Parasites of *Loligo gahi* from waters off the Falkland Islands, with a phylogenetically based identification of their cestode larvae

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Abstract: One thousand and ninety-six longfin Patagonian squid, *Loligo gahi*, were collected from waters off the Falkland Islands over a period of 1 year and 4 months and examined for helminths. Cestode and *Anisakis* sp. (Nematoda) larvae were present throughout the study, but occurred at low rates. The pooled data showed prevalences of 5.75 and 2.46%, respectively. An adult digenean, *Derogenes varicus*, was found in a single instance and was thus considered an accidental infection. Parasite parameters were not significantly correlated with either host sex or seasonality. Stomach contents of the squid revealed a highly varied diet, although krill, amphipods, and chaetognaths were the dominant prey items. To aid in the identification of the cestode larvae, a subsample of 14 plerocercoids was characterized for the D2 variable region of the nuclear lsrDNA gene and compared with both published cestode lsrDNA sequences as well as that of six additional adult tetraphyllidean cestode species sequenced herein. Direct sequence comparison showed that 12 of the 14 plerocercoids were identical with each other and differed by a single transition (of a total of 658 base pairs) from the tetraphyllidean *Clistobothrium montaukensis*, and another plerocercoid differed by two transitions from the trypanorhynch *Grillotia erinaceus*. The remaining plerocercoid sequence was identified through phylogenetic analysis as being closer to the tetraphyllidean *Ceratobothrium xanthocephalum* than to any other taxon analyzed, but may not be congeneric, given its relatively high degree of divergence from *C. xanthocephalum*.

Résumé : Mille quatre-vingt-seize Calmars communs, *Loligo gahi,* de Patagonie, ont été recueillis dans les eaux entourant les îles Falkland au cours d'une période de 1 an et 4 mois; nous y avons dénombré les helminthes parasites. Nous avons trouvé des larves de cestodes et d'*Anisakis* sp. (Nematoda) pendant toute la période de l'étude, mais leur prévalence était faible. À partir des données combinées, la prévalence a été estimée à 5,75 % dans le cas des cestodes et à 2,46 % dans celui du nématode. Un adulte du digène *Derogenes varicus* a été trouvé une seule fois et est considéré comme un cas d'infection accidentelle. Les paramètres des parasites ne sont liés ni au sexe de l'hôte, ni à sa saisonnalité. Les contenus stomacaux des calmars démontrent que leur régime alimentaire est très varié, mais le krill, les amphipodes et les chétognathes, sont les proies les plus communes. Pour faciliter l'identification des larves de cestodes, la région variable D2 du gène de la grande sous-unité de l'ADN ribosomique (ADNlsr) nucléaire a été caractérisée dans un sous-échantillon de 14 plérocercoïdes et comparée aux séquences publiées de l'ADNlsr de cestodes ainsi qu'aux séquences trouvées chez les adultes de six espèces additionnelles de cestodes tétraphyllidiens. La comparaison directe des séquences a révélé que 12 des 14 plérocercoïdes sont identiques et diffèrent par une seule transition (sur 658 paires de bases possibles) du cestode tétraphyllidien *Clistobothrium montaukensis* et qu'un autre plérocercoïde diffère par deux transitions du tripanorhynque *Grillotia erinaceus*. Le dernier plérocercoïde a été identifié par analyse phylogénétique et il est plus apparenté à *Ceratobothrium xanthocephalum* qu'à tout autre taxon analysé, mais il n'appartient pas nécessairement au même genre, puisque la divergence d'avec *C. xanthocephalum* est relativement importante.

[Traduit par la Rédaction]

Introduction

The longfin Patagonian squid, *Loligo gahi* (d'Orbigny, 1835), is found off the Pacific and Atlantic coasts of South America from southern Peru to Argentina. It is a neritic species occurring from the surface to a depth of 600 m and, like many other teuthoid squid (Boyle 1983), it is semelparous and has a maximum life-span of about a year (Arkhipkin 1993; Hat-

field 2000). The *Loligo* fishery in the Falkland Islands is one of the most productive loliginid fisheries in the world, averaging 60 000 t per annum (Falkland Islands Government 2000). Despite this, almost nothing is known about the host–parasite relationships of squid in these waters.

