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PHYLOGENETIC RELATIONSHIP OF SPECIES IN THE GENUS *ASPIDOGASTER* (ASPIDOGASTRIDAE, ASPIDOGASTRINAE) IN CHINA AS INFERRED FROM ITS rDNA SEQUENCES

CHEN Ming-Xiu[1, 2], ZHANG Li-Qiang[2], WEN Chun-Gen[3], SUN Jun[4] and GAO Qian[2]

(1. *College of Fishery*, *Huazhong Agricultural University*, *Wuhan* 430070, *China*; 2. *State Key Laboratory of Freshwater Ecology and Biotechnology*, *Institute of Hydrobiology*, *Chinese Academy of Sciences*, *Wuhan* 430072, *China*; 3. *Department of Bioscience and Technology*, *Nanchang University*, *Nanchang* 330047, *China*; 4. *Institute of Hydrobiology*, *Jinan University*, *Guangzhou* 510632, *China*)

Abstract: Five species in the genus *Aspidogaster* have been reported in China, and four species, i.e. *A. conchicola* Bare, 1827, *A. limacoides* Diesing, 1834, *A. ijimai* Kawamura, 1913 and *A. chongqingensis* Wei, Huang et Dai, 2001, were obtained to examine their phylogenetic relationship by comparing ITS rDNA sequences. The length of the ITS-1 and ITS-2 sequences ranged from 728 to 877 bp and 518 to 645 bp, and the G + C content from 50.1 to 52.3% and 49.2 to 52.2%, respectively. Both maximum likelihood (ML) and neighbour-joining (NJ) phylogenetic trees revealed three well-supported main clades, which correspond respectively to *A. limacoides* and *A. chongqingensis*, *A. ijimai*, and *A. conchicola*, with *A. conchicola* located at the base of the phylogenetic tree. The group containing *A. chongqingensis* and *A. limacoides* formed the sister taxon to *A. ijimai* with 100% of bootstrap value. It was considered that *A. chongqingensis* and *A. limacoides* should be the closest, and they should be closer to *A. ijimai* than to *A. conchicola*. In addition, host specificity of *Aspidogaster* spp. in China was discussed.

Key words: ITS rDNA; *Aspidogaster*; Phylogenetic relationship; Host specificity
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The Aspidogastrea is an archaic and minor group among platyhelminths[1], with about 80 species reported throughout the world[2]. According to a recent systematic revision, the subclass Aspidogastrea comprises 4 families, of which the family Aspidogastridae is divided into three subfamilies whereas other three families only contain a single genus each[3]. Apart from *Cotylaspis sinenesis* Faust et Tang, 1936 in Cotylaspidinae and *Lophotaspis arientalis* Faust et Tang, 1936 in Aspidogastrinae, five of seven aspidogastrean species reported in China belong to the genus *Aspidogaster* Baer, 1827 (Aspidogastridae, Aspidogastrinae), including *A. conchicola* Bare, 1827, *A. limacoides* Diesing, 1834, *A. ijimai* Kawamura, 1913, *A. indica* Dayal, 1943 and *A. chongqingensis* Wei, Huang et Dai, 2001. However, up to now, nothing is known about the phylogenetic relationship among these species in the genus *Aspidogaster*.

The internal transcribed spacer of ribosomal DNA (ITS rDNA) has been proved to be a reliable marker for determining the phylogenetic relationship at and below species level for platyhelminth parasites [4, 5]. The present study thus aims to illustrate the phylogenetic relationship of the species of *Aspidogaster* collected in China by comparing their ITS rDNA sequences in order to clarify their identity.

1 Materials and methods

A total of 4 formerly described *Aspidogaster* species were collected from two lakes and two reservoirs and one river in China. Live specimens of *Aspidogaster* were isolated from the intestines of fish hosts, and identified according to the previous description[6, 7]. Specimens were washed in double-distilled water before being fixed in 90% ethanol. The parasite species and their host species were listed in Tab. 1, with the sample localities, the number of fish individuals, and infection levels also given.

