são crípticos e dificultam a replicação de seus resultados. Por exemplo, o grupo externo de suas análises não foi explicitado e as matrizes de caracteres morfológicos empregados nunca foram publicadas integralmente. Ademais, a própria representação dos cladogramas obtidos dá margem a críticas. Por exemplo, o cladograma proposto para *Acanthobothrium* mostra um clado contendo as quatro espécies então conhecidas para o gênero e parasitas de potamotrigonídeos (*Acanthobothrium terezae* Rego & Dias, 1976; *Acanthobothrium amazonensis* Mayes, Brooks & Thorson, 1978; *Acanthobothrium quinonesi* Mayes, Brooks & Thorson, 1978; e *Acanthobothrium regoi* Brooks, Mayes & Thorson, 1981a) como grupo-irmão de *Acanthobothrium cartagenensis* Brooks & Mayes, 1980 do Caribe e *Acanthobothrium holorhini* Alexander, 1953 do sul da Califórnia. As demais 59 espécies de *Acanthobothrium* analisadas por Brooks *et al.* foram representadas como grupo-irmão do clado *A. holorhini* + linhagens dulcícolas de *Acanthobothrium* em um nó sem resolução.

A primeira hipótese concorrente a Brooks *et al.* foi levantada por Lovejoy (1996, 1997) e Lovejoy *et al.* (1998) com base em dados provenientes dos próprios hospedeiros. Estes autores empregaram dados morfológicos de diversos grupos de arraias para proporem que os potamotrigonídeos derivaram de um ancestral comum compartilhado com linhagens amfi-Americanas de *Himantura*, habitantes do mar do Caribe (“hipótese Caribe/*Himantura*”). Marques (2000) corroborou esta hipótese com dados morfológicos e moleculares de parasitas e arraias, respectivamente. Segundo estes autores, transgressões marinhas durante o Mioceno teriam possibilitado a invasão do América do Sul há aproximadamente 20 Ma. O subsequente isolamento do ancestral potamotrigonídeo teria sido resultado de alterações no escoamento do

paleo-Orinoco-Amazonas, há aproximadamente 11 Ma.

A hipótese mais recente quanto à origem dos potamotrigonídeos foi proposta por de Carvalho *et al.* (2004) com base na morfologia de arraias viventes e fósseis. Os autores concordam que transgressões marinhas na região do Caribe seriam o mecanismo responsável pela colonização da América do Sul pelos potamotrigonídeos. Entretanto, a data do evento proposta pelos autores é muito mais antiga, no mínimo durante o Eoceno, há 50 Ma ou mais. Os autores ainda refutaram a hipótese de que o clado *Himantura* pacifica + *H. schmardae* é grupo irmão de Potamotrygonidae, propondo que as arraias de água doce compartilham um ancestral comum com (“Dasyatidae”+(Gymnuridae+Myliobatidae)).

Atualmente, há consenso na literatura quanto à monofilia de Potamotrygonidae assim como quanto a transgressões marinhas na região do Caribe terem sido o mecanismo responsável pela invasão da América do Sul pelas arraias de água doce. Entretanto, a data de origem e o grupo-irmão desta família de elasmobrânquios permanecem em disputa. Dentro deste contexto, acredita-se que a resolução da taxonomia e filogenia de tetrafilídeos pode vir a esclarecer a origem dos potamotrigonídeos (Reyda, 2007) e ainda que *Acanthobothrium* poderá ser empregado como modelo nos estudos de associações históricas entre parasitas e hospedeiros (Fyler, 2009).

1.2 Presente estado do conhecimento taxonômico e filogenético de *Acanthobothrium*

Os avanços mais recentes em relação à taxonomia de *Acanthobothrium* de água doce se devem às descrições de *A. ramiroi* por Ivanov (2005) e *A. peruvienne* por Reyda (2008) e pela revisão ainda não publicada de Cardoso (2010). Segundo Cardoso, a maioria das espécies de *Acanthobothrium*, em particular as espécies parasitas de potamotrigonídeos, foi descrita com base em um número restrito de exemplares provenientes da amostragem de poucos hospedeiros de localidades geograficamente distantes umas das outras. Para Cardoso, esta prática tem conduzido pesquisadores a denominar novos táxons sob a premissa de que estas linhagens exibem pouca variação morfológica, uma vez que algumas espécies foram erigidas com base em descontinuidades morfométricas em relação a outras já atribuídas ao gênero. Afim de averiguar a real plasticidade fenotípica de espécies dulcícolas de *Acanthobothrium*, Cardoso amostrou 10 espécies válidas e 14 possíveis espécies novas de potamotrigonídeos, totalizando 649 espécimes de potamotrigonídeos provenientes de 10 sub-bacias hidrográficas da América do Sul. Esta ampla amostragem permitiu que Cardoso observasse uma variação morfológica intraespecífica jamais documentada para membros de *Acanthobothrium*.

Cardoso advoga que somente cinco espécies de *Acanthobothrium* são discerníveis morfológicamente no ambiente de água doce: *A. terezae* (sinônimo júnior: *A. ramiroi*), *A. quinonesi* (sinônimos: *A. regoi* e *A. peruviense*), *A. amazonensis* e duas novas espécies ainda não descritas, às quais o autor se refere como *Acanthobothrium* sp. 1 e *Acanthobothrium* sp. 2. Cardoso verificou ainda que estas cinco espécies de *Acanthobothrium* parasitam 27 espécies de potamotrigonídeos em quase todas as sub-bacias hidrográficas da América do Sul, demonstrando um padrão distinto de seus congêneres marinhos que são considerados altamente específicos a seus hospedeiros (Ghoshroy & Caira, 2001; Campbell & Beveridge, 2002). A alta plasticidade fenotípica e baixa especificidade observadas em espécies de *Acanthobothrium* de água doce pode ser resultado de características específicas do sistema potamotrigonídeos/parasitas (ver Reyda & Marques, 2011) ou um artefato taxonômico relacionado ao número restrito de espécimens empregados na descrição de espécies marinhas, muitas vezes obtidos por coletas distantes umas das outras e/ou com baixa representatividade de espécies de hospedeiros. Entretanto, Cardoso recomenda que novas fontes de dados (e.g., molecular) sejam adicionadas para circunscrever de forma mais robusta as espécies de água doce de *Acanthobothrium* e entender melhor as peculiaridades deste sistema em relação ao ambiente marinho.

Impressionantemente, três décadas após o trabalho seminal de Brooks *et al.* sobre este sistema parasita/hospedeiro, as linhagens de *Acanthobothrium* parasitas de potamotrigonídeos permanecem negligenciados dentro de um contexto filogenético. Olson *et al.* (2001), Caira *et al.* (2005) e Healy *et al.* (2009) são os únicos autores a terem publicado filogenias incluindo membros de *Acanthobothrium*, no entanto, restringindo-se à inclusão de uma única espécie não descrita parasita de *Dasyatis longus* (Garman, 1980). Fyler (2009) foi a primeira autora a fazer uma análise extensa de *Acanthobothrium* dentro de um contexto filogenético, porém a representatividade de linhagens dulcícolas em sua análise estava restrita a apenas três terminais representando duas espécies nominais parasitas de potamotrigonídeos (*A. terezae* e *A. ramiroi*).

1.3 Objetivos gerais

Dentro deste contexto, este trabalho visa:

1. Avaliar o status taxonômico das espécies nominais de *Acanthobothrium* parasitas de potamotrigonídeos com base em sua monofilia recíproca e consistência morfológica; and
2. Identificar a posição relativa de linhagens monofiléticas de *Acanthobothrium* dulcícolas em relação a seus congêneres marinhos.

1.4 Organização da dissertação

Além da contextualização apresentada neste capítulo, a dissertação está dividida em mais três capítulos com objetivos independentes, interrelacionados e complementares.

O **Capítulo 2**, “The forgotten origin of *Acanthobothrium* Blanchard, 1848 (Eucestoda: Tetraphyllidea: Onchobothriidae)”, procura resolver conflitos nomenclaturais em torno do nome genérico *Acanthobothrium* afim de delinear adequadamente o gênero em estudo.

O **Capítulo 3**, “Diversification and species boundaries of *Acanthobothrium* (Eucestoda: Tetraphyllidea: Onchobothriidae) in South American freshwater stingrays (Myliobatiformes: Potamotrygonidae)”, aborda os objetivos específicos desta contribuição através da análise do posicionamento filogenético de linhagens de *Acanthobothrium* com base na otimização direta de dados moleculares.

Finalmente, o **Capítulo 4** traz as “Considerações finais” deste estudo, destacando questões taxonômicas e evolutivas levantadas durante os capítulos anteriores.

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The forgotten origin of *Acanthobothrium* Blanchard, 1848 (Eucestoda: Tetraphyllidea: Onchobothriidae)

The genus *Acanthobothrium* consists of an assemblage of cosmopolitan onchobothriid tapeworm parasites of chondrichthyan fishes. Despite its clear diagnostic features, which includes a scolex with four septate bothridia bearing three loculi and a pair of as/symmetric bipronged hooks, this genus is represented by worms that possess a range of morphologies and host lineages (e.g., rays, skates, guitarfishes, and sharks; Campbell and Beveridge, 2002; Zschoche *et al.*, 2011). The wide distributional range of this group, its diverse morphology, and a somewhat long and intricate taxonomic history may account for the fact that to date no absolute number of valid species, of potentially more than 200 nominal species, can be assigned to this genus with certainty. Therefore, a taxonomic revision of this group is overdue; a task that certainly will only be accomplished by collaborative efforts of cestodologists given the widespread distribution of its members and the necessity of collecting new material to verify host identity and/or re-access species' morphology. It is not our purpose to provide such a revision. However, it is our intention to clarify the authorship, date of publication, type species, and type host of *Acanthobothrium*. To the best of our understanding, these have been mistakenly assigned throughout the taxonomic history of the genus.

Despite several taxonomic reviews (e.g., Linton, 1925; Baer, 1948; Goldstein, 1967; Williams, 1969), there is still no consensus on the authority of *Acanthobothrium*. Nomenclatur Zoologicus (Neave, 1939, Vol. I, p. 11) registers two entries for the genus-group name *Acanthobothrium*, attributed to Blanchard (1849) and Van Beneden (1849), which means that at least since 1939 the authorship of the genus is ambiguous. Contemporary authors disregard Blanchard as the author of *Acanthobothrium* and assign two possible dates for Van Beneden's

authorship of the genus. For some (e.g., Khalil *et al.*, 1994; Reyda and Caira, 2006; Vardo-Zalik and Campbell, 2011), *Acanthobothrium* should be assigned to Van Beneden (1849), whereas others (e.g., Jensen and Caira, 2008; Twohig *et al.*, 2008; Fyler, 2011; Zschoche *et al.*, 2011) implicitly assume that the genus should only be recognized as valid by the more detailed description provided by Van Beneden (1850).

To our knowledge, the first usage of the name *Acanthobothrium* was by Blanchard (1848: 364) in the legend of Plate 12, Fig. 9, in which it reads “ACANTHOBOTHRIE COURONNÉ (*Acanthobothrium coronatum*), pour montrer la forme de la tête, la forme générale du corps, et le trajet des tubes intestinaux.” We consider that the name *A. coronatum* was made available by Blanchard since this nomenclatural act satisfies the provisions of Article 11 and 12 of the ICZN (see ICZN Art. 12.2.7 for information about species-group names published before 1931 in association with an illustration). Despite the absence of a detailed description for the new species, Blanchard (1848) withholds the authorship of *A. coronatum* for being the first published work to name it (ICZN Art. 23.1). It is important to note that the availability of a new species-group name is not dependent on the availability of the genus-group name (ICZN Art. 11.9.3.1). However, if this species-group name is available, so is the genus-group name to which it is related (see ICZN Art. 12 and 12.2.5). Thus, Blanchard (1848) also withholds the authorship of the genus *Acanthobothrium*.

In February of the next year, Blanchard (1849: 121-122) provided the first detailed description of the “Genre ACANTOBOTHRIE (*Acanthobothrium*)” in which the author cited “*Bothriocephali onchobothrii* Rud.” as its type species. In the same month (date confirmed by Muquardt, 1849), Van Beneden (1849: 191) indicated a different type species for the genus using the term “*Acanthobothrium* n. gen. *Bothr. bifurcatus*” in a list of genera he included in “Bothroïdes.” On the second revision of *Acanthobothrium* and *A. coronatum*, Van Beneden (1850: 129) cited “*A. coronatum* Rud.” as the type of *Acanthobothrium*. With such confusion on the first works dealing with *Acanthobothrium*, it is not surprising that the type species of the genus is currently ambiguous. For example, while Yamaguti (1959: 83) claims that the type species of *Acanthobothrium* is “*A. coronatum* (Rud., 1819) van Beneden, 1849 (Pl. 21, Fig. 163), syn. *Bothriocephalus bifurcatus* Leuckart, 1819”, Campbell and Beveridge (2002: 239) state that the type species is “*Bothriocephalus coronatus* Rudolphi, 1819 by original designation.” However, none of the aforementioned works published after Blanchard (1848) are valid nomenclatural acts regarding the typification of *Acanthobothrium*. Since there were no other species attributed to *Acanthobothrium* in 1848, it is clear that *A. coronatum* shall be considered the type species for being the only species name related to the genus when it was created (ICZN Art. 68).

The above leads us to another issue, which concerns the current valid name of *A. coronatum*. We understand that the stability of the name-bearing type implies that the type species must

always be referred to as its original combination (ICZN Art. 61; see also Art. 67.1.2 and Recommendation 67B). Nevertheless, understanding the intricate taxonomic history of *A. coronatum* is crucial for understanding the concept of *Acanthobothrium* and of this species itself.

The first revision of *A. coronatum* was provided by Blanchard (1849: 122-124), with a list of synonyms including “*Taenia raiae batis*” Rudolphi (1810), *Bothriocephalus coronatus* Rudolphi, 1819, and *Bothriocephalus bifurcatus* Leuckart, 1819. *Bothriocephalus bifurcatus* was erected by Leuckart (1819: 30-32) who considered *Taenia corollata* Abildgaard, 1790 as its senior synonym. Abildgaard (1790: 62-63) provided a description of *T. corollata* consistent with a member of *Acanthobothrium* and identified “*Rajæ Batis*” (*Raja batis* L.) as the type host of his new species (see Abildgaard, 1790: 63; in which you should read “*LOCUS. Inter valvulas cochlidiales intestini Rajæ Batis*”). The name *B. corollatus* was previously used by Rudolphi (1819: 485-486). This may have provoked Leuckart to assign his species in a different species group name. *Bothriocephalus bifurcatus* was selected for the new species-group name giving into consideration its bipronged hooks. The establishment of *B. bifurcatus* as a junior synonym of *A. coronatum* results in a new combination, *A. corollatum* (Abildgaard, 1790), following the oldest available name for this taxon, *T. corollata* (ICZN Art. 23.3).