With one possible exception, adult cestodes have never been reported from cephalopods (Hochberg 1990), although a diversity of larval and postlarval stages has been described

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from decapod and octopod cephalopods (e.g., Dollfus 1936, 1958; Threlfall 1970; Gaevskaya 1977, 1978; Stunkard 1977; Naidenova and Zuev 1978). Such a diversity of larval parasites indicates that cephalopods are important as second intermediate or paratenic hosts of cestodes, particularly those cestodes that mature in elasmobranchs and are transferred from host to host through the food chain (Hochberg 1983; Caira 1990). Cestodes are excellent parasites for use as biological tags in studying trophic interactions in the marine environment (Caira 1990). MacKenzie (1987) suggested that the use of parasites as biological tags in population studies in invertebrates, particularly crustaceans and cephalopods, would reward further investigation, and Pascual and Hochberg (1996) have reviewed the use of cephalopod parasites as biological tags specifically.

A number of scientists working in the Falkland Islands have reported small tetraphyllidean plerocercoids infecting *L. gahi* that were assumed to be a species of the genus *Phyllobothrium* van Beneden, 1850. Consequently in 1999 and 2000, samples of *L. gahi* were collected from commercial and research cruises for analysis onshore with the aims of quantifying the metazoan parasites and more accurately identifying the cestode larvae through molecular techniques, which in turn will help with the elucidation of their life cycles.

The use of molecular data for larval identification is efficient and effective in cases where larval morphology itself is insufficient to differentiate among closely related species or genera (Mariaux and Olson 2001), and although there are relatively few examples in the literature (e.g., Schulenburg and Wägele 1998; Jousson et al. 1999), the rapidly increasing number of helminth parasites characterized genetically (e.g., Mollaret et al. 2000; Nadler and Hudspeth 2000; Cribb et al. 2001; Littlewood and Olson 2001; Olson et al. 2001) will help to improve the accuracy and utility of this method in the years to come.

Materials and methods

Collection and examination of squid

One thousand and ninety-six post-recruit *L. gahi* were collected from fishing grounds off the coast of the Falkland Islands from February 1999 to June 2000. Three hundred and ninety-six squid were collected by the commercial fleet, while the remainder were collected during research cruises carried out by the Falkland Islands Fisheries Department (FIFD). Squid were examined both frozen and fresh. Fresh material was examined during the research cruises, whereas frozen material collected from the commercial fleet was examined in the FIFD laboratory in Stanley. For each individual squid, dorsal mantle length (DML, cm) and total body mass (g) (where possible) were recorded. The animals were opened longitudinally on their ventral side and the sex and range of gonad maturity according to an established scale of maturity were then determined (Lipinski 1979). Each individual was examined thoroughly using a dissecting microscope, and the numbers of metazoan parasites in the caecum, stomach, digestive gland, rectum, gonads, and reproductive organs were recorded. Representative samples of parasites were collected for the FIFD reference collection. The stomach contents of each squid were analysed by noting the presence of prey items. Prevalences were compared between the sexes as proportions using a χ^2 test. To eliminate possible geographical and seasonal effects (as prevalences were low) the relationship between host factors (length and maturity) was investigated using pooled data for all stations sampled, as sample sizes were similar (Fig. 1). The decline in prevalence of both cestode and nematode

larvae in squid longer than 18 cm was examined using Fisher's exact test. Data analysis was performed using GraphPad Prism (version 2.01). Use of the terms prevalence, mean intensity, and mean abundance follows the recommendations of Bush et al. (1997).

Molecular identification of cestode larvae

Because of the morphological uniformity of cestode plerocercoids, the specific identities of these worms were not recorded. However, a subsample of 14 cestode larvae, each from a different *L. gahi* individual, was collected for genetic analysis at The Natural History Museum in London. These were preserved in 95% EtOH and their genomic DNA was extracted using standard techniques described in Olson et al. (2001). The work of Olson et al. (2001) indicated that the D2 region of the large subunit rDNA (lsrDNA) gene exhibited sufficient variability to be useful for species-level identification (see also Mariaux and Olson 2001). Thus, a ~700 base pair (bp) portion of the nuclear lsrDNA gene encompassing this region was determined via direct cycle sequencing of polymerase chain reaction (PCR) products produced using the primers LSU5 and 1200R, and sequenced using the internal primers 300F and ECD2 (for primer definitions see Littlewood et al. 2000 and for sequencing techniques see Olson et al. 2001).