The genomic DNA were extracted from pooled samples of 3-5 individual specimens and resuspended in 30 μL distilled water as described previously[8]. Universal eukaryotic primers, BD1 (5′ - GTC GTA ACA AGG TTT CCG TA - 3′) and BD2 (5′ - TAT GCT TAA (G/A) TT

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Brief introduction of the author: Chen Ming-Xiu (1978—), female; major in hydrobiology and parasitology
Corresponding author: Gao Qian, E-mail: gaoqian@ihb.ac.cn

CAG CGG GT - 3′) [9], were used to amplify the fragment spanning ITS-1, 5.8S and ITS-2. PCR amplification reaction was performed in 50 μL volume containing 2 μL of DNA template, 5 μL of 10 × PCR buffer (TaKaRa), 200 μM of each dNTP, 0.5 μM of each primer, 1.25 units of *Taq* polymerase (TaKaRa), under the following condition: 5 min at 94℃, 30 cycles of 30s at 94℃, 30s at 54℃ and 1 min at 72℃, followed by a final elongation of 10 min at 72℃ (PTC - 100™ Programmable Thermal Controller, MJ Research). Purified PCR products were cloned into pMD18-T vector (Omega) and sequenced in both directions.

Sequences were aligned automatically using Clustal X (1.8) with default gaps and weighing values[10]. Boundaries of coding and spacer regions were deduced by comparison with previously published ITS sequences of *Bothriocephalus acheilogathi* (Cestoda)[11] and *Haplorchis taichui* (Digenea)[12]. Base composition data were generated using MEGA version 3.4[13].

As the ITS-1 and ITS-2 represent separate regions in the rDNA sequence and may evolve independently at different rates, the sequence data were first analyzed separately to infer the relationship. Then, ITS-1, 5.8S and ITS-2 sequence data were combined to maximize the number of characters, and phylogenetic analyses were performed using two different phylogenetic methods, maximum likelihood (ML) and neighbour-joining (NJ) methods as implemented in PHYLIP [14], to verify if alternative topologies support each other. The ML tree was constructed using the program DNAML in PHYLIP, with the effect of default option in which all characters were equally weighed. A bootstrap was performed with 100 replicates to evaluate the significance of clades in the consensus tree using the program SEQBOOT of PHYLIP. A consensus tree was computed from the 100 inferred trees using CONSENSE. NJ tree was constructed by using NEIGHBOR in PHYLIP based on the distance matrices of sequence divergences calculated on a bootstrapped data set (100 replicates). The DNADIST of PHYLIP was applied to perform pairwise distances which were corrected by using the Kimura two-parameter model, and then a consensus tree was computed from the 100 inferred trees. In all cases, *Multicalyx elegans* (Olsson, 1869) (Aspidogastrea, Multicalycidae), from the gall bladder of elephant shark, *Callorhinchus millias* was used as the outgroup for rooting trees. ITS rDNA of only one individual of *M. elegans*, which was kindly given by Professor Klaus Rohde, University of New England, Australia, was amplified and sequenced.

2 Results

The ITS-1 and ITS-2 sequences of the 9 samples representing 4 formerly described *Aspidogaster* species (Tab. 1) and the outgroup *M. elegans* were determined, including 44 bp 18S 3′ end, 160 bp 5.8S and 44 bp 28S 5′ end. Nucleotide sequences obtained were deposited in the GenBank database. All accession numbers, excluding DQ345325 of *M. elegans*, were listed in Tab. 1.

Overall, the length of the ITS-1 and ITS-2 sequences ranged from 728 bp to 877 bp and 518 bp to 645 bp, and the G + C content from 50.1% to 52.3 % and 49.2% to 52.2 %, respectively. Little variation was detected in the base composition of each of the two regions within species. The complete alignment consisted of 1,743 characters, excluding respective 44 bp fragment of 18S and 28S rDNA, of which 407 were variable and 397 were potentially phylogenetically informative within the ingroup. 0-26.9% variations in the level of sequence divergence were observed. However, the 5.8S rDNA sequences were almost identical within the *Aspidogaster* specimens examined in the present study. In addition, a high level of similarity (99.8 %) was observed between ITS rDNA sequences of *A. chongqingensis* and *A. limacoides* (Tab. 2).