Williams (1969: 5) had previously suggested that “in view of the numerous problems connected with the specific name ‘*corollatus*’ relative to Tetrarhynchide (...) it may be advisable to ignore the name *T. corollata* Abildgaard, 1793 in studies of *A. coronatum*” (sic). Note that Williams (1969: 5) is probably referring to Abildgaard (1790) when he writes “Abildgaard, 1793”. Moreover, Williams (1969: 5) advocated that “the host list given by Abildgaard (...) indicates that he may have been dealing with more than one species under the same name.” Whether or not Abildgaard (1790) was dealing with a complex of several species has no nomenclatural relevance, since it neither interferes with the availability of the name *T. corollata* nor stands as an impediment for the appointment of this name as a senior synonym of *A. coronatum*.

Once the type species of *Acanthobothrium* and its current valid name is clarified, the identity of the type host may now be addressed. According to Williams (1969: 9), “*A. coronatum* should be regarded as a cestode of *Scyliorhynchus stellaris* only.” Williams’ (1969) statement ignores that the concept of *B. bifurcatus* is correlated with Leuckart’s (1819: 31-32) to *T. corollata*, which according to Abildgaard (1790: 63), had only been found in *R. batis*. The corollary of this historical event is that the type species of *Acanthobothrium* is associated with *R. batis*.

In an effort to more completely understand the concept of *A. corollatum*, we attempted to locate the type materials used in the original descriptions of its older synonyms. Unfortunately, all of Abildgaard’s material was lost throughout the years (Buchmann, per. comm.) and our efforts to locate Leuckart’s original material have also been fruitless. At this point, it seems

that the type series of *A. corollatum* (including older synonyms) could have been lost. In this case, assigning a neotype for *A. corollatum* would not be recommended since the type material would have to be consistent with the original concept of its synonyms (ICZN Art. 74-75), which are related to parasites of *R. batis*. According to Campbell and Beveridge (2002), there are at least five nominal species of *Acanthobothrium* known to occur in *R. batis* (*A. coronatum*, *A. icelandicum*, *A. parvum*, *A. rajaebatis*, and *A. septentrionale*) and the original description of *T. corollata* may not suffice the requirements to identify which one is Abildgaard's concept of *T. corollata*.

We conclude that the authorship of *Acanthobothrium* belongs to Blanchard (1848). The type species of *Acanthobothrium* is *Acanthobothrium coronatum* Blanchard, 1848, a junior synonym of *Acanthobothrium corollatum* (Abildgaard, 1790) n. comb. The type host of *A. corollatum* and therefore *Acanthobothrium* is *Raja batis* L.

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Diversification and species boundaries of *Acanthobothrium* (Eucestoda: Tetraphyllidea: Onchobothriidae) parasites of freshwater stingrays (Myliobatiformes: Potamotrygonidae)

3.1 Introduction

Acanthobothrium Blanchard, 1848 comprises an ubiquitous and specious genus of elasmobranch parasites characterized by tricolulate bothridia, each one armed with a pair of bifurcated hooks (Southwell, 1925). Since the erection of the genus, approximately 200 *Acanthobothrium* species have been described from elasmobranchs in all major oceans (e.g., Campbell and Beveridge, 2002; Ivanov, 2005; Reyda and Caira, 2006; Jensen and Caira, 2008; Twohig *et al.*, 2008; Fyler, 2009; Fyler and Caira, 2006, 2010; Fyler, 2011; Zschoche *et al.*, 2011). Few species of *Acanthobothrium* have also been reported from the rivers of South America since the 1970's (Rego and Dias, 1976; Mayes *et al.*, 1978; Brooks *et al.*, 1981a; Ivanov, 2005; Reyda, 2008). Until 2010, a total of six South American freshwater species of *Acanthobothrium* were recognized: *Acanthobothrium terezae* Rego and Dias, 1976; *A. amazonensis* Mayes, Brooks and Thorson, 1978; *A. quinonesi* Mayes, Brooks and Thorson, 1978; *A. regoi* Brooks, Mayes and Thorson, 1981a; *A. ramiroi* Ivanov, 2005; and *A. peruvienne* Reyda, 2008. However, recent evidence suggests that the taxonomic status of these species must be reviewed (Cardoso, 2010).

Historically, most species of *Acanthobothrium* have been described based on a restricted number of specimens from distant localities and host species. This practice has led researchers to describe new species based on the premises that those lineages present low morphological variation. Recently, Cardoso (2010) addressed the morphological variation within and between freshwater species of *Acanthobothrium* using considerable numbers of specimens collected

from many species of potamotrygonids from all major drainages of South America. This unprecedented sampling effort unveiled a morphological variation within *Acanthobothrium* species, which has never been reported in the taxonomic history of the genus. According to Cardoso (2010), only five freshwater species of *Acanthobothrium* should be recognized as valid: *A. terezae* (syn. *A. ramiroi*), *A. quinonesi* (syn. *A. regoi* and *A. peruviense*), *A. amazonensis*, and two new species still undescribed. However, Cardoso (2010) claimed that new sources of data (e.g., molecular) would be necessary to better understand the taxonomic status of species of this genus in Neotropical freshwater systems.

The intention of this contribution is to evaluate the taxonomic status and phylogenetic position of the nominal species of *Acanthobothrium* in the freshwater environment. To achieve this goal, we associate morphological cohesion with phylogenetic patterns of monophyly recovered from nucleotide data of multiple loci. This is the first attempt to include all freshwater species of *Acanthobothrium* in a broader phylogenetic context which includes representatives of several marine species of *Acanthobothrium* as well as their closest relatives. In doing so, we address conflicting patterns of host specificity and morphological variability between freshwater and marine species of *Acanthobothrium*. We also discuss conflicting hypothesis of colonization of South American freshwater by *Acanthobothrium* species.

3.2 Material and methods

3.2.1 TAXONOMIC REPRESENTATION OF MARINE TETRAPHYLLIDEANS

The position of *Acanthobothrium* within tetraphyllidean cestodes is currently unclear. Also, the monophyly of the freshwater lineages still awaits phylogenetic testing. Thus, for the purposes of this study, sequences from 10 members of Proteocephalidea and three specimens of *Acanthobothrium* were obtained from GenBank (Appendix A.7, Table A.3). Other sequences from hooked tetraphyllideans, including several additional species representing marine lineages of *Acanthobothrium*, were kindly provided by Dr. Caroline A. Fyler (Fyler, 2009; unpublished data) (Appendix A.8, Table A.4). Our data set for marine tetraphyllideans is believed to be representative of the taxa more closely related to *Acanthobothrium* based on the results of a larger analysis by Caira (per. comm.) with representation of 80 percent of tetraphyllidean genera. Trees were rooted with a species of an undescribed tetraphylliden genus as they consistently grouped outside *Acanthobothrium*, *Uncibilocularis* Southwell, 1925, *Potomotrygonocestus* Brooks and Thorson, 1976 and Proteocephalidea in the trees generated by Caira (per. comm.). The collect of biological material and sequencing of DNA data from parasites followed the above strategies and protocols in order to expand the representativity of freshwater lineages of *Acanthobothrium* on Fyler's (2009) dataset, thus allowing the evaluation of their reciprocal monophyly and phylogenetic position.

3.2.2 COLLECTION OF FRESHWATER STINGRAY SPECIMENS

Freshwater stingrays were collected from multiple localities (see Appendix A.1, Table A.1) throughout the Amazon and La Plata River basins in South America from July of 2004 to January of 2011 (see Fig. 3.1). These localities represent most of the major river systems of South America from which potamotrygonids have been historically reported (de Carvalho and Ragno, 2011; de Carvalho *et al.*, 2011) and/or included type localities for tetraphyllideans found in potamotrygonids (e.g., two different collections at the Salobra River in Mato Grosso do Sul State, Brazil, the River referred to as the type locality of *A. terezae*). Collecting, fixation, identification, and deposition of biological material from stingrays follows the procedures and guidelines of Reyda and Marques (2011).

Unidentified species were assigned either by regional common names (e.g., *Potamotrygon* sp. “jabuti” or “negra”). Images of each stingray specimen from which cestodes were collected are available on-line (see Marques and Domingues, 2011) and can be searched in the database using the field code for each locality that is provided in Table A.1

3.2.3 COLLECTION OF CESTODE SPECIMENS

The spiral intestine of 198 stingray specimens was removed, opened with a mid-ventral incision, and examined for parasites with a dissecting microscope and/or immediately immersed in 95-100% ethanol. The spiral intestines were examined in the laboratory, and cestodes were removed, and placed in vials to be stored at low temperature (approximately -20°C). Members of *Acanthobothrium* selected for molecular study were fragmented in 3 parts of which the scoleces and terminal portions were prepared as whole mounts to serve as vouchers (hologenophores, sensu Pleijel *et al.*, 2008). Material prepared as whole mounts were hydrated in a graded ethanol series, stained in Delafield's hematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted on glass slides in Canada balsam (see Appendix A.3).

Identification of hologenophores, for freshwater species of *Acanthobothrium*, follows the concept of Cardoso (2010). Each hologenophore received a field code, a molecular code (see Appendix A.4, Table A.2), and has been deposited at the Museu de Zoologia da Universidade de São Paulo (MZUSP) under the accession numbers 7349 to 7480.

3.2.4 DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Fragments of cestodes were allowed to air-dry, and prepared for genomic DNA extraction, using one of the following two protocols: (1) InstaGene^{TM} Matrix (Bio-Rad) kit; in which the reactions were performed on a total volume of 30 μl , and other conditions follows the manufacturer's specifications; or (2) Agencourt® DNAdvance^{TM} DNA Isolation Kit optimized

Figure 3.1: Collection sites of potamotrygonid specimens in South America. Details of field codes shown here are given in Table A.1. Figure modified from Luchetti (2011)

for 200µl tubes and very small amounts of cestode tissue (<3mm³). The concentrations of each reagent follows manufacturer's specifications. We employed the Agencourt® SPRIPlate Super Magnet Plate (see Appendix A.5) to better suit the optimized protocol for 200 µl tubes.

PCR was performed on 3 genes, ITS-1, 16S and 28S rDNA. Table 3.1 shows the size of each of the sequenced genomic fragments as well as the primers employed for amplification and/or cycle sequencing.

Table 3.1: Primers used for PCR amplification and cycle sequencing.

Primers used for each gene		Author(s)
ITS1 rDNA (~300 base pairs)		
ITS1A (5')	GTAACAAGGTTTCCGTAGGTG	Matejusová <i>et al.</i> (2001)
ITSR3A (3')	GAGCCGAGTGATCCACC	Matejusová <i>et al.</i> (2001)
Partial 16S rDNA (~900 base pairs)		
Cyclo-16SF (5')	TGCCTTTTGCATCATGCT	Marques (unpublished data)
Cyclo-16SR (3')	AATAGATAAGAACCGACCTGG	Marques (unpublished data)
Partial 28S rDNA (D1-D3 region, ~1300 base pairs)		
LSU5F (5')	TAGGTCGACCCGCTGAAYTTAAGCA	Littlewood <i>et al.</i> (1997)
LSU330F (5')	CAAGTACCGTGAGGGAAAGTTG	Williams and Ozawa (2006)
LSU1500R (3')	GCTATCCTGAGGGAAACTTCG	Tkach <i>et al.</i> (1999)

Thermal cycles were as follows: initial denaturation for 5 min at 95°C, followed by 35-40 cycles of 30 secs at 94°C, 40 secs at 50–60°C, and 1-2 min at 72°C, followed by a final extension of 7 min at 72°C. The specific annealing temperatures for each gene are: 55°C for 16S rDNA, 60°C for 28S rDNA, and 50°C for ITS1 rDNA.

PCR reactions were performed using Techne® TC-5000™ thermocycler. PCR products were purified using an Ampure™ kit (see Appendix A.6). Products were subsequently cycle-sequenced directly from forward, reverse, and, in some cases, internal strands, using ABI Big-Dye™ chemistry, cleaned with sephadex beads, and sequenced on an ABI 3100 Genetic Analyzer at the Centro de Sequenciamento do Departamento de Química, Institute of Chemistry, USP.

The quality of each chromatogram was checked using FinchTV version 1.3.1 (Geospiza Inc.). Contiguous sequences were assembled and edited using the package Consed/Phred Phrap (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998, 2001).

3.2.5 PHYLOGENETIC ANALYSIS

Phylogenetic reconstruction was performed with parsimony analysis of a combined data set of 16S, 28S, and ITS1. The phylogenetic hypothesis was obtained from the simultaneous analysis of all data partitioned under the assumption that all available evidence maximizes explanatory power of the working hypothesis (Kluge, 1989; Nixon and Carpenter, 1996). Partial analyses

of each gene fragment were employed to assess character incongruity between individual and combined analysis.

Different partitions were created on BioEdit version 7.0.9.0 (Hall, 1999) using pre-aligned sequences by Muscle version 3.7 (Edgar, 2004). The phylogenetic analysis was performed in POY version 4.1.2 (Varón *et al.*, 2010) employing the concept of dynamic homology (Wheeler, 2001a, 2001b). This concept allows characters and the characters states within those characters to be discovered simultaneously on the bases of a single optimality criterion and takes into account insertion/deletion events (i.e., INDELs) as historical evidence (Wheeler, 1996, 2002). All data partition albeit pre-aligned during partition definition were treated as unaligned and non-additive and analyzed under nine different cost ratios for gap opening, gap extension, transversions, and transitions (2:1:1:1, 2:1:1:2, 2:1:2:1, 4:2:1:1, 4:2:1:2, 4:2:2:1, 8:4:1:1, 8:4:1:2, and 8:4:2:1) by POY.

POY was run on the Zoology parallel computing cluster using 8 processors for partial analysis and 16 processors for combined analysis. For the sequence character optimization, two direct optimization (DO; Wheeler, 1996) algorithms were employed as part of the same search strategy as shown in Figure 3.2 and specified above.

The first part of the search strategy employed Optimization Alignment (OA), generally called DO (Wheeler, 1996). Optimization Alignment optimizes hypothetical ancestral sequences based on descendant sequences. The search strategy under OA comprised tree building, branch swapping, perturbing using ratchet, and tree fusing. The first part of the strategy was set on a script using three sequential “search” commands with maximum allowed memory of two Gigabytes per processor and maximum execution time of three hours for partial analysis and nine hours for combined analysis. Each script for OA searches was run five times storing the shortest trees of each one.