To complement the relatively few published lsrDNA sequences of tetraphyllideans (see Olson et al. 2001) available for comparison with the larval sequences, six additional tetraphyllidean taxa were characterized for the lsrDNA D1–D3 region (~1400 bp): *Ceratobothrium xanthocephalum* Monticelli, 1892 (ex *Isurus oxyrinchus*, Atlantic Ocean, Montauk, N.Y., U.S.A.), *Calyptrobothrium* sp. Monticelli, 1893 (ex *Apristurus atlanticus*, North Sea, U.K.), *Echeneibothrium maculatum* Woodland, 1927 (ex *Raja montagui*, North Sea, U.K.), *Orygmatobothrium musteli* (van Beneden, 1849) Diesing, 1863 (ex *Mustelus asterias*, North Sea, U.K.), *Spongiobothrium* sp. Linton, 1889 (ex *Dasyatis brevis*, Puertecitos, Gulf of California, Baja California, Mexico), and *Trilocularia* sp. Olsson, 1867 (ex *R. montagui*, North Sea, U.K.).
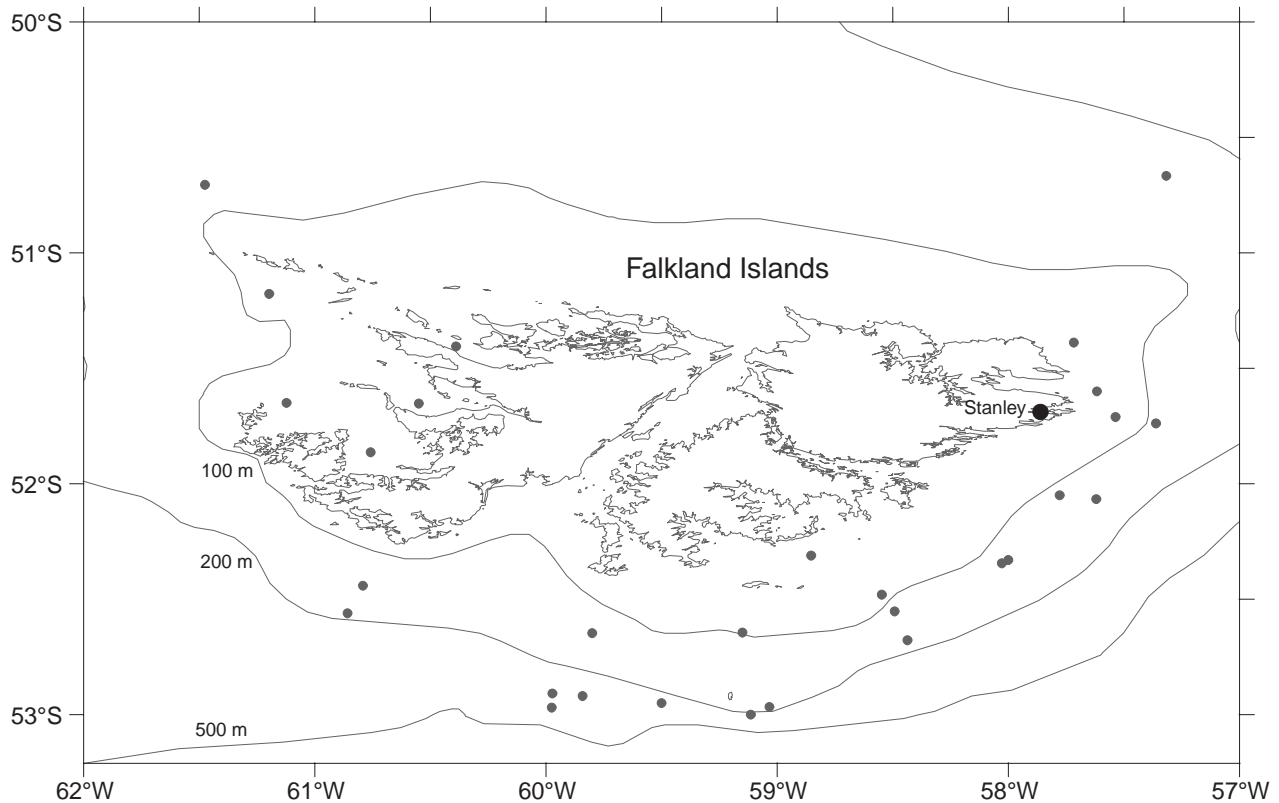
Larval sequences were aligned initially using MacClade version 4 (Maddison and Maddison 2000) with both published and new (P.D. Olson, unpublished data) lsrDNA sequences of cestodes to determine their affinities within the class, and 13 of the 14 larval sequences were subsequently realigned with only the tetraphyllidean taxa to maximize the number of informative positions for analysis. Regions where homology was not readily apparent were removed (32 of the total of 658 sites), and parsimony analysis of the tetraphyllidean sequences was carried out using PAUP* (Swofford 2001; for details of analysis see Olson et al. 2001). The full alignment is available by anonymous FTP (accession No. ALIGN_000143) from ftp.ebi.ac.uk in directory /pub/databases/embl/align.