Phylogenetic analyses of the entire ITS region with *M. elegans* as the outgroup by ML and NJ methods produced two same topological trees (Fig. 1). Both ML and

Tab. 1 Host and sample site of *Aspidogaster* spp. from China for determining ITS rDNA sequences, with the number of fishes sampled and examined (N), prevalence (P), mean intensity (MI), and the GenBank accession numbers (AN)

Parasite	Sample site	Host	N	P (%)	MI (mean ± S. D.)	AN
A. conchicola Baer, 1827	Danjiangkou Reservoir, Danjiangkou, Hubei	*Mylopharyagodon pieus*	7	85.7	113.5 ± 87.8	DQ345317
	Liangzi Lake, E'zhou, Hubei	*M. pieus*	5	100	26.6 ± 9.5	DQ345318
A. limacoides Diesing, 1834	Jialing River, Beibei, Chongqing	*Coreius guickenoti*	3	33.3	4.0 ± 0.0	DQ345319
A. ijimai Kawamura, 1913	Danjiangkou Reservoir, Danjiangkou, Hubei	*Cyprinus carpio*	20	55	4.1 ± 3.4	DQ345320
	Jiangkou Reservoir, Xinyu, Jiangxi	*C. carpio*	21	23.8	20.7 ± 18.0	DQ345321
	Niushan Lake, Wuhan, Hubei	*C. carpio*	24	20.8	3.0 ± 2.6	DQ345322
	Jialing River, Beibei, Chongqing	*C. carpio*	7	42.9	3.3 ± 2.1	DQ345323
A. chongqingensis Wei, 2001	Jialing River, Beibei, Chongqing	*Spinibarbus sinensis*	11	36.4	1.0 ± 0.0	DQ345324

Tab. 2 Kimura 2-parameter distance matrix of the complete ITS region rDNA sequences across all samples of *Aspidogster* spp.

	A. chongqingensis DQ345324	*A. limacoides* DQ345319	*A. ijimai* DQ345323	*A. ijimai* DQ345322	*A. ijimai* DQ345321	*A. ijimai* DQ345320	*A. conchicola* DQ345317	*A. conchicola* DQ345318
DQ345324	—							
DQ345319	0.2	—						
DQ345323	9.3	9.4	—					
DQ345322	9.3	9.4	0.0	—				
DQ345321	9.4	9.5	0.1	0.1	—			
DQ345320	9.3	9.4	0.1	0.1	0.1	—		
DQ345317	26.6	26.8	23.4	23.4	23.6	23.4	—	
DQ345318	26.8	26.9	23.4	23.4	23.6	23.4	0.2	—

Note: Numbers represent percentage in nucleotide difference

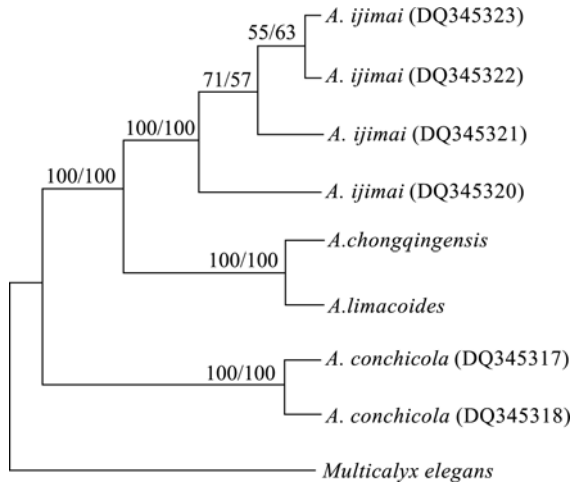

Fig. 1 Phylogenetic tree inferred from ITS rDNA sequences of *Aspidogaster* spp. collected in China with *Multicalyx elegans* as outgroup using Maximum Likelihood method. Bootstrap values are shown on the tree branch, and values obtained using Neighbor-Joining method on the right of the slash

NJ trees strongly supported three main clades, the first comprising *A. ijimai* parasitic on the common carp from Danjiangkou Reservoir, Jiangkou Reservoir, Niushan Lake and Jialing River, the second *A. limacoides* and *A. chongqingensis* respectively parasitic on *Coreius guichenoti* and *Spinibarbus sinensis* from Jialing River, and the third *A. conchicola* on the black carp from Danjiangkou Reservoir and Liangzi Lake. All bootstrap values for the three clades were 100%. Moreover, the first and the second clades formed a large, well-supported clade (100% in both ML and NJ trees) as a sister group of the third clade at the base of phylogenetic trees (Fig. 1). Phylogenetic trees based on either ITS-1 or ITS-2 (data not shown) were similar in overall topology to those based on the whole ITS rDNA sequence, but the latter produced higher resolution.

3 Discussion

The present study represented the first by using ITS rDNA sequences to reveal phylogenetic relationship of aspidogastreans. According to the tree topology, *A. chongqingensis* and *A. limacoides* appeared as the sister taxa with 100% of bootstrap value, and the group containing the above two species formed the sister taxon to *A. ijimai* with 100% of bootstrap value. Moreover, *A. conchicola* was located at the base of the phylogenetic tree and formed the sister taxon to all others. Thus, it was suggested that *A. chongqingensis* and *A. limacoides* should be the closest, and that they should be closer to *A. ijimai* than to *A. conchicola*.