On the second part of the search strategy, the shortest topologies selected during each OA search were subjected to a final round of branch swapping under Iterative Pass (IP). Iterative Pass optimizes hypothetical ancestral sequences based on both descendant and ancestral sequences. Therefore, IP is a three-sequence DO with iterative improvement that, although much more computationally demanding, holds the potential to find shorter solutions (Wheeler, 2003).

The selection of the working hypothesis followed the criteria of minimization of character incongruity via congruence analysis. With this purpose, the Mickevich–Farris Extra Steps Index (MFES) (MFES; Mickevich and Farris, 1981) were calculated using the length of the combined analysis to normalize the number of extra steps (see equation below).

$$MFES = \frac{\text{Length}_{Combined} - \sum \text{Length}_{Partials}}{\text{Length}_{Combined}}$$

Figure 3.2: Flow chart showing the main steps of the combined DO search strategy. This strategy was repeated for the combined and partial analysis of each parameter.

As character incongruence among data partitions increases, MFES increases. The parameter set with the lowest MFES value is selected as the working hypothesis as it means that the minimum character incongruity has been achieved (but see Dowton and Austin, 2002).

The sensitivity analysis (sensu Wheeler, 1995; Giribet, 2001) were employed using Cladescan (Sanders, 2009) to test the robustness of various clades to perturbations in the relative weighting parameters. If a clade is stable throughout the parameter space (i.e., different weighting parameters) it is considered better supported¹ by the data than a clade that was recovered on fewer parameters.

The support for branches was achieved by using Branch Support values, also referred to as decay indices and Goodman-Bremer (GB) support values (Goodman *et al.*, 1982; Bremer, 1988; 1994; see also Grant and Kluge, 2008a). Goodman-Bremer values provide measures of the objective support of a clade according to the number of extra steps required to collapse a branch in the consensus of nearly most parsimonious trees (Grant and Kluge, 2008b). Goodman-Bremer values vary from zero to infinity. The higher the GB value, the more contradictory evidence would be required to be added to the matrix to erode support for a given clade. The support was calculated in the Department of Zoology – IB/USP parallel computer cluster using 24 processors. The search for suboptimal trees were executed using four different search strategies with increasing heuristic aggressiveness: (1) combined DO search with relative low aggressiveness (OA: search for 3 hours, IP: swap for 1 hour, repeat 9 times); (2 and 3) two combined DO searches with intermediate aggressiveness (OA: search for 6 or 9 hours, IP: swap for one hour); and (4) combined DO search strategy with higher aggressiveness (OA: 3 times search for 9 hours, IP: swap for one hour). Goodman-Bremer values were used to track the efficiency of each search strategy to calculate support. Goodman-Bremer calculations were made for each subset of trees found. Selected GB value for each branch represent the minimum GB values encountered, which is the GB value calculated with all four tree sets. For further details, see data flow in Figure 3.3.

Trees with an approximation of branch lengths approximately proportionals to the numbers of transformations were build parsing the diagnose (.xml) POY files by the Perl script `newick4poy5.pl` (Marques, unpublished script). All tree edition was performed on Dendroscop version 3.0.10 beta (Hudson and Scornavacca, in prep.) or FigTree version 1.3.1 (Rambaut, 2006-2009) and vectorial SVG image files were further edited on Inkscape version 0.47 (Harrington *et al.*, 2004-2011).

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¹Support sensu (Brower, 2006: 378): “a measure of the net amount of evidence that favors the appearance of that branch in a most-parsimonious topology”).

Figure 3.3: Main steps of the Goodman-Bremer calculation.

3.3 Results

3.3.1 MOLECULAR DATASET

The dataset generated for this analysis includes 258 new sequences from three different regions (16S, 28S, and ITS1). The complete molecular dataset employed in this study represents 10 species of Proteocephalidea and 50 species of *Acanthobothrium* collected from a total of 45 species and 22 genera of hosts.

3.3.2 WORKING HYPOTHESIS

The IP optimization was able to find shorter alignments for each of the first runs under DO. However IP was not able to find different topologies than those selected under DO. It is also interesting to note that in several cases the shortest tree under IP was considered suboptimal under DO (Table 3.2).

Table 3.2: Tree lengths from the OA combined analysis. Shaded cells indicates trees selected under IP. * Trees selected under IP that were considered sub-optimum under OA.

Rounds	Weighting parameters								
	2:1:1:1	2:1:1:2	2:1:2:1	4:2:1:1	4:2:1:2	4:2:2:1	8:4:1:1	8:4:1:2	8:4:2:1
1 st	7520	12328	11557	9101	15272	14611	11911	20383	20056
2 nd	7520	12328	11563	9104*	15272	14608	11909	20388	20085
3 rd	7520	12331	11554	9102	15257	14616	11910	20378	20058
4 th	7520	12331	11558	9101	15265*	14616	11907	20379	20059*
5 th	7520	12332	11555	9102	15263	14612	11906	20377	20058

The working hypothesis selected by MFES corresponds to parameter 2:1:1:1. The results of the MFES calculations are shown in Table 3.3. Six different topologies were found with 7507 weighted steps for this parameter set. Our working hypothesis is the strict consensus of those trees, which has 166 dichotomic nodes and only five polytomies (i.e., 97% of resolved nodes). All polytomies refer to external branches and the consensus tree is considered well resolved. Figure 3.4 shows the consensus tree with collapsed nodes without the terminals. The complete consensus tree is on Appendix A.9: Fig. A.1. The first tree from parameter 2:1:1:1 was selected to generate the tree with branch length proportional to the number of transformations (Appendix A.10: Fig. A.2).

3.3.3 MEASURE OF SUPPORT AND STABILITY

The sensitivity analysis employed all 31 trees selected by IP for nine weighting parameters. The percentage of trees in which each clade is present is shown in Figure A.9 at the top left of each branch. Figure 3.5 shows the sensitivity plots for selected clades not present in all parameters.

Figure 3.4: Collapsed nodes are to facilitate visualization. Open circles indicates freshwater clades. Original data are indicated by *. See complete consensus tree in Fig. A.1

Table 3.3: Mickevich-Farris Extra Steps Index (MFES) calculations. The first parameter (shaded line) was selected as it minimizes character incongruity.

Weighting parameters					
Parameter	ITS1	28S	16S	Total	MFES
2:1:1:1	1146	3043	3121	7507	0.026242174
2:1:1:2	1865	5326	4714	12257	0.028718283
2:1:2:1	1922	4363	4924	11537	0.028430268
4:2:1:1	1587	3674	3550	9080	0.029625551
4:2:1:2	2646	6504	5506	15199	0.035726035
4:2:2:1	2790	5586	5775	14584	0.029690071
8:4:1:1	2324	4823	4306	11886	0.036429413
8:4:1:2	4031	8530	6871	20301	0.042805773
8:4:2:1	4215	7821	7258	20035	0.036985276

For GB calculations, suboptimal tree searches with increasing OA search time yielded 4318, 826, 869, and 646 trees, respectively. Goodman-Bremer values (see Fig. A.1) calculated using each of these tree sets varied in different manners between 14 and 248. The final round of branch swap under IP found cladograms as parsimonious as the selected hypothesis independently of how aggressive had been of the previous search under OA. This add support to the potential of IP to find more parsimonious cladograms (Wheeler, 2003). GB calculations using the tree set from the least aggressive OA searches provided most of the lower GB values for longer branches or branches depicting relationships between different genera. On the other hand, minimum GB values for shorter branches or branches representing inter or intra-specific relationships were only achieved using tree sets from more aggressive OA searches (see support and sensitivity measures on Appendix A.9: Fig. A.1).

3.4 Discussion

3.4.1 ON THE RELATIONSHIP BETWEEN *Acanthobothrium* AND *Uncibilocularis*

Although our primary concern in this contribution was to test the phylogenetic position of the freshwater lineages of *Acanthobothrium* based on molecular data, the phylogenetic pattern we recovered suggests that this genus as a whole requires revision.

The genus *Acanthobothrium* was not recovered as a monophyletic group either by Fyler (2009) or by any of the trees within our parameter space. The monophyly of the genus is undermined by position of two specimens (CF75 and CF77) initially identified by Fyler (2009) as “*Acanthobothrium* sp. 31” and one specimen (CF68) of *Uncibilocularis* Southwell, 1925. The species represented by terminals CF75 and CF77 have not been formally described and

Figure 3.5: Sensitivity plot for all discussed clades which are not present in all parameters. Shades of gray that some trees within a given parameter set contained the clade. Numbers below each plot refer to percentage of trees with the clade and GB value, respectively. See measures of support and sensitivity in Appendix A.9: Fig. A.1.

may show to belong to a different genus within Tetraphyllidea. However, a clade comprised by the remaining specimens of *Acanthobothrium* is not monophyletic unless *Uncibilocularis squireorum* Jensen and Caira, 2008 is included, in which case an *Acanthobothrium* spp. + *U. squireorum* is recovered in all the of the first six parameter sets (2:1:1:1, 2:1:1:2, 2:1:2:1, 4:2:1:1, 4:2:1:2, and 4:2:2:1; see Fig. 3.5a).

The close relationship between *Acanthobothrium* and *Uncibilocularis* may be explained by the intricate taxonomic history of these genera and its relationship with *Prosthecobothrium* Diesing, 1863. The genus *Prosthecobothrium* was erected to accommodate onchobothriids that bear bipronged hooks, as *Acanthobothrium*, but also possessed bothridium divided into two rather than three loculi. The type species of *Prosthecobothrium* is *Acanthobothrium dujardinii* (Van Beneden, 1850). According to Southwell (1925:94), closer examination of this species revealed that “the number of loculi in *A. dujardinii* either vary, or one of then is so small that it cannot always be seen”. Based on these observations, the author concluded that “it is obvious that the species cannot be relegated to Diesing’s genus *Prosthecobothrium*. This genus accordingly becomes a *nomen nudum*”. Southwell proposed *Uncibilocularis* as a replacement name for *Prosthecobothrium*, designating *Uncibilocularis trygonis* (Shiple and Hornell, 1906) (syn. *P. trygonis*) as its type species. *Uncibilocularis* was originally described by Southwell (1925) as an Onchobothriidae in which each bothridium is divided into two loculi by a single septum, and it is armed with either simple or “compound” (i.e., bifurcated) hooks. Since then, the concept of *Uncibilocularis* has been redefined to include only biloculate onchobothriids bearing a single pair of bipronged hooks per bothridium (e.g., see Euzet, 1994; Khalil *et al.*, 1994).

Jensen and Caira (2008) provided the latest review of *Uncibilocularis*. These authors made 12 emendations to Euzet’s (1994) description of *Uncibilocularis* but stated that “these generic attributes of *Uncibilocularis* cannot be considered as synapomorphies of the genus. Only in combination do these features allow species of *Uncibilocularis* to be distinguished from species of *Acanthobothrium*”. Finally, Jensen and Caira (2008: 172) concluded that “the monophyly of these 2 genera relative to one another remains uncertain until a broad scale phylogenetic analysis can be performed to explore this particular question”.

Our analysis suggests that *Uncibilocularis* may represent a derived lineage of *Acanthobothrium* which lost one loculi on its bothridium. The implication would be that *Uncibilocularis* should be considered junior synonym of *Acanthobothrium* and that the current description of this genus should be emended to include biloculated species. New molecular and morphological information for different species of *Uncibilocularis* are needed to test the reciprocal monophyly of these genus.

3.4.2 FRESHWATER *Acanthobothrium* AND INTRA-SPECIFIC VARIATION

This study represents the first attempt to evaluate the reciprocal monophyly of members of *Acanthobothrium* parasites of potamotrygonids. In doing so, we added support to the morphological concepts of most of these species as proposed by Cardoso (2010), in agreement with the high levels of intra-specific morphologic variation observed by the author.

Our molecular data corroborated the morphological concept of *Acanthobothrium* sp. 1 and *Acanthobothrium* sp. 2 as the clades representing these species were recovered with high support and stability (see Fig. 3.4). *Acanthobothrium* sp. 1 has a conspicuous barb at the posterior end of each hook prong which can be used to unambiguously diagnose this species. *Acanthobothrium* sp. 2 can be easily sorted distinguished from other potamotrygonid *Acanthobothrium* species by its scolex and hook morphology (i.e., slender cephalic peduncle, canoe-shaped bothridia; and long and slender hooks with prongs approximately symmetrical). The clade attributed to *Acanthobothrium* sp. 1 is represented by 22 specimens from four different potamotrygonids collected in Brazil and Venezuela and it is divided into a sub-clade represented by specimens collected in Barinas-Apure basin and its sister group from the Amazon foreland basin (see sensitivity plot in Fig. 3.5d). However, morphological features related to both clades overlap and therefore do not offer evidence to divide the species. In the absence of information to the contrary, we interpret these two clades composed by members of *Acanthobothrium* sp. 1 as resulting from populational differences rather than a recent speciation process. All quantitative and meristic characters from both these species are highly variable and many of them overlap in some degree with other freshwater species (Cardoso, 2010:44-55)

We also recovered a clade representing the *A. terezaelramiroi* complex. *Acanthobothrium ramiroi* constitutes a separate clade from *A. terezae* in six parameters (Fig. 3.5e) but the reciprocal monophyly of *A. terezae* was never recovered. This is in conformity with Cardoso (2010), which showed that the morphometric and meristic characters originally employed by Ivanov (2005) to distinguish between *A. terezae* and *A. ramiroi* (such as total length, scolex dimensions, size of lateral and medial hook, number of proglottids, and number of testes) are actually much more variable than Ivanov could have anticipated. The congruence between the morphological concept of *A. terezae* and the molecular data presented here adds evidence to the idea that *A. terezae* is senior synonym of *A. ramiroi*. This was proposed by Cardoso (2010) based on the larger length of the posterior loculi (165-370 μ m, 280.96 $\pm$ 53.85, 16; all measurements are presented respectively as ranges, mean, standard deviation, and number of measured specimens)² for *A. terezae* compared to all other potamotrygonid *Acanthobothrium* spp. *Acanthobothrium terezae* differs from all other *Acanthobothrium*

²For further information, refer to text and Tables 5-6 on Cardoso (2010) .

parasites of potamotrygonidae for the bigger length of its posterior loculi. *Acanthobothrium terezae* differs further from *Acanthobothrium* sp. 1 and *Acanthobothrium* sp. 2 by having more proglottids (70-175 μm , 112 ± 45 , 11), a wider scolex (555-12155 μm (958.50 ± 206.63 , 10), and a longer scolex (590-12,055 μm (955.83 ± 126.59 , 18), bothridium (390-860, 759.23 ± 147.67 , 13), and anterior loculi (185-4545 μm , 324.44 ± 66.84 , 17).

