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Molecular evidence for the synonymy of two species of *Apatemon* Szidat, 1928, *A. gracilis* (Rudolphi, 1819) and *A. annuligerum* (von Nordmann, 1832) (Digenea: Strigeidae) parasitic as metacercariae in British fishes

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Abstract

The current study examined rDNA (internal transcribed spacer regions, ITS1 and ITS2) and cytochrome c oxidase subunit 1 (CO1) sequence data of *Apatemon annuligerum* (originating from two geographical locations) and *A. gracilis* metacercariae (originating from four natural piscine hosts) to determine the systematic status of these two strigeid digeneans. With the exception of short repeat motifs, the ITS1 regions sequenced demonstrated no intra- or inter-specific sequence variation. ITS2 sequences were 292 bp and CO1 sequences 366 bp in length and identical for both nominal *Apatemon* species. These sequence data provide strong evidence that the two species are con-specific and that *A. annuligerum* should be regarded as a junior synonym of *A. gracilis*.

Introduction

Only two species of the strigeid subgenus *Apatemon* (*Apatemon*) sensu Sudarikov, 1959, *Apatemon gracilis* (Rudolphi, 1819) and *A. annuligerum* (von Nordmann, 1832), are known to infect British fishes (Bell, 1996). *Apatemon annuligerum* is poorly characterized, with few life-cycle data available since only the adult and metacercarial stages have been described. Both of these life-stages demonstrate only minor morphological differences from those of *A. gracilis*. Consequently, *A. gracilis* and *A. annuligerum* are discriminated solely on the basis of their piscine (i.e. metacercarial) and avian (i.e. adult) host specificities. Many early records of *A. gracilis* were made under the synonyms *A. cobitidis* (Von Linstow, 1890) Vojtek, 1964, *A. pellucidus* (Yamaguti, 1933) and *A. gracilis pellucidus* (Yamaguti, 1933) Dubois, 1953. According to Blair (1976) the fish hosts of *A. gracilis* include members of six families: the Salmonidae, Eleotridae, Cobitidae, Cottidae, Gasterosteidae and Gobiidae, with their scattered records suggesting at least a Holarctic circumpolar distribution. The location of cysts within the fish host appears to vary according to the host family; the Salmonidae harbour metacercariae predominantly within the pericardial cavity, the Cottidae within the body cavity and the Gasterosteidae within the eyes. Adults develop within fish-eating ducks, primarily those belonging to the genus *Mergus* (see Yamaguti, 1933; Dubois, 1968; Odening, 1978; McDonald, 1981; Shigin, 1983; Sudarikov, 1984; Watson & Pike, 1993). Von Nordmann (1832) described an encysted larva, which he called *Distomum annuligerum*, from the eyes of perch *Perca fluviatilis* in Germany. These larvae were later identified by Kozicka (1961) as being of the tetracotyle type and renamed *Tetracotyle annuligerum*. In a series of experimental infections, Odening (1970) discovered that the metacercariae were those of a strigeid which he called *Apatemon* (*Apatemon*) *annuligerum*. *Apatemon annuligerum* is thought to be specific to both its piscine (the eyes of perch) and avian (the buzzard *Buteo buteo*; see Odening, 1970) hosts. Attempts at experimental infections with metacercariae recovered from the eyes of perch, and considered to be *A. annuligerum*, have typically failed in various experimental hosts in which *A. gracilis* adults have been raised (Odening, 1970; Blair, 1974; Bell, 1996). Nevertheless, Luk'iantseva (1976) was able to raise gravid *A. annuligerum* adults in ducklings; the parasite identity being based upon metacercarial host. There is also some

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evidence that *A. gracilis* metacercariae may display definitive host specificities relative to their piscine hosts. Bell (1996) found that metacercariae originating from stoneloach *Barbatula barbatulus* (L.) successfully established in domestic ducklings and yielded collectable quantities of viable eggs. Similarly, most other records of successful experimental *A. gracilis* infections in domestic duckings or mallards have utilized metacercariae originating from the Cobitidae or Cottidae (see Crocombe, 1959; Vojtek, 1964). Whereas both Watson & Pike (1993) and Bell (1996) noted that metacercariae from salmonids failed to establish in domestic ducklings, mature, gravid *A. gracilis* adults were obtained from eider ducklings fed with metacercariae from rainbow trout (Watson & Pike, 1993).