Results

Individual *L. gahi* ranged from 3 to 31 cm DML, with a mean of 10.55. Squid maturity levels ranged from immature to mature. Both cestode and nematode larvae were found to infect *L. gahi* throughout the study area, although both occurred at low rates of infection (Table 1). The majority of cestodes encountered were found in the caecum, with fewer found in the intestine, mantle, stomach, and gonads. *Anisakis* sp. was found only in the stomach (Fig. 2). A single occurrence of an adult derogenid digenean, *Derogenes varicus* (Müller 1784), was found infecting the stomach of a male *L. gahi*.

No significant difference in prevalence of infection was found between the sexes of squid for either the cestode ($\chi^2_{[1]}$ = 2.38, $P > 0.05$) or nematode larvae ($\chi^2_{[1]}$ = 0.88, $P > 0.05$), therefore sex was not considered in subsequent analyses and the samples were pooled. Figure 3 shows the relationship

Fig. 1. Sampling locations around the Falkland Islands where 1096 *Loligo gahi* were collected for this study between February 1999 and June 2000.

Table 1. Parasites recovered during examination of 1096 *Loligo gahi* from around the Falkland Islands between February 1999 and June 2000.

	Cestode plerocercoids	*Anisakis* sp.	*Derogenes varicus*
Range of numbers per host	1–2	1–3	0–1
Prevalence (%)	5.75	2.46	0.09
Mean intensity*	1.08 (0.27)	1.41 (0.03)	1
Mean abundance	0.06	0.03	0.0009

*Values in parentheses are standard deviations.

between prevalence of infection with parasites and host length and maturity. There was an increase in the prevalence of both types of larvae with host maturity until DML reached 18 cm, at which point prevalence decreased, but the differences were not statistically different (Fisher's exact test, $P > 0.05$).
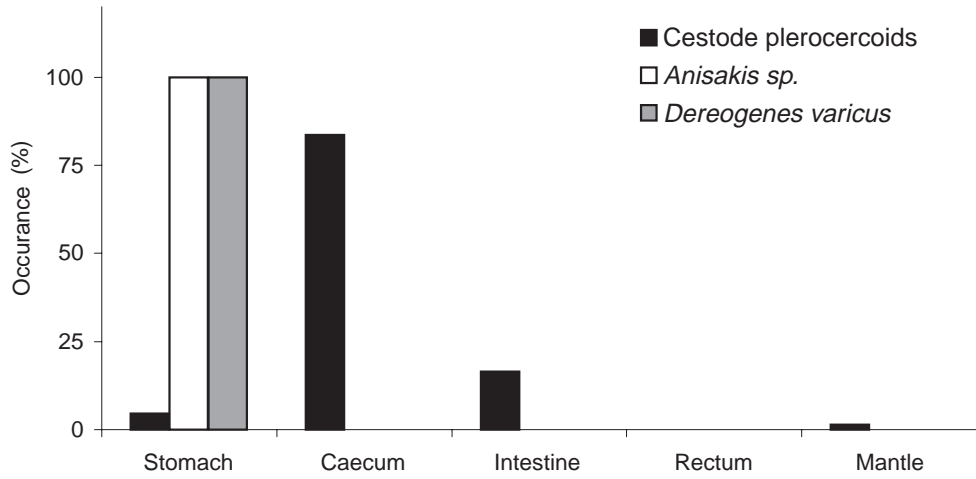
Table 2 lists the prey items encountered in *L. gahi* stomachs. *Loligo gahi* itself was the most dominant prey item in terms of percent occurrence, but was not considered an important natural prey item, because it is thought that a significant amount of net feeding occurs during the trawl (P. Schroeder, unpublished data). Thus, euphausiids (krill), *Themisto gaudichaudi* (Amphipoda), and Chaetognatha (arrow worms) were found to be the most abundant components of the diet of *L. gahi*.