However, the reciprocal monophyly of *A. quinonesi* and *A. amazonensis* is not recovered in any of our parameter sets. Cardoso (2010) considered *A. quinonesi* a synonym of both *A. regoi* and *A. peruvienne* since all characters that were used to identify each of these nominal species were based on measurements of only a few specimens or on the shape of variable morphological structures, not unique to any of them. All morphometric and merist characters used to distinguish *A. quinonesi* from *A. amazonensis* overlap, but Cardoso (2010) was able to distinguish *A. amazonensis* from *A. quinonesi* based on the shape of the cirrus sac.

The morphology of the cirrus sac has been traditionally used in the taxonomy of freshwater lineages of *Acanthobothrium* (e.g., Mayes *et al.*, 1978; Reyda, 2008). However, the morphology of this structure may vary within each taxon. These observations depend on a large number of samples. Reyda (2008: 695) distinguished *A. peruvienne* from *A. quinonesi* based on meristic characters (i.e., total length, width of the terminal proglottids, width of the bothridia, and length of the anterior and posterior bothridial loculi) and on the format of the cirrus sac of 9 specimens. Cardoso (2010), to whom 24 specimens of this species complex were available, was able to observe that the characters used by Reyda (2008) varied and overlapped, supporting the conclusion that *A. quinonesi* and *A. peruvienne* should be considered the same species. According to Cardoso (2010), the cirrus sac in *A. amazonensis* is piriform and straight, where as the cirrus sac of *A. quinonesi* is curved or J-shaped in the young proglottids and assumes a triangular or T-shape at maturity. However, Cardoso (2010) had only 4 specimens of *A. amazonensis* available, which according to the author would have immediate impact over the observed phenotypic variability observed. Our results indicate that a revision of the taxonomic status of *A. quinonesi* and *A. amazonensis* is required and that, in the absence of information to the contrary, they may be considered synonyms.

The clade comprised by haplotypes attributed to *A. quinonesi* is roughly divided into specimens from La Plata River basin and others from Amazon River basin and Venezuela (see Fig. A.1). However, the monophyly of the clade comprising species of *A. quinonesi* from La Plata River is challenged by the position of terminal A039. The terminal A039 is weakly supported as the sister group of the remaining specimens of the *A. quinonesi/amazonensis* complex. The clade consisted of all specimens of the *A. quinonesi/amazonensis* complex except A039 is present in only two of nine parameters (see Fig. 3.5f) and is also one of the least supported nodes in the tree (GB value of 14). Although the clade comprising members of *A. quinonesi* from La Plata is not present in the selected hypothesis, it was recovered

on 3 other parameter sets (Fig. 3.5i). The clade consisting of haplotypes of *A. quinonesi* from La Plata (except terminal A039) is unstable (it was only recovered in the trees of the selected parameter set; see Fig. 3.5g) and also one of the clades with lowest support. The clade representing members of *A. quinonsei* from Amazon River basin is also unstable (two of nine parameter sets; Fig. 3.5h). As the nodes representing intra-specific relationships between *A. quinonesi/amazonensis*, the clade comprising all haplotypes from *A. amazonensis* and *A. quinonesi* has low support, but it is present in all parameter sets.

The phylogenetic information provided here corroborates the high phenotypic variation observed by Cardoso (2010) since most of the species he considered valid were recovered as monophyletic clades. This leads us to reiterate Cardoso's (2010) main contention that careful assessment of morphological variability is a prior requirement for the justification of its use to describe new species; and that the assessment of this variability is highly dependent on sample size and/or biogeographical representation. It is puzzling that the same variation in morphological characters have not yet been reported for marine species of *Acanthobothrium*, in particular, or tetraphyllidenas in general. The same pattern has however been reported for marine and freshwater members of *Rhinebothrium* Linton, 1890 (Reyda and Marques, 2011). Despite different levels of phenotypic variation, freshwater and marine tetraphyllideans further differ on their host specificity levels.

3.4.3 CONFLICTING PATTERNS OF HOST SPECIFICITY BETWEEN MARINE AND FRESHWATER ENVIRONMENT

Marine species of *Acanthobothrium* are remarkably host specific, exhibiting essentially oioxenous specificity (sensu Euzet and Combes, 1980) for their definitive hosts, i.e., each tapeworm species parasitizes a single host species (e.g., *Acanthobothrium woodsholei* Baer, 1948 only parasitizes *Dasyatis centroura*; *Acanthobothrium holorhini* Alexander, 1953 only parasitizes *Myliobatis californicus*). This is consistent with other onchobothriid genera, which are among “the most host specific parasite taxa in existence” (Caira and Jensen, 2001: 968). The same level of host specificity is not, however, seen in species of *Acanthobothrium* parasitizing potamotrygonids.

Each recognized freshwater lineage attributed to species of *Acanthobothrium* was found in at least three species of potamotrygonids, and their host list is growing. Our survey expanded the known host list of each of these lineages, except for *Acanthobothrium* sp. 2, which remains known to inhabit *Potamotrygon orbigny*, *Po. schroederi* (type host), and an unidentified *Potamotrygon* species (“negra” or “tpj2” from Tapajós river). We added *Po. orbigny*, *Po. schroederi*, and *Po. yepezi* to the list of known hosts of *Acanthobothrium* sp. 1 which already included *Paratrygon aiereba* (type host) and *Potamotrygon* sp. (“cururu”). We

also included *Po. orbigny* to the host list of *A. terezae*, which also comprises *Pa. aiereba*, *Po. brachyura*, *Po. motoro* (type host), and *Po. scobina*. But the lowest level of host specificity in *Acanthobothrium* still belongs to the *A. quinonesilamazonensis* species complex. Cardoso (2010) encountered specimens of *A. amazonensis* species in its type host (*Po. motoro*) and in two additional host species (*Potamotrygon* cf. *motoro* and *Potamotrygon* sp. “toc2”). According to the same author, *A. quinonesi* can be found infecting those three species of hosts plus 20 other species: *Pa. aiereba*, *Po. brachyura*, *Po. falkneri*, *Po. henlei*, *Po. histrix*, *Po. leopoldi*, *Po. magdalena* (type host), *Po. orbignyi*, *Po. schroederi*, *Po. scobina*, *Potamotrygon* cf. *2 motoro*, *Potamotrygon* cf. *castexi*, *Potamotrygon* cf. *orbignyi*, *Potamotrygon* sp. (“tar1”), *Potamotrygon* sp., *Potamotrygon* sp. (“toc1”), *Potamotrygon* sp. (“toc3”), *Potamotrygon* sp. (“toc4”), *Potamotrygon* sp. (“jabuti”), and *Potamotrygon* sp. (“mar1”). In addition to that, we found mature specimens of the *A. quinonesilamazonensis* species complex in the spiral valves of *Potamotrygon* sp. (“negra”), *Potamotrygon* sp. (“tpj1”), and *Po. yepezi*.

Reyda and Marques (2011) reported the same disparity regarding levels of host specificity between freshwater and marine lineages of *Rhinebothrium*. These authors suggested that ecological and historical factors could have played a role in defining the patterns we see presently in this system. These factors could easily be invoked to explain low specificity in *Acanthobothrium* and other genera of tetraphyllideans that are exclusive to the Neotropical freshwater system, such as *Potamotrygonocetus* Brooks and Thorson, 1976 (Luchetti *et al.*, 2008) and *Rhinebothroides* Mayes, Brooks and Thorson, 1981 (Bueno, 2010). From a historical standpoint, a low degrees of host specificity could result from a combination of host switching and diversification of host lineages without the corresponding speciation in their associated parasite lineage(s) (Poulin, 2007), which may or may not be related to the age of this potamotrygonid/parasite system (for conflicting hypothesis regarding the age of this system, see Lovejoy *et al.*, 1998; de Carvalho *et al.*, 2004). From a ecological point of view, multiple potamotrygonid species occur sympatrically (de Carvalho *et al.*, 2003) and many different potamotrygonid species have an overlapping diet (Shibuya *et al.*, 2009), which may have allowed trophic exchange of cestode fauna between hosts, possibly facilitating host switching. Nevertheless, none of these explanations can be tested until a phylogenetic hypothesis for the Potamotrygonidae is available.

One should consider that the high specificity and low morphological variation within marine species of *Acanthobothrium* could be an artifact of the restricted number of specimens traditionally used in species descriptions. For example, the last 11 described species of *Acanthobothrium* were based on the examination of 2-28 specimens of tapeworms from no more than one locality for each one (Vardo-Zalik and Campbell, 2011; Zschoche *et al.*, 2011; Fyler, 2011; Fyler and Caira, 2010). This phenomenon remains to be demonstrated. The number

of specimens used in descriptions of marine *Acanthobothrium* species should be increased in order to test this phenomenon under a taxonomic and phylogenetic framework.

3.4.4 PHYLOGENETIC RELATIONSHIPS AMONG FRESHWATER *Acanthobothrium*

Brooks *et al.* (1981b) provided the first phylogenetic analysis of freshwater species of *Acanthobothrium*. At the time, four species of freshwater *Acanthobothrium* were recognized, and hypothesized to be related as follows: (((*A. quinonesi*, *A. regoi*), *A. amazonensis*), *A. terezae*). Since then, two new species of *Acanthobothrium* parasitizing potamotrygonids have been described (*A. ramiroi* and *A. peruvienne*) and two more await formal description (referred to here as *Acanthobothrium* sp. 1 and *Acanthobothrium* sp. 2; see Cardoso (2010)). To date, *A. terezae* is considered senior synonym of *A. ramiroi*, and *A. quinonesi* is considered senior synonym of *A. regoi* and *A. peruvienne* (Cardoso, 2010). Thus, our results provide the first comprehensive phylogenetic analysis of freshwater lineages of *Acanthobothrium* encompassing the current taxonomic diversity of this genus.

Freshwater lineages of *Acanthobothrium* are not monophyletic. Topologies from all parameter sets support this assertion. The first freshwater clade is comprised by haplotypes of *Acanthobothrium* sp. 2, which is more closely related to marine species of *Acanthobothrium* from Senegal than any other freshwater lineage (Fig. 3.5b). *Acanthobothrium* sp. 2 share many morphological similarities with these Senegal species, which are absent in the remaining freshwater lineages of its genus: medial and lateral hooks similar in size, straight cirrus sac, and total number of testes equal to or less than 80. At the same time, *Acanthobothrium* sp. 2 can be distinguished from its sister group by presenting hooks with axial prongs longer than abaxial prongs, apolysis apolytic, symmetric ovary, and total length greater than 15 mm.

The remaining species of *Acanthobothrium* from the South American freshwater system (*A. quinonesi*, *A. amazonensis*, *A. terezae*, and *Acanthobothrium* sp. 1) comprise a monophyletic group (this clade is present on trees from the nine parameters, but with low support). Cardoso (2010) distinguished the species complex of *A. terezae/ramiroi* from the remaining freshwater lineages by the presence of more conspicuous musculature extending from the scolex to the strobila, larger number of testes, and larger hooks. The conspicuous morphological differences that immediately distinguishes *A. terezae/ramiroi* from the remaining freshwater species of *Acanthobothrium* and the resemblance of these species could be naively be interpreted as an indication that *Acanthobothrium* sp. 1 is more closely related to the species complex of *A. quinonesi/amazonensis* than to *A. terezae/ramiroi*. Our results contradict this idea, suggesting that *A. terezae/ramiroi* and *Acanthobothrium* sp. 1 are sister group of the clade comprised by *A. amazonensis* and *A. quinonesi*. Although this second clade of freshwater *Acanthobothrium* is not sensitive to alignment parameters, its relationship with its marine sister group is. Despite

the observation that the clade is not one of the least supported clades (GB=15), it was only recovered for the selected parameter set (Fig. 3.5i).

Freshwater species of *Acanthobothrium* have low specificity of the parasites and widespread geographical distribution, which overcomes the distribution of many of their hosts. In this context, our data could further be interpreted as a suggestion that the evolutionary history of species of *Acanthobothrium* in Neotropical river systems could be more related to paleogeographical history of the Neotropical region than to the diversification patterns of their hosts. This hypothesis is supported by the distribution and population relationships between freshwater lineages. For example, *Acanthobothrium* sp. 1 is divided into two clades representing the Orinoco and the Amazon River basins, which could be interpreted as a result of the separation of the Llanos basin from the Amazon system by the Vaupes-Guaviare region around 10.5 Ma followed by episodic connections between those drainages via episodic connections similar to the current Casiquiare Canal or megafans in the foreland basins (Wilkinson *et al.*, 2010; Lima and Ribeiro, 2011). *Acanthobothrium quinonesi* haplotypes collected in the Amazon River basin are more related to the fauna in the Orinoco than to the La Plata basin, resembling patterns observed in biogeographical analysis of several fish taxa in tropical South America (Albert and Carvalho, 2011) and the observation of semipermeable barriers dividing these drainages (Albert *et al.*, 2004). Wide distributions such as these span the divides of one or more basins and thus may provide evidence of historical relationships among foreland basins (see Lima and Ribeiro, 2011), or simply as evidence of dispersal routes (Hubert and Renno, 2006; Domingues and Marques, 2010). Thus, expanding the data on freshwater lineages of *Acanthobothrium* may thus add valuable insight into the diversification of the freshwater *Acanthobothrium* fauna in South American river systems. Unfortunately, our current data does not allow further inference into the history of *Acanthobothrium* diversification after the colonization of South American freshwater. Nevertheless, it surely allows us to position freshwater *Acanthobothrium* lineages within a broader concept, supporting the hypothesis that these lineages do not share an exclusive common ancestor, which ushers us to debate the implications about how *Acanthobothrium* colonized Neotropical river systems.

3.4.5 ON THE POLYPHYLY OF FRESHWATER *Acanthobothrium* LINEAGES AND ITS IMPLICATIONS

Brooks *et al.* (1981b) suggested that the non/monophyly of potamotrygonid parasites, such as freshwater *Acanthobothrium* lineages, should indicate the non/monophyly of their hosts. Although this information may provide some insight about the evolutionary history of their hosts, Lovejoy (1997) pointed out that the non/monophyly of the parasites is not sufficient to support or reject host monophyly unless supporting evidence is provided (e.g., degree of

specificity, potential frequency of host switching, sampling error probability). Lovejoy (1997) discussed why the monophyly of the parasites is not by itself evidence of the monophyly of their hosts and provided an illustrative example in his Figure 1. We modified this illustration to show two distinct scenarios in which a polyphyletic group of freshwater *Acanthobothrium* lineages could be formed (Fig. 3.6).