Bell *et al.* (2002) utilized the multidimensional approach of principal components analysis (PCA) to determine the intra- and inter-specific morphological variation that exists for metacercariae of these species. Analyses revealed that metacercarial morphometrics cannot confidently discriminate between *A. gracilis* and *A. annuligerum* specimens. Indeed, levels of intra-specific variation present between *A. gracilis* metacercariae originating from different piscine hosts were as marked for certain populations as those between the two species. These findings and the lack of strong life-cycle data for *A. annuligerum* place some doubt on its validity as a species discrete from *A. gracilis*.

Molecular investigations, dependent upon the gene/gene-region analysed, have proved to be invaluable in establishing systematic relationships at all taxonomic levels (Hillis & Moritz, 1990). The current study examined rDNA (internal transcribed spacer regions, ITS1 and ITS2) and cytochrome c oxidase subunit 1 (CO1) sequence data of *A. annuligerum* (from two geographical locations) and *A. gracilis* (from four natural piscine host species) metacercariae to determine the systematic status of these two strigeid digeneans. Sequence data of the rRNA gene, in particular the two highly variable internal transcribed spacer regions, are of considerable utility for determining phylogenetic affinities between closely related digenean species (Luton *et al.*, 1992; Adlard *et al.*, 1993; Bowles *et al.*, 1995; Morgan & Blair, 1995; Blair *et al.*, 1997a,b; Van Herwerden *et al.*, 1998). Indeed, such data have been used previously to demonstrate the validity of disputed species (Després *et al.*, 1995; Bell *et al.*, 2001), or as evidence for the synonymy of species (Blair *et al.*, 1997a). The mitochondrial protein-coding gene, cytochrome c oxidase subunit 1 has similarly been shown to be expedient for the discrimination of closely related digenean species (Bowles *et al.*, 1995; Blair *et al.*, 1997b). Congruent phylogenies derived from both nuclear and mitochondrial genomes, which exhibit different patterns of inheritance and consequently evolution, provide for particularly robust analyses.

As well as establishing the systematic placement of *Apatemon gracilis* and *A. annuligerum*, the sequence data obtained in the current study will enable the future confident identification of all life-cycle stages of these parasites. Additionally, these data may be used for subsequent studies on the two *Apatemon* Szidat, 1928 subgenera, *Apatemon* (*Apatemon*) and *Apatemon* (*Australapatemon*) sensu Sudarikov, 1959, the status of which remain uncertain (see Bell, 1996).

Materials and methods

Collection of metacercariae

Apatemon gracilis and *A. annuligerum* metacercariae were obtained from the sources listed in table 1. Wild fish were collected with rod and line, fyke nets, gill nets or using electro-fishing techniques; those obtained from fish farms were hand-netted out of the ponds and cages. Live fish were transported back to the laboratory in aerated local water. All fish were examined immediately after killing. The body surface, body cavity and all organs contained therein, the heart and pericardium, eyes, brain and cranial cavities were examined for the presence of metacercarial cysts. Excised tissues were dissected in 0.85% physiological saline and metacercarial cysts removed using a fine pipette.

Artificial digestion of cysts

Newly excised, encysted, metacercariae were freed by subjecting them to pepsin and trypsin-tauroglycholate solutions, as described by Blair (1974, 1976). Cysts were placed in a 0.8% solution of pepsin in Hanks' saline (buffered at pH 1.7–2.0) for 10 min at 40°C. They were then washed in several changes of saline and incubated at 40°C in a solution of 0.5% trypsin and 0.3% sodium tauroglycholate adjusted to pH 7.8.

DNA isolation, PCR amplification and sequencing

Live, identified, metacercariae were washed in warm physiological saline and placed into microcentrifuge tubes containing 0.5 ml of high concentration urea buffer (TNES-urea: 10 mM Tris-HCL, pH 7.5; 125 mM NaCl;

Table 1. Sources of *Apatemon gracilis* and *A. annuligerum* metacercariae and the location of cysts within the fish host.