Initial screening of the larval cestode sequences via parsimony analysis (results not shown) clearly indicated that all species but one were members of the order Tetraphyllidea,
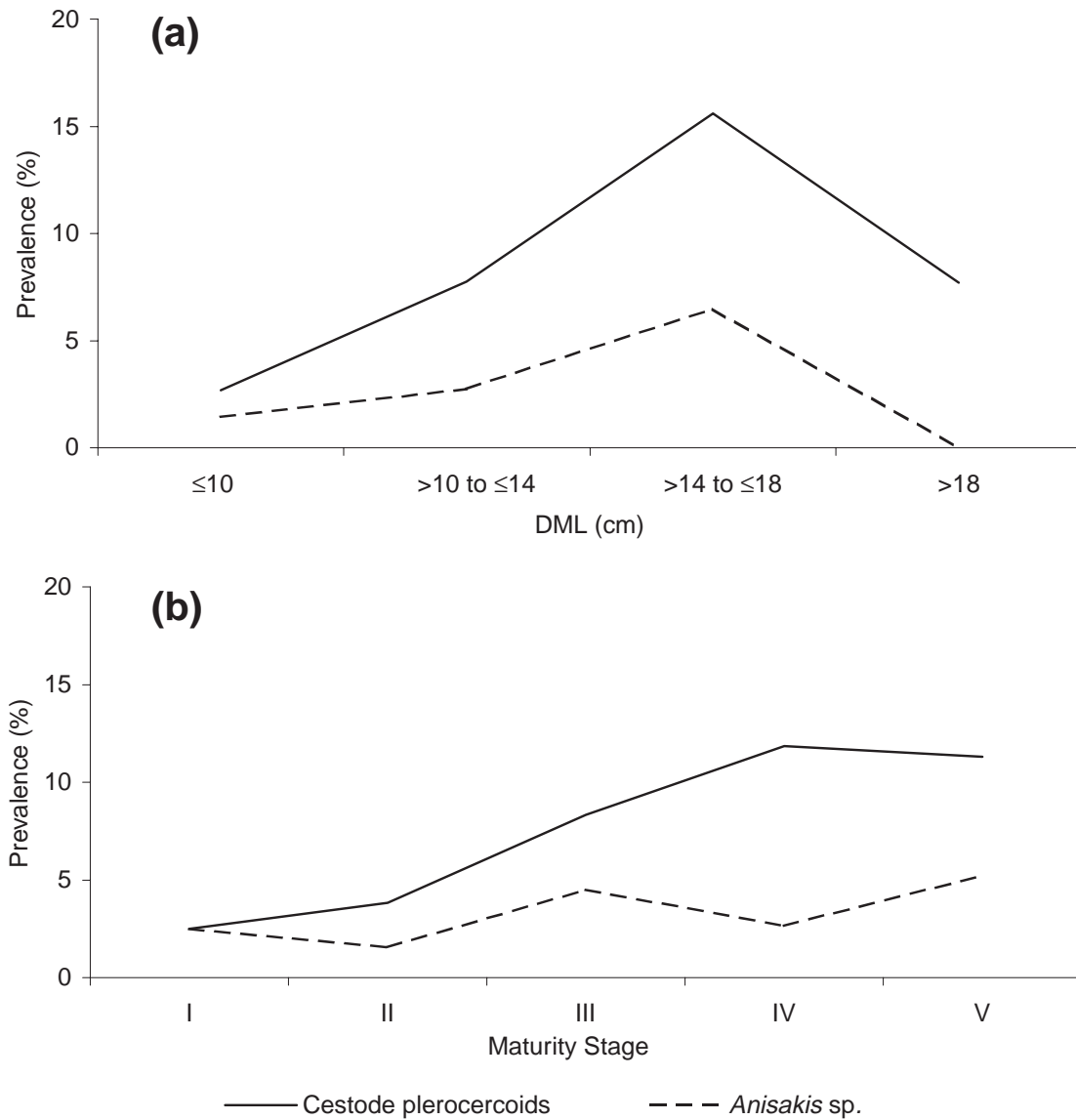
whereas the remaining larval sequence represented a species in the order Trypanorhyncha. Direct comparison of the latter larval sequence showed that it differed by 2 C-T transitions (651 bp compared) from the sequence (AF286967) of *Grillotia erinaceus* (van Beneden, 1858) Guiart, 1927, a common trypanorhynch in rays throughout the Atlantic Ocean (Schmidt 1986).

