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Evidence for the co-existence of separate strains or species of *Ligula* in Lough Neagh, Northern Ireland

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Abstract

Ligula (Cestoda: Pseudophyllidea) infections in gudgeon (*Gobio gobio*) and roach (*Rutilus rutilus*) differ markedly in the pathology that is observed in the host, particularly with respect to a tissue response and the extent of inhibition of gonadal development. The entire internal transcribed spacer (ITS) region (ITS-1, 5.8S and ITS-2) and the large subunit domains D1–D3 were sequenced and compared in parasites from these fish from Lough Neagh, Northern Ireland, together with a single specimen from minnow (*Phoxinus phoxinus*) from Wales. Sufficient differences were observed between parasites from *R. rutilus* and *G. gobio* to support the suggestion that they may represent different strains/species. In contrast, *Ligula* from *P. phoxinus* closely resembled those from *R. rutilus*. *Ligula* infections in *G. gobio* were recorded prior to the introduction of *R. rutilus*. The co-existence of separate strains or species of *Ligula* in Lough Neagh probably resulted from the introduction of *R. rutilus* to these waters, correlated with an increase in the number of great crested grebes (*Podiceps cristatus*).

Introduction

Members of the genus *Ligula* are pseudophyllidean cestodes with a three-host life cycle. In terms of parasite longevity and host involvement, the dominant phase of the life cycle is the plerocercoid, which inhabits the body cavity of fish. Infected fish are eaten by fish-eating birds and adult worms develop in the bird intestine. Eggs are passed in bird faeces and develop in water. Eventually, a free-swimming coracidium is released, which must be eaten by a suitable first intermediate host, a copepod. Here, the first parasitic stage of the life cycle, the procercoid, develops in the haemocoel. Fish are the second intermediate hosts, and they become infected by ingesting parasitized copepods.

In the United Kingdom, members of the Cyprinidae are the usual fish hosts, and all publications on the genus from the UK have referred to the species of parasite involved as *Ligula intestinalis*. However, in the absence of detailed taxonomic studies, it is not known whether this assumption is correct.

Arme (1997) discussed *Ligula* infections of two cyprinid fish, roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*). He drew attention to certain marked differences in host pathology associated with infection. In roach, infection is invariably associated with a pronounced host tissue response in the body cavity and a disruption of the pituitary-gonadal axis. This latter results in inhibition of gonadal development and hence an inability of parasitized fish to reproduce. In contrast, no host tissue

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response is elicited in infected gudgeon. The limited observations that exist on gonadal development in parasitized gudgeon indicate that gonadal maturation proceeds much further than in infected roach, although it is not known whether mature gametes are produced.

These, and other features of infection discussed by Arme (1997), led him and others (see Discussion) to suggest that the species or strains of *Ligula* infecting roach and gudgeon may differ. The terms species and strain are here used loosely – see Thompson & Lymbery (1988) and Thompson *et al.* (1995) for discussion. Here we examine the question of status using a molecular approach, and conclude with comments on the possible ecological significance of these findings.

Materials and methods

Collection of specimens

Plerocercoids of *Ligula* were collected from *R. rutilus* and *G. gobio* from Lough Neagh, Northern Ireland, and preserved in 95% ethanol for genetic analysis. In addition, a single specimen of *Ligula* was collected from *Phoxinus phoxinus* in Aberystwyth, Wales, for comparison with those from *R. rutilus* and *G. gobio*.