Metacercarial identity	Host species	Source*	Cyst location
A. gracilis	Rainbow trout *Oncorhynchus mykiss*	River Almond fish farm	Pericardial cavity
A. gracilis	Salmon parr *Salmo salar*	River Almond fishery	Body cavity/pericardial cavity
A. gracilis	Stoneloach *Barbatula barbatulus*	Loch Carron beck	Body cavity
A. gracilis	Brown trout *Salmo truttae*	River Almond	Body cavity
A. annuligerum	Perch *Perca fluviatilis*	Loch Lomond	Humour of eye
A. annuligerum	Perch *Perca fluviatilis*	Loch Leven	Humour of eye

* All sources within Scotland.

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10 mM EDTA; 0.5% SDS; 4 M urea). In the case of *A. gracilis*, four excysted metacercariae from a single infected fish were pooled for DNA extraction. This was repeated for four individuals of each fish species, yielding four samples per species. In the case of *A. annuligerum*, samples consisted of six pooled metacercariae and this was done for at least three individual fish from each sample site. The pooled metacercariae were homogenized using a sterile plastic rod, proteinase K added to a concentration of 100 $\mu g\,ml^{-1}$ and digestion allowed to occur overnight in a 37°C waterbath. DNA was subsequently extracted with phenol:chloroform:isoamyl-alcohol (25:24:1) and diethyl ether, then precipitated at −80°C with 2.5 × volume of 95% cold ethanol and 0.1 × volume of 3 M sodium acetate, and finally re-suspended in sterile water.

Targeted DNA was amplified using PCR primers to conserved bordering regions described previously for other digenean species: ITS1, BD1 (5′ GTCGTAACAAG-GTTTCCGTA 3′) and 4S (5′ TCTAGATGCGTTCGAAGT-GTCGATG 3′) (Bowles & McManus, 1993); ITS2, 3S (5′ GGTACCGGTGGATCACTCGGCTCGTG 3′) and BD2 (5′ ATCTAGACCGGACTAGGCTGTG 3′) (Bowles *et al.*, 1995); CO1, JB3 (5′ TTTTTTGGGCATCCTGAGGTTTAT 3′) and JB4.5 (5′ TAAAGAAAGAACATAATGAAAATG 3′) (Bowles *et al.*, 1995). Each 25 μl PCR reaction contained ~10 ng of genomic DNA, 25 pmol of each primer and utilized Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech UK Limited) which comprise ~1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and stabilizers including BSA. With the exception of annealing temperatures, reaction conditions employed were the same regardless of primer sets used. After an initial denaturation at 95°C for 5 min, samples were subjected to 30 cycles of amplification (denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s [ITS1 and ITS2] or at 50°C for 30 s [CO1], and extension at 72°C for 1 min), followed by a 10 min terminal extension at 72°C. All amplifications were performed on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The PCR product obtained was visualized in an ethidium bromide-stained 1% agarose gel, the DNA band excised and purified using the Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, California, USA).

All sequences were determined directly from the PCR products. Cycle sequencing reactions using the BigDye Terminator™ Sequencing Kit (Perkin-Elmer Corporation) and incorporating the same primers as those used in the initial PCRs were performed according to the manufacturer's instructions. Sequencing products were run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corporation). Sense and anti-sense strands were sequenced for all targeted products (amplified from each DNA sample), performed for each gene region and each species sample. Putative 5′ and 3′ ends of the gene regions sequenced were identified by comparison with published data for other digenean groups (ITS1 and ITS2, Luton *et al.*, 1992; CO1, Bowles *et al.*, 1995). CLUSTAL W (Thompson *et al.*, 1994) was used for initial sequence alignments with default settings for gap and weighting values. No subsequent editing was necessary as alignments proved unambiguous. Sequences determined in the current study were assigned the accession numbers: *Apatemon gracilis* (ex rainbow trout; ITS1; AJ301888), *Apatemon gracilis* (ex stoneloach; ITS1; AJ314759), *Apatemon gracilis* (ex salmon parr; ITS1; AJ314760), *Apatemon annuligerum* (ITS1; AJ314761), *Apatemon gracilis* (ITS2; AJ301893), *Apatemon annuligerum* (ITS2, AJ314762), *Apatemon gracilis* (CO1, AJ301894), *Apatemon annuligerum* (CO1, AJ314763).