To say that a non-monophyletic group of freshwater *Acanthobothrium* indicate the para/polyphyly of the potamotrygonids would require evidence that each host taxon holds only one parasite taxon or a monophyletic assemblage at any taxonomic level. In this case, the para/polyphyly of freshwater *Acanthobothrium* would only be possible via multiple invasions of South American freshwater by separated host taxa (Fig. 3.6a). This hypothesis is unlikely given the evidence (including shared morphological structures, such absence of fossa or foramen in the scapular process and presence of an extended pelvic process, which are not ecologically correlated with the freshwater environment) that support the monophyly of Potamotrygonidae (Lovejoy, 1996; de Carvalho *et al.*, 2004). Given the monophyly of Potamotrygonidae, it is likely that two or more independent parasite lineages have invaded freshwater via the common ancestor of the freshwater stingrays. It is common for more than one marine species of *Acanthobothrium* to show specificity toward the same host species. In fact, it is not uncommon for two to four different species of *Acanthobothrium* to be found parasitizing a single host species (e.g., see Alexander, 1953; Marques *et al.*, 1997; Caira and Burge, 2001; Ghoshroy and Caira, 2001; Fyler and Caira, 2006; Reyda and Caira, 2006). Recently, Fyler *et al.* (2009) reported five new species of *Acanthobothrium* from a single specimen of *Himantura* sp. Furthermore, synhospitalic congeners often represent multiple independent lineages (Fyler, 2009). Therefore, the monophyly of Potamotrygonidae as well as the observations of multiple independent lineages of *Acanthobothrium* in the same host specimen add support to the hypothesis that at least two separate lineages of *Acanthobothrium* could have invaded the South American freshwater via the exclusive common ancestor of the potamotrygonids, as exemplified in Figure 3.6b. The colonization of the Neotropical freshwater system by members of *Acanthobothrium* was followed by extensive diversification of this genus, and the resulting lineages are now well-established components of South American river systems.

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Figure 3.6: Representation of the *Acanthobothrium* invasion of South American freshwater. A polyphyletic group of freshwater *Acanthobothrium* lineages result in both scenarios, exemplifying that polyphyletic parasite clades do not necessarily indicate host polyphyly. (a) Invasion by two stingray host lineages, each infected with a separate *Acanthobothrium* lineage. (b) Invasion by single stingray host lineage infected with two separate *Acanthobothrium* lineages. Modified from Lovejoy (1997: Fig.1).

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Considerações finais

4.1 A origem de *Acanthobothrium*

A revisão taxonômica de *Acanthobothrium* está além do escopo deste trabalho. Porém, inconcistências nomenclaturais (i.e., a falta de consenso a respeito da autoria do gênero, sua data de publicação, sua espécie-tipo e seu hospedeiro-tipo) atestam a necessidade de se revisar os atos nomenclaturais responsáveis pela disponibilização e tipificação de *Acanthobothrium*. Tal revisão foi impresssindível para delinear-mos da melhor maneira possível nosso objeto de estudo. Verificou-se que a autoria de *Acanthobothrium* deve ser atribuída a Blanchard, 1848. A espécie-tipo do gênero é *Acanthobothrium coronatum* Blanchard, 1848. O nome válido de *A. coronatum* é *Acanthobothrium corollatum* (Abildgaard, 1790) n. comb. O hospedeiro-tipo de *Acanthobothrium* corresponde ao hospedeiro-tipo do sinônimo mais antigo de *A. corollatum*, *Raja batis* L. Esforços recentes em localizar o material tipo de *A. corollatum* provaram-se infrutíferos. No caso de que este material seja considerado perdido, não se aconselha a designação de um neótipo. Isto porque o neótipo deve estar relacionado ao conceito original de *T. corollata*, i.e., helmintos parasitas de *R. batis* portadores de quatro botrídeos, cada um portando um par de ganchos bifurcados. Como atualmente são conhecidas ao menos cinco espécies nominais de *Acanthobothrium* parasitas de *R. batis* (*A. coronatum*, *A. icelandicum*, *A. parvum*, *A. rajaebatis*, and *A. septentrionale*), a identificação daquela a qual Abildgaard (1790) atribuiu o nome de *T. corollata* é problemática, se não impossível.

4.2 *Acanthobothrium* é monofilético?

Apesar de já ter sido alvo de várias revisões taxonômicas (e.g., Linton, 1925; Baer, 1948; Goldstein, 1967; Williams, 1969; Campbell & Beveridge, 2002) e uma hipótese filogenética representativa de suas espécies (Fyler, 2009), a monofilia de *Acanthobothrium* permanece discutível. Nossa análise não corrobora a monofilia do gênero devido em parte a seu

relacionamento com helmintos biloculados com ganchos bifurcados do gênero *Uncibilocularis*. Nossos resultados sugerem a necessidade de se expandir a amostragem de membros de *Uncibilocularis* em uma análise filogenética que permita avaliar a delimitação deste táxon com base em sua consistência morfológica e monofilia em relação aos demais gêneros de Tetraphyllidea. É provável que os botrídios biloculados de *Uncibilocularis*, única característica que o distingue inequivocamente de *Acanthobothrium*, venha a ser considerada um caráter derivado dentro de *Acanthobothrium*. Neste caso, *Uncibilocularis* deverá ser considerado sinônimo júnior de *Acanthobothrium*, que deverá ser redescrito para acomodar espécies com um ou dois septos transversais por botrídio.

4.3 Clados negligenciados

A discussão das relações filogenéticas entre as espécies marinhas de *Acanthobothrium* foi extensivamente comentada por Fyler (2009) e foge do escopo desta contribuição. Entretanto, ao menos dois casos merecem destaque. Primeiramente, os terminais CF75 e CF77, identificados como “*Acanthobothrium* sp. n. 31” por Fyler (2009), estão mais relacionados com Proteocephalidea do que com as demais espécies de *Acanthobothrium*, indicando que é preciso cuidado na futura descrição desta espécie (veja resultados e Fyler, 2009). Em segundo lugar, é preciso chamar atenção para o clado formado pelos representantes de *A. masniae* Fyler & Caira, 2006 e *A. saliki* Fyler & Caira, 2006. A monofilia recíproca de todas as espécies marinhas de *Acanthobothrium* é recuperada, com a exceção destas duas. Isto sugere que a delimitação destas duas espécies devem ser estudadas com maior cuidado. Este mesmo resultado é encontrado na hipótese filogenética proposta por Fyler (2009). A descrição destas espécies por Fyler & Caira (2006) conta com respectivamente 17 e 14 espécimes coletados na mesma localidade e em um único hospedeiro. Todas as medidas relacionadas à escólex, botrídios e ganchos destas duas espécies apresentam sobreposição. Seu comprimento total e várias outras medidas associadas às proglótides também não são taxonomicamente informativas para distinguir *A. masniae* de *A. saliki*. Entretanto, ambas diferem quanto ao número de proglótides (respectivamente $31,4 \pm 5,1$ e $14,4 \pm 2,9$)¹, comprimento da proglótide terminal (respectivamente $373,5 \mu\text{m} \pm 57,2$ e $587,2 \mu\text{m} \pm 117,6$), número de testículos (respectivamente $7,1 \pm 1,3$ e $20,6 \pm 1,9$), comprimento dos testículos (respectivamente $19,5 \mu\text{m} \pm 2,5$ e $27,4 \mu\text{m} \pm 5,0$), largura dos testículos (respectivamente $33,1 \mu\text{m} \pm 4,4$ e $47,3 \mu\text{m} \pm 8,5$), largura do saco do cirro (respectivamente $55,2 \mu\text{m} \pm 4,7$ e $63,8 \mu\text{m} \pm 3,5$) e largura do ovário (respectivamente $70,4 \mu\text{m} \pm 7,0$ e $98,3 \mu\text{m} \pm 8,0$). *A. masniae* ainda difere de *A. saliki* por apresentar testículos parcialmente ou completamente atrofiados nas proglótides maduras, campo de testículos pós-ovarianos e ovário assimétrico.

¹As medidas são dadas como média e desvio padrão; mais detalhes em Fyler & Caira (2006:107, Tabela 1).

Apesar de não recuperarmos a monofilia recíproca de *A. masnihae* e *A. saliki*, nossa análise não deve ser tomada como uma indicação de que ambas as espécies devam ser sinonimizadas, especialmente dadas suas diferenças morfológicas. Nossa sugestão neste caso é apenas que se expandam as amostragens para ambas as espécies de modo a se aprimorar o registro de sua plasticidade fenotípica.

4.4 Relações filogenéticas entre *Acanthobothrium* dulcícolas

Brooks *et al.* (1981) e Fyler (2009) foram os únicos autores a analisarem espécies dulcícolas de *Acanthobothrium* dentro de um contexto filogenético. Entretanto, Brooks *et al.* jamais descreveram os caracteres empregados em sua análise nem mesmo especificaram os métodos empregados em suas análises filogenéticas, o que dificulta a avaliação de seus resultados. O trabalho de Fyler é muito mais robusto neste sentido, entretanto apenas três terminais de duas espécies nominais de *Acanthobothrium* parasitas de potamotrigonídeos foram contemplados pela autora. Este trabalho, portanto, apresenta a primeira avaliação explícita do posicionamento filogenético de linhagens de *Acanthobothrium* parasitas de potamotrigonídeos.

Com base no posicionamento filogenético de novas linhagens descobertas por Cardoso (2010), chegamos à conclusão de que, ao contrário do sugerido por Brooks *et al.* e reiterado por Fyler, as linhagens dulcícolas de *Acanthobothrium* não constituem um grupo monofilético. *Acanthobothrium* sp. 2 está mais relacionada à congêneres marinhos da costa do Senegal do que com o restante das espécies de água doce (veja Fig. 4). Com base nestes resultados, podemos discutir aspectos do processo de colonização do sistema fluvial neotropical, padrões de variação morfológica intraespecífica e padrões de especificidade ao hospedeiro.

A mono/para/polifilia dos parasitas não necessariamente implica na monofilia/polifilia do hospedeiro (Lovejoy, 1997). Atualmente, a monofilia dos potamotrigonídeos é fortemente corroborada (Rosa, 1985; Lovejoy, 1996; de Carvalho *et al.*, 2004) e há diversos trabalhos registram duas ou mais linhagens separadas de *Acanthobothrium* ocorrendo em simpatria dentro de uma única espécie de hospedeiro (Fyler & Caira, 2006; Reyda & Caira, 2006; Fyler *et al.*, 2009; ver relacionamentos filogenéticos em Fyler, 2009, e em nosso cladograma, Apêndice A.9: Figura A.1). Assim, propomos que a para/polifilia de *Acanthobothrium* parasitas de arraia de água doce é explicada por duas ou mais linhagens independentes de *Acanthobothrium* infectando o ancestral dos potamotrigonídeos.

Recuperamos a monofilia recíproca de *Acanthobothrium* sp. 1, *Acanthobothrium* sp. 2 e *A. terezae* (sin. *A. ramiroi*) conforme a delimitação destas espécies proposta por Cardoso. Este resultado sugere que a grande variabilidade morfológica observada dentro destas espécies assim como sua baixa especificidade ao hospedeiro não são artefatos taxonômicos. A variabilidade morfológica documentada e os padrões de especificidade observado se repetem dentre as

demais linhagens de tetrafilídeos parasitas de potamotrigonídeos (como em *Rhinebothrium*; Reyda & Marques, 2011) e difere significativamente do que se reporta para seus congêneres marinhos. Neste momento, não está claro se os padrões conflitantes são causados por características específicas do sistema potamotrigonídeos/parasitas (ver Reyda & Marques, 2011) ou um reflexo do baixo número de espécimes empregados na descrição de espécies de tetrafilídeos marinhos, que frequentemente não ultrapassa 20 exemplares.

Ademais, não fomos capazes de recuperar a monofilia recíproca de *A. quinonesi* (sin. *A. regoi* e *A. peruvienne*) e *A. amazonensis*. Hoje, a única característica que distingue estas espécies é o formato do saco do cirro: triangular ou em forma de “T” em *A. quinonesi* e reto em *A. amazonensis*. Entretanto, Cardoso já demonstrou que esta característica é variável e a diferença observada atualmente pode ser um reflexo do pequeno número de espécimes de *A. amazonensis* que foram examinados por Cardoso (apenas 4 exemplares, em comparação com mais de 300 exemplares examinados para *A. quinonesi*). A decisão a respeito da sinonimização destas duas espécies carece, portanto, da examinação material adicional para *A. amazonensis*.

Reiteramos a conclusão central de Cardoso (2010), de que taxonomistas devem ter cautela no uso de dados morfométricos e merísticos na diagnose de espécies que são descritas com base um material biológico limitado. Para se acessar a variabilidade morfológica de tetrafilídeos, em geral, é requerido o aumento do tamanho amostral considerando diferentes hospedeiros e áreas, uma vez que a representatividade biogeográfica é fundamental para a compreensão da biodiversidade.

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Machado, D.J. Dissertação de Mestrado, 2012

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Resumo

Primeiramente, apresentamos esclarecimentos sobre a origem do táxon *Acanthobothrium*. A autoria do gênero deve ser creditada à Blanchard em 1848, ocasião na qual este autor disponibilizou a primeira espécie válida para o gênero, *Acanthobothrium coronatum*. A espécie tipo de *Acanthobothrium* é *A. coronatum* Blanchard, 1848, um sinônimo júnior de *A. corollatum* (Abilgaard, 1790). Também foi identificada *Raja batis* L. como hospedeiro-tipo do gênero. A seguir, empregamos a otimização direta de 16S, 28S, and ITS1 rDNA para avaliar o posicionamento filogenético das linhagens de *Acanthobothrium* parasitas de arraias de água doce (Potamotrygonidae). As delimitações morfológica das espécies *Acanthobothrium* sp. 1, *Acanthobothrium* sp. 2 e *A. terezae* (sin. *A. ramiroi*) são validadas pela monofilia recíproca destas linhagens. São apresentados indícios de que *A. amazonensis* é sinônimo de *A. quinonesi*. Nossos resultados reforçam a ideia de que tetrafilídeos parasitas de potamotrigonídeos apresentam grande variação morfológica intraespecífica e baixa especificidade (em contraste com seus congêneres marinhos). *A. quinonesi* é grupo irmão do clado que compreende *A. terezae* e *Acanthobothrium* sp. 1. *Acanthobothrium* sp. 2 está mais relacionados com seus congêneres marinhos do que com as demais espécies parasitas de potamotrigonídeos, o que sugere que a colonização da água doce por *Acanthobothrium* se deu através de pelo menos duas linhagens ancestrais independentes que infectavam o ancestral exclusivo comum das arrais de água doce.