DNA isolation, PCR amplification and sequencing

Three plerocercoids of *Ligula* from different host individuals were sequenced for each of the host species, *R. rutilus* and *G. gobio*, and one plerocercoid was sequenced from *P. phoxinus*. Ethanol in the tissue samples was replaced with 1 M Tris-EDTA (pH 8) buffer via repeated washings and genomic DNA was extracted using a Qiagen® DNeasy™ tissue kit following manufacturer-recommended protocols. Polymerase chain reaction (PCR) amplifications were performed as described by Olson *et al.* (2001). Two regions of the rDNA array were amplified separately: one encompassing the entire internal transcribed spacer (ITS) region (ITS-1, 5.8S and ITS-2 genes), and another encompassing the large subunit (LSU) domains 1–3. The ITS region was amplified using primers ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) and sequenced with the PCR primers and internal primers DIO2 (5′ CCTATGGCCACGTCTGGCCGAGGG 3′) and DIO3 (5′ AGTTGGCTGCACTCTTCATC 3′). The LSU region was amplified using primers LSU5 and 1200R and sequenced with the PCR primers and internal primers 300F and ECD2 (see Littlewood *et al.* (2000), for primer definitions). PCR amplicons were purified using Qiagen® Qiaquick™ columns, cycle-sequenced directly from both strands using ABI BigDye™ chemistry, alcohol-precipitated, and run on an ABI Prism 377™ automated sequencer.

Sequences were assembled using Sequencher™ ver. 3.1.1 (GeneCodes Corp.) and aligned manually using MacClade 4.0 (Maddison & Maddison, 2000). Gene regions were delineated with reference to annotated, published ribosomal sequences of *Echinococcus granulosus* (AF132701), *Peltidocotyle rugosa* (AJ238841) and *Crepidobothrium* sp. (AJ238838). Novel sequences of the LSU rDNA and ITS rDNA regions, reported below, are available in the GenBank™ under the accession numbers AF382090–96 and AF385760–69, respectively.

Results

Table 1 shows a summary of the differences observed among the sequences. No differences were detected among sequences from plerocercoids collected from the same host species, with the possible exception of multiple copies of the ITS-2 gene within individuals, as inferred from multiple peaks in some sequencing reactions; the dominant signal in this region, however, was identical among the samples collected from the same host species. The 5.8S gene (106 bps) was invariant among all sequences, and the D1–D3 region (1282 bps) of its functional counterpart LSU gene showed a single transition differentiating plerocercoids from *G. gobio* with those from both *P. phoxinus* and *R. rutilus*. The ITS-1 gene (574 bps) showed two transitions, a transversion and an insertion/deletion (indel) also differentiated the plerocercoids according to host species as above. The most variable of the genes sequenced, the ITS-2, was consistent with the results above showing four transitions differentiating plerocercoids of *G. gobio* from the other host species. Two gapped regions within the gene, however, showed indels unique to the sequences from each of the three hosts.

Discussion

There are several species recognized within the genus *Ligula* (Dubinina, 1980), the plerocercoids of which infect a variety of fish worldwide (Orr, 1967). In the United Kingdom, members of the Cyprinidae are the usual, but

Table 1. Sequence comparisons among *Ligula* sp. from *Phoxinus phoxinus*, *Rutilus rutilus* and *Gobio gobio*.

Comparison	rDNA region No. bases compared, no. ti/tv, no. gaps*				Total (2485 bps)† uncorrectd 'p' distance
	ITS-1	5.8S	ITS-2	28S (D1–D3)	
P. phoxinus vs. *R. rutilus*	574, 0/0 (0)	106, 0/0 (0)	500, 0/1 (18)	1282, 0/0 (0)	0.00040
P. phoxinus vs. *G. gobio*	574, 2/1 (1)	106, 0/0 (0)	500, 4/0 (17)	1282, 0/0 (0)	0.00322
R. rutilus vs. *G. gobio*	574, 2/1 (1)	106, 0/0 (0)	500, 4/2 (19)	1282, 1/0 (0)	0.00362

*Number of bases compared, number of transitional/transversional substitutions, number of positions containing gaps.
†Total number of bases compared, excluding all gapped positions.

not exclusive, hosts and all publications on the genus from the UK have referred to the species of parasite involved as *L. intestinalis*.

A number of authors have discussed the possible existence of strains/species within the genus *Ligula* in the United Kingdom (for example, Arme, 1975, 1997; Kennedy & Burrough, 1981; McManus, 1985). Evidence from host pathology (Arme, 1997) confirms that differences exist between gudgeon and roach *Ligula*, although the possibility that these differences may be related to host effects has not been totally excluded. Arme (1975) reported unpublished observations by Arme and Ferguson. They used iso-electric focusing in pH 3.5–10.0 gels of phenoxyethanol extracts of parasites from *G. gobio* and *R. rutilus*, with subsequent Coomassie Brilliant Blue staining. The general protein pattern differed between parasites in seven out of 43 bands, demonstrating some degree of genetic difference between them. McManus (1985) studied enzyme polymorphism in ligulids from *R. rutilus*, *G. gobio*, *Alburnus alburnus* and *Abramis brama*. In contrast, he found that there were no differences consistent with the existence of different strains or species of the parasite.