Species description and identification of aspidogastreans were in general based on morphological characters, and in several instances on very small morphological differences, thus leading to some disputes over the identity of several species. For instance, Timofeeva[15] considered that *A. armurensis* Achmerov, 1956 should be the synonym of *A. conchicola*. In respect of platyhelminthes, the internal transcribed spacers of ribosomal DNA (ITS1 and ITS2) have been shown to be the reliable markers for species determination in digeneans, monogeans, and cestodes[11, 16, 17]. The divergences of ITS sequences among the three species, i.e. *A. ijimai*, *A. limacoides*, and *A. conchicola* varied between 9.3 and 26.9 % confirming that they are separate species. However, the lower divergence of ITS rDNA sequences between *A. chongqingensis* and *A. limacoides* (0.2%) might suggest a need for morphological re-examination of the newly described species, *A. chongqingensis*, which was found in a cyprinid fish *Spinibarbus sinensis* from Jialing River in upper reaches of Yangtze River in China[7].

In the present study, molecular variations of individuals for *A. conchicola* and *A. ijimai* from different geographical area have been presented primarily, and it is of significance to further study the population variations of the parasites, and to examine more species in the genus *Aspidogaster* and other aspidogastreans as to clarify the species composition and the phylogenetic relationship.

Most aspidogastrean parasites have been regarded as having a low degree of host specificity in terms of definitive mollusk and vertebrate hosts[2]. However, within about 50 fish species investigated in the Dangjiangkou Reservoir (data not shown), only common carp and black carp were found to harbour *A. ijimai* and *A. conchicola*, respectively. In Jialing River, 12 fish species were examined (data not shown), and three *Aspidogaster* species, i.e. *A. ijimai*, *A. limacoides* and *A. chongqingensis* were found respectively in common carp, *Coreius guickenoti*, and *Spinibarbus sinensis*. It is thus shown, at least in this particular case, that one fish host species only harbours a single species of *Aspidogaster*. The result of the present study strongly supports the hypothesis by Gao, *et al*.[18] that *A. ijimai* may be a specialist parasite for common carp, and *A. conchicola* a specialist parasite for black carp at least in the flood-plain lakes of the Yangtze River, nevertheless the host specificity should then be interpreted cautiously in different species of aspidogastreans.

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依据 ITS rDNA 序列探讨我国盾腹虫属种类的系统发育关系

陈明秀[1,2] 张立强[2] 文春根[3] 孙 军[4] 高 谦[2]

(1. 华中农业大学水产学院, 武汉 430070; 2. 中国科学院水生生物研究所, 淡水生态与生物技术国家重点实验室, 武汉 430072; 3. 南昌大学生命科学学院, 南昌 330047; 4. 暨南大学水生生物研究所, 广州 510632)

摘要: 盾腹吸虫为寄生扁形动物中一小的类群。我国已报道 7 种盾腹吸虫, 其中 5 种隶属于盾腹虫属(Aspidogastridae, Aspidogastrinae)。研究测定了在我国采集到的 4 种盾腹虫属吸虫的核糖体 DNA 转录内间隔区(ITS rDNA)序列, 并分别采用邻接法和最大似然法构建分子系统发育树。结果显示, 这 4 种盾腹吸虫的 ITS-1 和 ITS-2 序列的长度分别在 728—877 bp 和 518—645 bp 之间, 其 G+C 含量分别在 50.1%—52.3 %和 49.2%—52.2%范围内。4 种盾腹吸虫的种间遗传距离在 0.2%—26.9%之间, 其中重庆盾腹吸虫(*Aspidogaster chongqingensis*)和似螺盾腹吸虫(*A. limacoides*)间仅为 0.2%。所构建的最大似然树和邻接树具有相同的拓扑结构, 均支持重庆盾腹吸虫和似螺盾腹吸虫亲缘关系最近, 它们与饭岛盾腹吸虫(*A. ijimai*)亲缘关系较近, 而与位于系统树基部的贝居盾腹吸虫(*A. conchicola*)关系较远。此外, 对我国盾腹属种类的宿主特异性进行了讨论。

关键词: ITS rDNA; 盾腹属; 系统发育关系; 宿主特异性