Palavras-chave: *Acanthobothrium*, sistemática, homologia dinâmica.

Abstract

We clarify some major points in the taxonomic history of *Acanthobothrium*. The authorship of the genus must be given to Blanchard in 1848, occasion in which its first species, *Acanthobothrium coronatum*, was created. Therefore, the type species of *Acanthobothrium* is *A. coronatum* Blanchard, 1848, a junior synonym of *A. corollatum* (Abildgaard, 1790). Both *Acanthothrothrium* and *A. corollatum* have their concepts attached to armed tapeworms parasites of *Raja batis* L., their type host. Following we provided a phylogenetic analysis based on the direct optimization of 16S, 28S, and ITS1 rDNA to access the phylogenetic position of freshwater *Acanthobothrium* lineages. The current morphological delimitations of *Acanthobothrium* sp. 1, *Acanthobothrium* sp. 2, *A. terezae* (syn. *A. ramiroi*) and *A. quinonesi* (syn. *A. regoi* and *A. peruvienne*) are corroborated. There are indications that *A. amazonensis* should be considered synonym of *A. quinonesi*. Our results provide additional evidence to support the idea that freshwater lineages of tetraphyllideans (as opposed to marine tetraphyllideans) presents great intra-specific morphological variation and low host specificity. *A. quinonesi* is sister group of a clade that comprises *A. terezae* and *Acanthobothrium* sp. 1. *Acanthobothrium* sp. 2 is more related to marine species of *Acanthobothrium* than to freshwater congeners. This suggests that the colonization of the freshwater system by *Acanthobothrium* is likely to have occurred via at least two separate lineages of *Acanthobothrium* infecting the exclusive common ancestor of the potamotrygonids, which would in time be responsible for the existence of two distinct clades of freshwater species of *Acanthobothrium*.

Keywords: *Acanthobothrium*, systematics, dynamic homology.

Anexos

A.1 Collection of freshwater stingrays

Table A.1: Potamotrygonid stingray species and localities sampled in this study.

Field code†	Sub-basin	Water body	Municipality, state and country	Lat.	Long.	Collection date	Species
<i>Amazon River System</i>							
MT05-009	Tapajós River	Teles Pires River	Alta Floresta, MT, Brazil	-8.88	-57.38	12/05/2005	<i>Potamotrygon sp.</i> (tpj2)
MT05-033		Jamanxim River	Novo Progresso, PA, Brazil	-7.16	-55.43	12/13/2005	<i>Potamotrygon sp.</i> (jabuti)
PA07-075	Lower Amazon	Igarapé do Urubu	Cachoeira do Arari, PA - BR	-1.00	-48.96	08/29/2007	<i>Po. motoro</i>
RN05-085	Negro/Branco	Negro River	Barcelos, AM, Brazil	-0.67	-63.20	02/04/2005	<i>Po. motoro</i>
RN05-087		Negro River	Barcelos, AM, Brazil	-0.98	-62.92	02/04/2005	<i>Po. motoro</i>
RN11-002		Negro River	Barcelos, AM, Brazil	-0.96	-62.94	01/09/2011	<i>Pa. aiereba</i>
RN11-023		Negro River	Barcelos, AM, Brazil	-0.96	-62.94	01/10/2011	<i>Po. schroederi</i>
RN11-029		Negro River	Barcelos, AM, Brazil	-0.96	-62.94	01/10/2011	<i>Po. orbignyi</i>
RN11-034		Negro River	Barcelos, AM, Brazil	-0.96	-62.94	01/12/2011	<i>Po. schroederi</i>
RN11-037		Negro River	Barcelos, AM, Brazil	-0.83	-63.04	01/12/2011	<i>Pa. aiereba</i>
RN11-039		Negro River	Barcelos, AM, Brazil	-0.83	-63.04	01/12/2011	<i>Pa. aiereba</i>
RN11-042		Negro River	Barcelos, AM, Brazil	-0.83	-63.04	01/12/2011	<i>Po. schroederi</i>
RN11-043		Negro River	Barcelos, AM, Brazil	-0.73	-62.93	01/13/2011	<i>Po. motoro</i>
RN11-044		Negro River	Barcelos, AM, Brazil	-0.73	-62.93	01/13/2011	<i>Po. motoro</i>
RN11-050		Negro River	Barcelos, AM, Brazil	-0.66	-63.02	01/14/2011	<i>Po. schroederi</i>
RN11-053		Negro River	Barcelos, AM, Brazil	-0.66	-63.02	01/14/2011	<i>Po. motoro</i>
RN11-054		Negro River	Barcelos, AM, Brazil	-0.66	-63.02	01/14/2011	<i>Po. motoro</i>
RN11-056		Negro River	Barcelos, AM, Brazil	-0.66	-63.02	01/14/2011	<i>Po. motoro</i>

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Table A.1 – Continued from previous page

Field code†	Sub-basin	Water body	Municipality, state and country	Lat.	Long.	Collection date	Species
RN11-057		Negro River	Barcelos, AM, Brazil	-0.66	-63.02	01/15/2011	<i>Po. motoro</i>
RN11-058		Negro River	Barcelos, AM, Brazil	-0.96	-62.94	01/16/2011	<i>Po. schroederi</i>
RN11-063		Negro River	Barcelos, AM, Brazil	-0.96	-62.94	01/16/2011	<i>Po. schroederi</i>
VZ11-012	Sinamaica lagoon	Limón River	Venezuela	11.04	-71.86	02/18/2011	<i>Po. yepezi</i>
VZ11-013		Limón River	Venezuela	11.04	-71.86	02/18/2011	<i>Po. yepezi</i>
VZ11-018		Limón River	Venezuela	11.04	-71.86	02/18/2011	<i>Po. yepezi</i>
VZ11-025	Apure	Apure River	Aguaro-Guaritico, Venezuela	7.89	-68.88	02/23/2011	<i>Pa. aiereba</i>
VZ11-026		Apure River	Aguaro-Guaritico, Venezuela	7.89	-68.88	02/23/2011	<i>Po. cf. orbignyi</i>
VZ11-029		Apure River	Aguaro-Guaritico, Venezuela	7.89	-68.88	02/23/2011	<i>Po. cf. orbignyi</i>
VZ11-031		Apure River	Aguaro-Guaritico, Venezuela	7.89	-68.88	02/23/2011	<i>Pa. aiereba</i>
VZ11-033		Apure River	Aguaro-Guaritico, Venezuela	7.89	-68.88	02/23/2011	<i>Pa. aiereba</i>
VZ11-034		Apure River	Aguaro-Guaritico, Venezuela	7.89	-68.88	02/23/2011	<i>Po. cf. orbignyi</i>
<i>de La Plata River System</i>							
MS10-005	Paraguay River	Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/11/2010	<i>Po. motoro</i>
MS10-007		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/11/2010	<i>Po. motoro</i>
MS10-014		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/11/2010	<i>Po. cf. motoro</i>
MS10-018		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/12/2010	<i>Po. motoro</i>
MS10-021		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/12/2010	<i>Po. cf. motoro</i>
MS10-026		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/13/2010	<i>Po. motoro</i>
MS10-028		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/13/2010	<i>Po. histrix</i>
MS10-035		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/13/2010	<i>Po. cf. motoro</i>
MS10-041		Salobra River	Miranda, MS, Brazil	-20.21	-56.50	07/13/2010	<i>Po. histrix</i>

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Table A.1 – Continued from previous page

Field code†	Sub-basin	Water body		Municipality, state and country	Lat.	Long.	Collection date	Species
MS10-043	Paraná River	Salobra River		Miranda, MS, Brazil	-20.21	-56.50	07/13/2010	<i>Po. falkneri</i>
PR09-001		Itaipu Dam		Paraguay/ Brazil	-25.53	-49.39	10/27/2009	<i>Po. motoro</i>
PR09-002		Itaipu Dam		Paraguay/ Brazil	-25.53	-49.39	10/27/2009	<i>Po. motoro</i>
PR09-005		Itaipu Dam		Paraguay/ Brazil	-25.53	-49.39	10/27/2009	<i>Po. motoro</i>
PR09-006		Itaipu Dam		Paraguay/ Brazil	-25.53	-49.39	10/27/2009	<i>Po. motoro</i>
PR09-007		Itaipu Dam		Paraguay/ Brazil	-25.53	-49.39	10/27/2009	<i>Po. motoro</i>
<i>Atlantic Ocean, Brazil</i>								
AL10-004	-	Easter Ocean	Atlantic	AL, Brazil	-9.67	-35.73	09/29/2010	<i>Dasyatis guttata</i>
AL10-005	-	Easter Ocean	Atlantic	AL, Brazil	-9.67	-35.73	09/29/2010	<i>Dasyatis guttata</i>

†Field codes correspond to locations shown in Figure 1. For further information, look the online Host-Parasite collection (www.ib.usp.br/hpc/hpc_index.html).

A.2 Coleta e fixação do material biológico

Os espécimes de potamotrigonídeos amostrados neste trabalho foram coletados com diversos métodos, tais como espinhéis, redes e arpão.

Após a coleta dos hospedeiros, aqueles submetidos à necropsia sofreram eutanásia pela injeção de solução saturada de benzocaína em etanol 92% na região cardíaca. As válvulas espirais foram removidas com hemostatos e bisturis, fixadas em etanol 95-100% e armazenadas em gelo.

Em laboratório, as válvulas foram armazenadas a -20°C até a triagem. As válvulas foram triadas sob esteromicroscópio para a remoção dos espécimes de parasitas, os quais foram posteriormente estocados a -70°C até a seleção para extração de DNA genômico e preparação de lâminas de hologenóforos (Anexo [A.3](#)).

A.3 Preparação de lâminas de hologenóforos

- Hologenóforos em etanol 100% foram hidratação em séries alcoólicas regressivas
  - Etanol 70%: 3 min
  - Etanol 50%: 3 min
  - Etanol 30%: 3 min
  - Água destilada I: 3 min
  - Água destilada II: 3 min
- Exemplos hidratados foram corados com Hematoxilina de Delafield por 30-120 min
- Amostras coradas foram hidratadas em séries alcólicas progressivas
  - Etanol 30%: 3 min
  - Etanol 50%: 3 min
  - Etanol 70%: 3 min
  - Neste ponto, sempre que necessário, o excesso de corante foi removido
    - * Manter a amostra corada em solução alcoólica (etanol 70%) de HCl 1% até a remoção do excesso de corante
    - * Transferir para solução alcóolica (etanol 70%) de hidróxido de amônia 2% por 1min
  - Etanol 100% I: 3min
  - Etanol 100% II: 3min
- Amostras coradas e desidratadas foram diafanizadas em Salicilato de Metila
  - Salicilato de Metila 75%: 3min
  - Salicilato de Metila 100% I: 3min
  - Salicilato de Metila 100% II: 3min
- Hologenóforos foram montados em lâmina e lamínula com Bálsamo do Canadá

A.4 Original cestode material collected in South America

Table A.2: Original cestode material collected in South America. Cestodes sequenced, with taxon names, hosts, collection localities, and museum voucher numbers for hologenophores.

Molecular code	Field code †	MZUSP	Species	Host	Locality	Genes		
						16S	28S	ITS1
A006	PA07-075.20	7349	<i>Acanthobothrium cf. amazonensis</i>	<i>Po. motoro</i>	Igarapé do Urubu, Cachoeira do Arari, PA - BR	•	•	
A017	MT05-009.06	7358	<i>Acanthobothrium sp.</i>	<i>Potamotrygon sp. (negra)</i>	Teles Pires River, Alta Floresta, MT - BR	•		•
A023	PR09-001.01	7362	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•		•
A026	PR09-006.01	7365	<i>Acanthobothrium sp.</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A027	PR09-007.01	7366	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A028	PR09-001.02	7367	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR		•	•
A029	PR09-001.03	No voucher	<i>Acanthobothrium sp.</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•		•
A030	PR09-002.02	7368	<i>Acanthobothrium sp.</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A032	PR09-005.02	7370	<i>Acanthobothrium sp.</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•

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Table A.2 – Continued from previous page

Molecular code	Field code †	MZUSP	Species	Host	Marine water body or freshwater river (system)	Genes		
						16S	28S	ITS1
A034	PR09-006.02	7372	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A035	PR09-006.03	7373	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A036	PR09-006.04	7374	<i>Acanthobothrium cf. amazonensis</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A037	PR09-006.05	7375	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A038	PR09-006.06	7376	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A039	PR09-006.07	7377	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Paraná River, Foz do Iguaçu, PR - BR	•	•	•
A045	MS10-005.01	7383	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS BR	-	•	•
A046	MS10-005.02	7384	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS BR	-	•	•
A047	MS10-014.01	7385	<i>Acanthobothrium cf. quinonesi</i>	<i>Potamotrygon sp.</i>	Salobra River, Miranda, MS BR	-	•	•
A048	MS10-014.02	7386	<i>Acanthobothrium cf. quinonesi</i>	<i>Potamotrygon sp.</i>	Salobra River, Miranda, MS BR	-	•	•
A049	MS10-018.01	7387	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS BR	-	•	•

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Table A.2 – Continued from previous page

Molecular code	Field code †	MZUSP	Species	Host	Marine water body or freshwater river (system)	Genes		
						16S	28S	ITS1
A050	MS10-021.01	7388	<i>Acanthobothrium cf. quinonesi</i>	<i>Potamotrygon sp.</i>	Salobra River, Miranda, MS - BR	•	•	•
A051	MS10-021.02	7389	<i>Acanthobothrium cf. quinonesi</i>	<i>Potamotrygon sp.</i>	Salobra River, Miranda, MS - BR	•	•	•
A052	MS10-028.01	7390	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. histrix</i>	Salobra River, Miranda, MS - BR	•	•	•
A053	MS10-041.01	7391	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. histrix</i>	Salobra River, Miranda, MS - BR	•	•	•
A054	MS10-041.02	7392	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. histrix</i>	Salobra River, Miranda, MS - BR	•	•	•
A055	MS10-043.01	7393	<i>Acanthobothrium quinonesi</i>	<i>Po. falkneri</i>	Salobra River, Miranda, MS - BR	•	•	•
A056	MS10-043.02	7394	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. falkneri</i>	Salobra River, Miranda, MS - BR	•	•	•
A062	MS10-026	7400	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS - BR	•	•	•
A063	MS10-005	7401	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS - BR	•	•	•
A064	AL10-004.01	7402	<i>Acanthobothrium sp.</i>	<i>Dasyatis guttata</i>	Atlantic Ocean, Maceió, AL - BR	•	•	•
A065	AL10-004.02	7403	<i>Acanthobothrium sp.</i>	<i>Da. guttata</i>	Atlantic Ocean, Maceió, AL - BR	•	•	•