Results

All targeted DNA were successfully amplified and sequenced, with the exception of ITS1 and ITS2 rDNA regions of *A. gracilis* obtained from brown trout. Twelve sequences (four samples from each of three piscine host species) were obtained for both the ITS1 and ITS2 rDNA regions of *A. gracilis*, as well as 13 sequences for the CO1 region (four samples from three piscine host species and one sample from one host species). Six sequences (three samples from two sample sites) were obtained for each of the ITS1, ITS2 and CO1 regions of *A. annuligerum*.

Internal transcribed spacer 2 (ITS2)

ITS2 sequences were 292 base pairs (bp) in length and identical for both *A. gracilis* (all samples regardless of piscine host) and *A. annuligerum* (from both sample sites).

Internal transcribed spacer 1 (ITS1)

Following PCR amplification, a number of different molecular weight products were visualized in ethidium bromide-stained agarose gels for all species samples. The amplicons ranged from less than 500 bp to almost 2000 bp in length. Only the most abundant, ubiquitous, product of *c.* 700 bp was isolated and subsequently sequenced for all samples.

The ITS1 sequences were 688 bp (*Apatemon gracilis* ex salmon parr and *A. annuligerum*), 694 bp (*A. gracilis* ex stoneloach) and 699 bp (*A. gracilis* ex rainbow trout) in length. The four samples of *A. gracilis* obtained from stoneloach each possessed three adjacent repeat motifs (TCGGCT) near the 5′ terminus, whereas all other species' samples exhibited only two such repeats at this position (fig. 1). Similarly, all samples of *A. gracilis* obtained from rainbow trout demonstrated a tandem repeat of the sequence (ATACCTCGACC), whereas no other sample exhibited such a repeat (fig. 1). Apart from differences in the numbers of short repeat motifs no intra- or inter-specific sequence variation was observed.

Cytochrome c oxidase subunit 1 (CO1)

CO1 sequences were 366 bp in length and were found to be identical for all species' samples.

Discussion

Apatemon gracilis and *A. annuligerum* were found to demonstrate no inter-specific variation in their ITS1, ITS2 or CO1 sequences. These sequence data provide strong evidence that the two species are con-specific and that

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Apatemon gracilis (RT)                                          CAAGTCCCTA TCTGAAACTG
Apatemon gracilis (SL)                                          .......... ..........
Apatemon annuligerum (AA) & Apatemon gracilis (SP)              .......... ..........
Ichthyocotylurus erraticus (IE)                                 .......... ..........


RT          ATCGAGTTCG GCTTCGGCT- -----GAGTT CGGAATTGGC GCGTTGGATC AGCAATTGCG
SL          .......... .........T CGGCT..... .......... .......... ..........
AA & SP     .......... .........- -----..... .......... .......... ..........
IE          ....G.CA.A .T.......G TGTTC..A.. AT--.....T .....A.GCA G.......A.


RT          TTGGTTCAGT GTGTCAAGGA ATTGACGGAA GGACTCCTT- ---GTGGGCC CGCGTATTAC
SL          .......... .......... .......... .........- ---....... ..........
AA & SP     .......... .......... .......... .........- ---....... ..........
IE          ..T..CT..T ..A....... .......... ..GT...C.T GAA.G.TT.. T...G..GG.


RT          AT---TGCTT ATATATGAAT GGA------- --ATTGACGG ATGAGTCT-- ----------
SL          ..---..... .......... ...------- --........ ........-- ----------
AA & SP     ..---..... .......... ...------- --........ ........-- ----------
IE          ..ATG..CA. .......C.. .TATATATAT AT...T.TTT ..G...A.AT ATATATATGA


RT          ---------- -----TCTGC ---------- -------TGA TGCAGGAACT CGCAAAACCT
Sl          ---------- -----..... ---------- -------... .......... ..........
AA & SP     ---------- -----..... ---------- -------... .......... ..........
IE          GTGTATATAT ACGGA.G....AGCTATTTGA ACGGAAT... C.G.T...T. .T.T.CTTG.


RT          CGACCAT--- -----ACCTC GACCATACCT GGGGACAGTA GAGTAACATC TGCTG-ACCT
SL          .......--- -----....- ---------- .......... .......... .....-....
AA & SP     .......--- -----....- ---------- .......... .......... .....-....
IE          A..GTT.TCG CAAAA....T .A........ .A..T....G ..TAT..T.. .....C.T..
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Fig. 1. Alignment of internal transcribed spacer region 1 (ITSI) sequences (first 320 bases). Nucleotides indentical to the top line are indicated by '.' and alignment gaps by '-'. Bold regions represent short repeat sequences. Abbreviations in parentheses correspond to fish hosts or parasite identities: RT, rainbow trout; SL, stoneloach; AA, *Apatemon annuligerum*; SP, salmon parr; IE, *Ichthyocotylurus erraticus*.