The results presented here provide additional evidence for strain/species differences between *Ligula* from gudgeon and roach in the UK. With the exception of two gapped regions in the ITS-2 gene (the most variable of the genes sequenced and potentially exhibiting differing copies within individuals), the single specimen from minnow is identical with the three specimens from roach. *Ligula* from gudgeon, however, are considerably and consistently different from those from either roach or minnow. There is little information on the pathology of *Ligula* infections of minnow. However, in the few specimens that have been examined by one of us (CA), there was evidence of a host tissue response and inhibition of gonadal development. That is, the pathology resembled that found in roach but not in gudgeon. However, it must be emphasized that more work is required on minnow to confirm these observations.

Since both roach and gudgeon are infected with *Ligula* in Lough Neagh, it might initially seem unlikely that two strains/species should co-exist in the same locality and one attain epizootic levels in roach. Indeed, the reports of the population dynamics of *Ligula* in both gudgeon (Bean & Winfield, 1989, 1992) and roach (Winfield *et al.*, 1992) imply that there is only a single species of *Ligula* infecting both hosts. However, the recent history of *Ligula* in Lough Neagh is unclear. The first report of the parasite in the Lough was that of Tobin (1986), who recorded it in both gudgeon and roach in 1984. Prior to this record, *Ligula* had only been reported from bream, *Abramis brama* in Ireland (Holland & Kennedy, 1997), where it is widespread. Kennedy & Fitzmaurice (1968) found it in bream in several localities, but never in gudgeon (Kennedy & Fitzmaurice, 1972). However, they examined no gudgeon from Lough Neagh. No *Ligula* was found in more than 50 bream, ranging in size from 50 to 300 mm and collected from the Lough between 1998 and 2000 (D. Griffiths & E. Bigsby, unpublished observations). Such data clearly do not rule out a low prevalence of the infection in bream.

Nevertheless, *Ligula* was present in Lough Neagh in gudgeon in 1974. The material Arme & Ferguson used for their iso-electric focusing study (Arme, 1975), which suggested that *Ligula* from gudgeon and roach were genetically different, was obtained from gudgeon from the Lough, although the precise locality was not recorded. Roach is not a native Irish species and they were absent from the Lough prior to *c.* 1973 (Cragg-Hine, 1973). The subsequent population expansion of *Ligula* throughout the late 1970s and early 1980s was correlated with an increase in the population of great crested grebes (*Podiceps cristatus*) overwintering in the Lough (Winfield *et al.*, 1992). Bean & Winfield (1989) also suggested causal links between the *Ligula* epizootic in Lough Neagh and the roach population expansion and that the parasite may well have been introduced into the Lough by the grebes that were attracted there by the increased numbers of roach.

The historical evidence, although incomplete, is therefore consistent with the possibility of two strains/species of *Ligula* in the Lough, each having arrived independently. *Ligula* was already present in gudgeon at the time of, and probably preceding, the roach introduction, whereas roach *Ligula* were probably introduced into the Lough by grebes at a later date from loughs elsewhere. However, the possibility that the parasite was already present in bream in the Lough and then spread to roach cannot be excluded. There are also differences in the population dynamics of *Ligula* in gudgeon and roach in the Lough (Bean & Winfield, 1989, 1992; Winfield *et al.*, 1992). For example, *Ligula* infects all length and age classes of gudgeon, which acquire infections throughout their life, and peak intensity occurs in older fish. In contrast, roach acquire most infections when young and infection levels decline in older and larger fish. The dynamics of *Ligula*–roach–grebes in Lough Neagh are typical of this system elsewhere (Kennedy *et al.*, 2001) and, whilst differences in the dynamics in gudgeon could be host determined, the very existence of such differences does support the view that the *Ligula* in gudgeon represents a different strain/species.

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