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Table A.2 – Continued from previous page

Molecular code	Field code †	MZUSP	Species	Host	Marine water body or freshwater river (system)	Genes		
						16S	28S	ITS1
A066	AL10-005.01	7404	<i>Acanthobothrium sp.</i>	<i>Da. guttata</i>	Atlantic Ocean, Maceió, AL - BR	•	•	•
A067	AL10-005.02	7405	<i>Acanthobothrium sp.</i>	<i>Da. guttata</i>	Atlantic Ocean, Maceió, AL - BR	•	•	•
A069	MS10-007.01	7406	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS - BR	•	•	•
A070	MS10-035.01	7407	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Salobra River, Miranda, MS - BR	•	•	•
A076	MT05-033.07	7408	<i>Acanthobothrium cf. amazonensis</i>	<i>Potamotrygon sp. (tpj1)</i>	Jamanxim River, Novo Progresso, PA - BR	•		•
A077	MT05-033.09	7409	<i>Acanthobothrium cf. quinonesi</i>	<i>Potamotrygon sp. (tpj1)</i>	Jamanxim River, Novo Progresso, PA - BR	•		•
A090	RN05-085.02	7414	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•		•
A092	RN05-087.02	7416	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A096	VZ11-025.17	7418	<i>Acanthobothrium sp. 1</i>	<i>Paratrygon aiereba</i>	Apure/Guaritico river - VE	•	•	•
A097	VZ11-026.20	7419	<i>Acanthobothrium cf. sp. 1</i>	<i>Po. yepezi</i>	Apure/Guaritico river - VE	•	•	•
A098	VZ11-013.01	7420	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. yepezi</i>	Limón River - VE	•		•
A099	VZ11-018.02	7421	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. yepezi</i>	Limón River - VE	•	•	•
A100	VZ11-018.04	7422	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. yepezi</i>	Limón River - VE	•	•	•
A101	VZ11-012.01	7423	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. yepezi</i>	Limón River - VE	•	•	•
A102	VZ11-012.04	7424	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. yepezi</i>	Limón River - VE	•	•	•
A103	VZ11-034.01	7425	<i>Acanthobothrium terezae</i>	<i>Po. orbignyi</i>	Apure River - VE			•

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Table A.2 – Continued from previous page

Molecular code	Field code †	MZUSP	Species	Host	Marine water body or freshwater river (system)	Genes		
						16S	28S	ITS1
A104	VZ11-034.04	7426	<i>Acanthobothrium sp. 1</i>	<i>Po. orbignyi</i>	Apure River - VE	•	•	•
A107	VZ11-025.39	7429	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Apure River - VE	•		•
A108	VZ11-025.40	7430	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Apure River - VE	•	•	•
A110	VZ11-033.03	7432	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Apure River - VE	•	•	•
A111	VZ11-033.06	7433	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Apure River - VE	•	•	•
A112	VZ11-031.01	7434	<i>Acanthobothrium terezae</i>	<i>Pa. aiereba</i>	Apure River - VE	•		•
A113	VZ11-031.13	7435	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Apure River - VE	•	•	•
A114	VZ11-026.04	7436	<i>Acanthobothrium sp. 1</i>	<i>Po. orbignyi</i>	Apure River - VE	•	•	•
A115	VZ11-029.01	7437	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. orbignyi</i>	Apure River - VE	•	•	
A116	RN11-043.02	7438	<i>Acanthobothrium amazonensis</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•		•
A117	RN11-043.03	7439	<i>Acanthobothrium amazonensis</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A118	RN11-058.04	7440	<i>Acanthobothrium sp. 2</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	
A120	RN11-058.01	7441	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A121	RN11-058.03	7442	<i>Acanthobothrium cf. sp. 2</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A122	RN11-058.06	7443	<i>Acanthobothrium sp. 1</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A124	RN11-002.02	7445	<i>Acanthobothrium cf. sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A125	RN11-034.02	7446	<i>Acanthobothrium amazonensis</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A126	RN11-034.03	7447	<i>Acanthobothrium sp. 1</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A127	RN11-034.04	7448	<i>Acanthobothrium sp. 1</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	
A129	RN11-057.05	7450	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A130	RN11-057.09	7451	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A131	RN11-039.01	7452	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•

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Table A.2 – Continued from previous page

Molecular code	Field code †	MZUSP	Species	Host	Marine water body or freshwater river (system)	Genes		
						16S	28S	ITS1
A132	RN11-039.05	7453	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A133	RN11-039.08	7454	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A134	RN11-039.09	7455	<i>Acanthobothrium sp.</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A135	RN11-039.11	7456	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	
A136	RN11-042.01	7457	<i>Acanthobothrium quinonesi</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A138	RN11-042.05	7459	<i>Acanthobothrium cf. sp. 2</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A139	RN11-042.06	7460	<i>Acanthobothrium sp. 1</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A140	RN11-037.01	7461	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A141	RN11-037.04	7462	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A142	RN11-037.08	7463	<i>Acanthobothrium sp. 1</i>	<i>Pa. aiereba</i>	Negro River, Barcelos, AM - BR	•	•	•
A144	RN11-023.01	7465	<i>Acanthobothrium cf. sp. 2</i>	<i>Po. schroederi</i>	Negro River, Barcelos, AM - BR	•	•	•
A146	RN11-053.04	7467	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A147	RN11-054.01	7468	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A148	RN11-054.03	7469	<i>Acanthobothrium cf. amazonensis</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A149	RN11-054.05	7470	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A150	RN11-050.01	7471	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A151	RN11-050.02	7472	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A152	RN11-044.01	7473	<i>Acanthobothrium quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A153	RN11-044.02	7474	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A154	RN11-056.01	7475	<i>Acanthobothrium amazonensis</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A156	RN11-056.05	7477	<i>Acanthobothrium cf. amazonensis</i>	<i>Po. motoro</i>	Negro River, Barcelos, AM - BR	•	•	•
A158	RN11-029.01	7479	<i>Acanthobothrium cf. quinonesi</i>	<i>Po. orbigny</i>	Negro River, Barcelos, AM - BR	•	•	•

Continued on next page

Table A.2 – Continued from previous page

Molecular code	Field code †	MZUSP	Species	Host	Marine water body or freshwater river (system)	Genes		
						<i>16S</i>	<i>28S</i>	<i>ITS1</i>
A160	RN11-063.02	7480	<i>Acanthobothrium cf. amazonensis</i>	<i>Po. orbignyi</i>	Negro River, Barcelos, AM - BR	•	•	•

†Field codes correspond to locations in Figure 1 (see the online Host-Parasite collection at www.ib.usp.br/hpc/hpc_index.html).

A.5 Protocolo otimizado para extração de DNA genômico com DNAdvance™

Nós testamos diferentes métodos para isolar DNA genômico de uma variedade de espécies de cestodas. Dentre todos os métodos averiguados, o melhor rendimento foi obtido para o kit de isolamento de DNA Agencourt® DNAdvance™, que forneceu extrações mais limpas e com maiores concentrações de DNA.

Este protocolo foi otimizado para tubos eppendorf de 200µL e pequenas amostras de tecidos de cestodas (<3mm³). É altamente recomendado que o tecido seja cortado em pedaços pequenos diretamente dentro dos eppendorfs de 200µL. O excesso de etanol deve então ser removido com a ajuda de uma micropipeta ou por outros meios. Antes de dar início ao protocolo, deixe as amostras secarem a temperatura ambiente ou a 37°C por aproximadamente 30 minutos. Este protocolo empregará a placa magnética Agencourt® SPRIPlate Super Magnet Plate (96-well format, produto no. A32782, a mesma placa empregada para a purificação com o kit Agencourt® AMPure™ XP)

- Mix para digestão (80µL por amostra).
  - Prepare o mix conforme a tabela abaixo. Feito isso, misture gentilmente no vórtex e dê um spin para que o material que estiver na tampa vá para o fundo do eppendorf.

Lysis Buffer	73µl
1M DTT	5µl
Proteinase K (20mg/ mL)	4µl
Volume Final	80µl

- É aconselhável preparar o mix de digestão no gelo. O mix deve ser preparado apenas em quantidade suficiente e ser usado imediatamente após o preparo.
- Adicione 80µl do mix de digestão em cada tudo. Deposite as amostras no vórtex por alguns segundos, em seguida centrifugue gentilmente.
- Incubar a 55°C
  - Mexa as amostras a cada 15 minutos até a digestão total do tecido (o que geralmente demora menos de uma hora mas depende da quantidade de tecido).
- Centrifugue a 15.7 RCF por 10s.
  - Neste momento as amostras poderão ser armazenadas a -70°C caso os passos subsequentes não forem ser realizados imediatamente.

- Adicione 50µl da solução tamponante Bind1 e pipete até ficar homogêneo.
- Misture o conteúdo do frasco de Bind2 até que todas as partículas estejam suspensas e a mistura esteja homogênea.
- Adicione 80µl de Bind2 em cada amostra. Pipete até a mistura ficar homogênea.
- Incube as amostras em temperatura ambiente por 3 minutos.
- Posicione as amostras na placa magnética e aguarde 10 minutos.
- Aspire e descarte o sobrenadante enquanto as amostras estão posicionadas sobre a placa magnética.
  - Ao aspirar, posicione a ponta da pipeta no centro do tubo e cuide para que o anel de partículas magnéticas não se desfaça.
- Retire as amostras da placa. Adicione 150µL de etanol 70% e pipete até que as partículas magnéticas estejam completamente suspensas.
  - Sugere-se que novas soluções de etanol 70% sejam preparadas a cada vez que for repetir este protocolo.
- Reposicione as amostras na placa magnética por 5 minutos.
- Repita os três passos anteriores até ter completado um total de três lavagens com etanol 70%.
- Remova o máximo de etanol possível antes de adicionar o Elution Buffer.
  - Sugere-se secar a temperatura ambiente ou a 37°C por não mais que 15 minutos.
- Retire as amostras da placa magnética. Adicione 100µL de Elution Buffer e misture com a pipeta até que todas as partículas estejam em suspensão.
- Reposicione as amostras sobre a placa magnética por 6 minutos.
- Transfira 80µL do sobrenadante para uma placa limpa ou tubo de armazenamento.
  - Aspire lentamente para não perturbar o anel de partículas magnéticas. Transferir todos os 100µL de produto final não é recomendável pois pode trazer algumas partículas magnéticas. Se partículas estiverem sendo aspiradas durante a transferência, dispense a amostra de volta ao seu tubo original e incube por mais 6 minutos na placa magnética antes de tentar a transferência novamente.

- Se as amostras forem ser armazenadas por um longo período antes do uso, mantenha-as a -70°C . Armazene as amostras entre 4 e -20°C enquanto as estiver usando.

A.6 Protocolo otimizado para purificação de DNA genômico com AMPure™ kit

- Adicione um volume de Agencourt® AMPure™ XP igual a 1,8 vezes o volume de amostra a ser purificada.
  - O volume final não deve ser menor do que 25µL ou a placa magnética não será capaz de separar as partículas magnéticas do retante da solução.
- Posicione as amostras no vórtex brevemente. Em seguida, centrifugue-as brevemente para que todo o material da tampa vá para o fundo do tubo.
- Incube em temperatura ambiente por 5 minutos.
- Posicione as amostras na placa magnética (Agencourt® SPRIPlate Super Magnet Plate, produto no. A32782) por 3 minutos.
- Ainda com as amostras sobre a placa magnética, aspire e descarte o sobrenadante.
  - Enquanto estiver aspirando, posicione a pipeta no centro do tubo para evitar que o anel de partículas magnéticas seja desfeito.
- Adicione gentilmente 150µl de etanol 70% para não desfazer o anel.
  - Recomenda-se fazer novas soluções de etanol 70% a cada vez que se for repetir este protocolo.
- Repita os dois passos anteriores até se completarem duas lavagens com etanol 70%.
- Remova o máximo de etanol possível com a ajuda da pipeta.
  - Após ter removido o etanol com a pipeta, deixe as amostras secarem a 37°C por 15 minutos.
- Adicione 15µl de água Milli-Q misture até que todas as partículas estejam em suspensão.
- Deixe as amostras descansarem a temperatura ambiente por 5 minutos e centrifugue a 15,7 RCF por 3 minutos.
- Transfira o sobrenadante para um tubo novo e armazene a -20°C. Caso as amostras devam ser armazenadas por um longo período antes do uso, mantenha-as a -70°C.

A.7 Sequence data acquired from GenBank

Table A.3: GenBank data for Proteocephalidea and *Acanthobothrium*.

Species	Host	Marine water body or freshwater river system	Gen Bank	
			16S	28S
<i>Acanthobothrium</i> spp.				
<i>A. brevissime</i>	<i>Dasyatis say</i>	Gulf of Mexico, Florida, U.S.A.	EU660532	-
<i>A. parviuncinatum</i>	<i>Urobatis maculatus</i>	Baja California, Mexico	-	EF095264
<i>Acanthobothrium</i> sp. 1	<i>Dasyatis logus</i>	Baja California, Mexico	-	AF286953
Proteocephalidea specimens				
<i>Acanthotaenia</i> sp.	<i>Varanus exanthematicus</i>	Ghana	AJ389480	AJ388593
<i>Gangesia parasiluri</i>	<i>Silurus asotus</i>	Nagano prefecture, Lake Suwa, Japan	AJ389477	AJ388590
<i>Silurotaenia siluri</i>	<i>Silurus glanis</i>	Orlik water reserve, Czech Republic	AJ389479	AJ388592
<i>Proteocephalus pirarara</i>	<i>Phractocephalus hemioliopus</i>	Amazonas, Itacoatiara, Brazil	-	AJ388616
<i>Rudolphiella piracatinga</i>	<i>Calophysus macropterus</i>	Amazonas, Itacoatiara, Brazil	-	AJ388627
<i>Ophiotaenia jarara</i>	<i>Bothrops jararaca</i>	Domingos Martins, Espirito Santo, Brazil	-	AJ388607
<i>Proteocephalus percae</i>	<i>Perca fluviatilis</i>	Neuchatel Lake, Switzerland	-	AJ388594
<i>Proteocephalus tetrastomus</i>	<i>Hypomesus nipponensis</i>	Nagano prefecture, Lake Suwa, Japan	-	AJ388635
<i>Nomimoscolex lenha</i>	<i>Sorubimichthys planiceps</i>	Amazonas, Itacoatiara, Brazil	-	AJ388611
<i>Zygobothrium megacephalum</i>	<i>Phractocephalus hemioliopus</i>	Amazonas, Itacoatiara, Brazil	-	AJ388621

A.8 Sequence data acquired from Fyler (2009)

Table A.4: Specimens of *Acanthobothrium* sequenced by Fyler (2009) with molecular codes, host codes, and localities.