A. annuligerum should be regarded as a junior synonym of *A. gracilis*.

Identical ITS2 sequences have been observed previously for different nominal digenean species. Such examples include three members of the *Schistosoma haematobium* group (see Després *et al.*, 1992), three *Echinostoma* spp. (see Morgan & Blair, 1995) and several members of the *Paragonimus ohirai* group (see Blair *et al.*, 1997a). However, in the two former cases the nominal species have been shown to be capable of producing viable hybrids, whereas Blair *et al.* (1997a) considered the identity among the *P. ohirai* group to be indicative of the potential for gene exchange. The three members of the genus *Echinostoma* that showed no intra-specific ITS2 variation also exhibited identity in their ITS1 sequences. Morgan & Blair (1995) suggested that these sequence data strongly supported the synonymy of *Echinostoma liei* and *Echinostoma* sp. II with *E. caproni*.

Of the two ITS regions, ITS1 has been found to display greater sequence variation among digeneans and is considered a more appropriate tool for systematics of closely related or sibling species (Luton *et al.*, 1992; Kane & Rollinson, 1994; Bell *et al.*, 2001). Repeat motifs embedded within the ITS1 region have been widely observed for the digenean species sequenced thus far (inter alia Luton *et al.*, 1992; Kane & Rollinson, 1994; Jousson *et al.*, 1998; van Herwerden *et al.*, 1998, 1999). Intra-specific and intra-individual variation has been recorded in the number of these repeats, as have sequence differences between repeats. Indeed, van Herwerden *et al.* (1999) identified sufficient intra-individual sequence variation, even in the region 3′ of the repeats, to confound phylogenetic studies within the *Paragonimus westermani* complex. No long (*c.* 100 bp) repeat motifs were observed within the single, ubiquitous, most abundant ITS1 product sequenced for the *Apatemon* samples in the current study. The absence of such repeats was also observed by Bell *et al.* (2001) in their phylogenetic examination of the strigeid genus *Ichthyocotylurus* Odening, 1969. However, intra-specific/intra-individual variation was observed in the number of short repeats present near the 5′ end of this region in both *Apatemon* (current study) and *Ichthyocotylurus* species (Bell *et al.*, 2001). This variation was limited to an additional (third) 6 bp repeat present in *A. gracilis* specimens from stoneloach and an 11 bp repeat (absent from all other samples) in *A. gracilis* specimens from rainbow trout. No variation was present between any of the samples in the region 3′ to these repeats and *A. gracilis* sequences from salmon parr samples were identical in all respects to those of *A. annuligerum*.

To the authors' knowledge there are no reports in the literature of identical CO1 sequences obtained from putatively distinct digenean species. Blair *et al.* (1997b)

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stated that the clonal, non-recombinant nature of mitochondrial genomes creates the expectation that their sequences will differ within and among populations. Indeed, these authors observed that geographically distant isolates of several *Schistosoma* spp. exhibited intra-specific variation in their CO1 sequences. Further, Blair *et al.* (1997a) recorded a maximum of two nucleotide differences (no amino acid changes) in CO1 sequences of three nominal species within the *Paragonimus ohirai* complex and concluded that these species were synonymous. The absence of any such variation between the two *Apatemon* species of the current study, even in point mutations that would still retain amino acid conservation, strongly suggest their synonymy.

It appears that perch represent another piscine host among the long list that already exists for *Apatemon gracilis* and that the rare host records in the buzzard that have been attributed to *A. annuligerum* were in fact *A. gracilis*. The minor morphological variation described for such specimens compared to *A. gracilis* adults recovered from the more widely recorded piscivorous duck hosts may in fact represent host-induced variation. It is notable that Olteanu *et al.* (1968) recovered a single *Apatemon* adult from within the gut of a buzzard from the Danube delta. These authors considered the worm to be morphologically identical with *A. gracilis* species described after Skryabin (1959). The identity of this worm was subsequently doubted by Dubois (1980), but the initial identity is likely to have been correct.

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