Molecular code	Species	Host	Host code†	Marine water body or freshwater river (system)	Coordinates
CF01	<i>Acanthobothrium</i> sp.	<i>Himantura leoparda</i>	NT-37	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 136.986
CF02	<i>Acanthobothrium</i> sp.	<i>Narcine entemedor</i>	BJ-107	Bahia de Los Angeles, Baja California, Pacific Ocean, Mexico	28.986, -113.537
CF03	<i>Acanthobothrium</i> sp.	<i>Rhinobatos rhinobatos</i>	SE-205	Atlantic Ocean, Senegal	14.678, -17.454
CF04	<i>Acanthobothrium</i> sp.	<i>Raja velezi</i>	BJ-243	Santa Rosalia, Baja California Sur, Pacific Ocean, Mexico	27.331, -112.254
CF05	<i>A. etini</i>	<i>Himantura polylepis</i>	BO-496	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.388
CF06	<i>A. cf. woodsholei</i>	<i>Dasyatis</i> sp.	SE-222	Mbour, Atlantic Ocean, Senegal	14.406, -16.971
CF07	<i>Acanthobothrium</i> sp.	<i>Raja miraletus</i>	SE-56	Atlantic Ocean, Senegal	14.678, -17.455
CF09	<i>Acanthobothrium</i> sp.	<i>Pastinachus atrus</i>	NT-105	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 136.988
CF24	<i>Acanthobothrium</i> sp.	<i>Brachaelurus waddi</i>	KJG-1	Coast of Sydney, Pacific Ocean, Australia	No data available
CF25	<i>A. cf. angelae</i>	<i>Hypnos monopterygius</i>	KJG-7	Coast of Sydney, Pacific Ocean, Australia	No data available
CF26	<i>A. cf. angelae</i>	<i>Hypnos monopterygius</i>	KJG-7	Coast of Sydney, Pacific Ocean, Australia	No data available
CF27	<i>A. cf. angelae</i>	<i>Hypnos monopterygius</i>	KJG-7	Coast of Sydney, Pacific Ocean, Australia	No data available

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Table A.4 – Continued from previous page

Molecular code	Species	Host	Host code†	Marine water body or freshwater river (system)	Coordinates
CF31	<i>A. mattaylori</i>	<i>Rhynchobatus laevis</i>	NT-7	Nhulunbuy (Gove), Northern Territory, Gove Harbor, Gulf of Carpentaria, Pacific Ocean, Australia	-12.194, 136.715
CF32	<i>A. jaenneae</i>	<i>Rhynchobatus laevis</i>	NT-7	Nhulunbuy (Gove), Northern Territory, Gove Harbor, Gulf of Carpentaria, Pacific Ocean, Australia	-12.194, 136.715
CF33	<i>A. hypermekkolpos</i>	<i>Rhynchobatus laevis</i>	NT-7	Nhulunbuy (Gove), Northern Territory, Gove Harbor, Gulf of Carpentaria, Pacific Ocean, Australia	-12.194, 136.715
CF34	<i>A. hypermekkolpos</i>	<i>Rhynchobatus laevis</i>	NT-7	Nhulunbuy (Gove), Northern Territory, Gove Harbor, Gulf of Carpentaria, Pacific Ocean, Australia	-12.194, 136.716
CF35	<i>A. herronorum</i>	<i>Rhynchobatus cf. laevis</i>	NT-66	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.016
CF37	<i>Acanthobothrium sp.</i>	<i>Dipturus batis</i>	AZ-10	Horta, Azores Islands, Faial Island, Atlantic Ocean, Portugal	38.517, -28.649
CF40	<i>Acanthobothrium sp.</i>	<i>Rhinobatos cemiculus</i>	SE-134	Atlantic Ocean, Senegal	14.678, -17.483
CF41	<i>Acanthobothrium sp.</i>	<i>Rhinobatos cemiculus</i>	SE-134	Atlantic Ocean, Senegal	14.678, -17.483
CF45	<i>Acanthobothrium sp.</i>	<i>Pteromylaeus bovinus</i>	SE-143	St. Louis, Atlantic Ocean, Senegal	16.024, -16.534
CF46	<i>Acanthobothrium sp.</i>	<i>Neotrygon cf. kuhlii</i>	BO-409	Sarawak, Borneo, South China Sea, Pacific Ocean, Malaysia	2.817, 110.901

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Table A.4 – Continued from previous page

Molecular code	Species	Host	Host code†	Marine water body or freshwater river (system)	Coordinates
CF47	<i>A. cf. brevissime</i>	<i>Dasyatis say</i>	MS05-402	Florida, Crooked Island Bay, Gulf of Mexico, Atlantic Ocean, U.S.A.	29.994, -85.551
CF48	<i>A. cf. brevissime</i>	<i>Dasyatis say</i>	MS05-402	Florida, Crooked Island Bay, Gulf of Mexico, Atlantic Ocean, U.S.A.	29.994, -85.551
CF50	<i>Acanthobothrium sp.</i>	<i>Dasyatis sabina</i>	MS05-431	Florida, Indian Pass, Gulf of Mexico, Atlantic Ocean, U.S.A.	29.669, -85.251
CF51	<i>Acanthobothrium sp.</i>	<i>Raja eglanteria</i>	MS05-421	Florida, Indian Pass, Gulf of Mexico, Atlantic Ocean, U.S.A.	29.669, -85.252
CF52	<i>Acanthobothrium sp.</i>	<i>Raja eglanteria</i>	MS05-421	Florida, Indian Pass, Gulf of Mexico, Atlantic Ocean, U.S.A.	29.669, -85.252
CF53	<i>Acanthobothrium sp.</i>	<i>Himantura lobistoma</i>	BO-268	Mukah, Sarawak, Borneo, South China Sea, Pacific Ocean, Malaysia	2.898, 112.119
CF61	<i>Acanthobothrium sp.</i>	<i>Dasyatis sp.</i>	SE-222	Mbour, Atlantic Ocean, Senegal	14.406, -17.003
CF65	<i>Acanthobothrium sp.</i>	<i>Paragaleus pectoralis</i>	SE-129	Ouakam, Atlantic Ocean, Senegal	14.715, -17.519
CF68	<i>Uncibilocularis squireorum</i>	<i>Himantura cf. urarnak</i>	CM03-3	Weipa, Queensland, Gulf of Carpentaria, Indian Ocean, Australia	-12.586, 141.736
CF69	<i>Acanthobothrium sp.</i>	<i>Brachaelurus waddi</i>	KJG-1	Coast of Sydney, Pacific Ocean, Australia	No data available
CF70	<i>A. cf. lasti</i>	<i>Himantura leoparda</i>	NT-117	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.020
CF72	<i>N. gen. n. sp. 1</i>	<i>Pristis clavata</i>	AU-36	Buffalo Creek, Timor Sea, Indian Ocean, Australia	-12.336, 130.937

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Table A.4 – Continued from previous page

Molecular code	Species	Host	Host code†	Marine water body or freshwater river (system)	Coordinates
CF73	<i>N. gen. n. sp. 1</i>	<i>Pristis clavata</i>	AU-36	Buffalo Creek, Timor Sea, Indian Ocean, Australia	-12.336, 130.938
CF75	<i>Acanthobothrium sp.</i>	<i>Gymnura altavela</i>	SE-275	Kafountine, Casamance, Atlantic Ocean, Senegal	12.928, -16.788
CF77	<i>Acanthobothrium sp.</i>	<i>Gymnura altavela</i>	SE-305	Joal, Atlantic Ocean, Senegal	14.175, -16.888
CF78	<i>Acanthobothrium sp.</i>	<i>Zanobatus schoenleinii</i>	SE-199	Atlantic Ocean, Senegal	14.678, -17.488
CF79	<i>Acanthobothrium sp.</i>	<i>Taeniura grabata</i>	SE-224	Mbour, Atlantic Ocean, Senegal	14.406, -17.005
CF80	<i>Acanthobothrium sp.</i>	<i>Dasyatis margaritella</i>	SE-234	Djifere, Atlantic Ocean, Senegal	13.931, -16.789
CF81	<i>N. gen. n. sp. 2</i>	<i>Pristis clavata</i>	AU-36	Buffalo Creek, Timor Sea, Indian Ocean, Australia	-12.336, 130.939
CF82	<i>N. gen. n. sp. 2</i>	<i>Pristis clavata</i>	AU-36	Buffalo Creek, Timor Sea, Indian Ocean, Australia	-12.336, 130.939
CF85	<i>A. cf. terezae</i>	<i>Potomotrygon cf. motoro</i>	PU-4	Boca Manu, Madre de Dios, Madre de Dios River, Peru	-12.267, -70.956
CF87	<i>A. rodmani</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.023
CF89	<i>A. romanowi</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.024
CF93	<i>A. oceanharvestae</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.024
CF96	<i>Potamotrygonocetus cf. fitzgeraldae</i>	<i>Potomotrygon castexi</i>	PU-23	Madre de Dios, Madre de Dios River, Peru	-12.283, -70.924

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Table A.4 – Continued from previous page

Molecular code	Species	Host	Host code†	Marine water body or freshwater river (system)	Coordinates
CF100	<i>A. margieae</i>	<i>Orectolobus japonicus</i>	TW-19	Magong, Penghu, Taiwan Strait, Pacific Ocean, Taiwan	23.564, 119.608
CF103	<i>Acanthobothrium sp.</i>	<i>Himantura undulata</i>	KA-43	Muara Pasir, East Kalimantan, Borneo, Makassar Strait, Pacific Ocean, Indonesia	-1.766, 116.425
CF105	<i>A. santarosaliense</i>	<i>Heterodontus mexicanus</i>	BJ-300	Santa Rosalia, Baja California Sur, Pacific Ocean, Mexico	27.331, -112.292
CF108	<i>A. cf. microcephalum</i>	<i>Myliobatis californica</i>	BJ-626	Bahia de Los Angeles, Baja California, Pacific Ocean, Mexico	28.986, -113.575
CF111	<i>A. popi</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.026
CF116	<i>A. romanowi</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.026
CF120	<i>A. popi</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.026
CF121	<i>A. zimmeri</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.026
CF122	<i>A. zimmeri</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.027
CF123	<i>A. romanowi</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.027
CF124	<i>A. rodmani</i>	<i>Himantura sp.</i>	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.027

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Table A.4 – Continued from previous page

Molecular code	Species	Host	Host code†	Marine water body or freshwater river (system)	Coordinates
CF126	<i>A. oceanharvestae</i>	<i>Himantura</i> sp.	NT-96	Northern Territory, east of Wessel Islands, Arafura Sea, Pacific Ocean, Australia	-11.296, 137.028
CF128	<i>A. margieae</i>	<i>Orectolobus japonicus</i>	TW-19	Magong, Penghu, Taiwan Strait, Pacific Ocean, Taiwan	23.564, 119.611
CF130	<i>Acanthobothrium</i> sp.	<i>Himantura</i> cf. <i>gerrardi</i>	KA-211	Sidu, West Kalimantan, Borneo, Java Sea, Pacific Ocean, Indonesia	-1.363, 110.111
CF134	<i>Acanthobothrium</i> sp.	<i>Himantura lobistoma</i>	BO-480	Mukah, Sarawak, Borneo, South China Sea, Pacific Ocean, Malaysia	2.898, 112.128
CF136	<i>A. etini</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.429
CF138	<i>A. masnihae</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.429
CF139	<i>A. saliki</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.429
CF140	<i>A. zainili</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.429
CF141	<i>A. etini</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.430
CF142	<i>A. masnihae</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.430
CF143	<i>A. masnihae</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.430

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Table A.4 – Continued from previous page

Molecular code	Species	Host	Host code [†]	Marine water body or freshwater river (system)	Coordinates
CF144	<i>A. etini</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.431
CF145	<i>A. saliki</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.431
CF146	<i>A. saliki</i>	<i>Himantura polylepis</i>	BO-108	Kampung Abai, Kinebatangan River, Sabah, Borneo, Kinabatangan River, Malaysia	5.686, 118.431
CF147	<i>Acanthobothrium sp.</i>	<i>Raja eglanteria</i>	TM-1	Cape Point, North Carolina, Western Atlantic Ocean, U.S.A.	35.250, -75.565
CF151	<i>A. ramiroi</i>	<i>Potamotrygon motoro</i>	VI-1	Colastiné River, Santa Fé, Argentina	-31.667, -60.815
CF152	<i>A. ramiroi</i>	<i>Potamotrygon motoro</i>	VI-2	Colastiné River, Santa Fé, Argentina	-31.667, -60.815
CF153	<i>Acanthobothrium sp.</i>	<i>Himantura cf. gerrardi</i>	KA-57	Kota Baru, South Kalimantan, Borneo, Makassar Strait, Pacific Ocean, Indonesia	-3.246, 116.266
CF154	<i>Acanthobothrium sp.</i>	<i>Himantura fai</i>	KA-436	Manggar, East Kalimantan, Borneo, Makassar Strait, Pacific Ocean, Indonesia	-1.215, 117.016

[†]For further information, please look for the host codes at the Global Cestode Database (<http://tapewormdb.uconn.edu>).

A.9 Strict consensus tree

Figure A.1: Complete strict consensus tree of the trees found for parameter set 2:1:1:1, including support and stability measures.

A.10 Cladogram with branch lengths representing the number of transformations

Figure A.2: Cladogram with branch lengths proportional to the number of transformations.

A.11 Descrição dos anexos eletrônicos

Junto a esta dissertação está anexo um CD-ROM com os seguintes arquivos:

1. Um arquivo contendo o [Resumo](#) da dissertação (formato RTF)
2. Um arquivo contendo o [Abstract](#) da dissertação (formato RTF)
3. Um arquivo contendo a [dissertação completa](#) (formato PDF);
4. Um arquivo contendo os dados das [sequências originais](#) deste trabalho (formato CSV);
5. Um arquivo contendo os dados das [sequências de Fyler \(2009\)](#) para espécimens marinhos de *Acanthobothrium* (formato CSV);
6. Um arquivo contendo os dados das [sequências do GenBank](#) (formato CSV);
7. Um arquivo contendo as [topologias](#) obtidas para o parâmetro 2:1:1:1 (formato TRE);