Of the 626 sites included in the phylogenetic analysis of the tetraphyllidean taxa, 247 (39%) were parsimony-informative and 90 (14%) were autapomorphic. Parsimony analysis resulted in two equal-length trees, one of which is shown as an unrooted phylogram (Fig. 4). The phylogenetic and taxonomic implications of these topologies, beyond the identities of the unidentified larval forms, are not considered herein. Of the tetraphyllidean larval sequences, 12 of the 13 were identical with each other and differed by two transitions (1 A-G and 1 C-T; total of 658 bp compared) from the sequence of *Clistobothrium montaukensis* Ruhnke, 1993. The

Fig. 2. Percent occurrence of cestode plerocercoids, *Anisakis* sp., and *Derogenes varicus* in organs of parasitized *L. gahi* collected during this study.

Fig. 3. Prevalence of helminth parasites infecting *L. gahi* as a function of dorsal mantle length (DML) (*a*) and stage of maturity (*b*).

Table 2. Percent occurrence of prey items recovered from stomach contents of *L. gahi* collected around the Falkland Islands between February 1999 and June 2000.

Prey item	Percent occurrence
Loligo gahi	42.88
Euphausiacea (krill)	31.70
Themisto gaudichaudi (Amphipoda)	16.23
Chaetognatha (arrow worms)	13.17
Unidentified Copepoda	7.81
Foraminifera	5.36
Munida spp. (Anomura: Galatheidae)	5.05
Fish scales	3.22
Kelp	2.91
Sprattus fuegensis (Pisces: Clupeidae)	2.14
Fish bones	0.92
Pteropoda (Mollusca)	0.92
Fish otoliths	0.61
Fish vertebrae	0.46
Unidentified Amphipoda	0.46
Gymnoscopolus sp. otoliths (Pisces: Myctophidae)	0.31
Octopod paralarvae	0.15

remaining tetraphyllidean plerocercoid showed a closer phylogenetic affinity (Fig. 4) to *C. xanthocephalum*, but differed from it by 23 bp (of 633 bp).

Discussion

Many reports have been published on the importance of squids in the life cycles of different groups of helminths (for review see Hochberg 1990) and on the roles of ommastrephids in the parasite fauna of the Atlantic Ocaen (Gaevskaya and Nigmatullin 1978; Pascual et al. 1995, 1996*a*), although few with respect to species of *Loligo* (e.g., Stunkard 1977; Nigmatullin 1989). However, Nigmatullin (1989) reported on parasites of *Loligo patagonicus* (syn. *L. gahi*) from populations inhabiting the northern part of the Patagonian shelf (international waters, 45–47°S). Only two helminths were reported, *Phyllobothrium* sp. and *Anisakis* sp., with prevalences of 5.6 and 1.4%, respectively (Nigmatullin 1989). During the present study, five parasites were encountered, of which the cestode plerocercoids (collectively) and *Anisakis* sp. had prevalences very near those reported in Nigmatullin (1989). These rates and the diversity of infection are extremely low compared with the results of studies carried out on squid of the family Ommastrephidae (Brown and Threlfall 1968; Threlfall 1970; Gaevskaya and Nigmatullin 1975, 1978; Pascual et al. 1995, 1996*b*). The prevalence of infection with both the tetraphyllidean plerocercoids and *Anisakis* sp. increased with squid maturity. A similar pattern emerged with host length, except that at a DML greater than 18 cm, the prevalence of both decreased. This decline in longer/older individuals has been observed in several fish species and may explain the pattern in *L. gahi*. Kabata (1963) and Arthur and Arai (1980) found that the prevalences of the protozoans *Goussia clupearum* and *Eimeria sardinae* appeared to decrease in herring (*Clupea harengus*) over 8 years old. Pennycuick (1971) observed a similar pattern with parasites in sticklebacks

(*Gasterosteus aculeatus*) and suggested that as the metabolic processes of older fish decline, their tolerance of parasitic infection might also decline, resulting in selective mortality of heavily infected individuals. Increasing parasite infection with size and maturity of marine fish hosts is also widespread. Cephalopods closely resemble fish in much of their way of life (Packard 1972). As size/maturity and age are correlated, a similar pattern could be expected for squid age (Pascual et al. 1995). In the case of longer lived parasites at least, differences in infection levels between squid in the various maturity groups may be due simply to an accumulation of worms over time as a result of the predatory nature of squid (Naidenova et al. 1985).
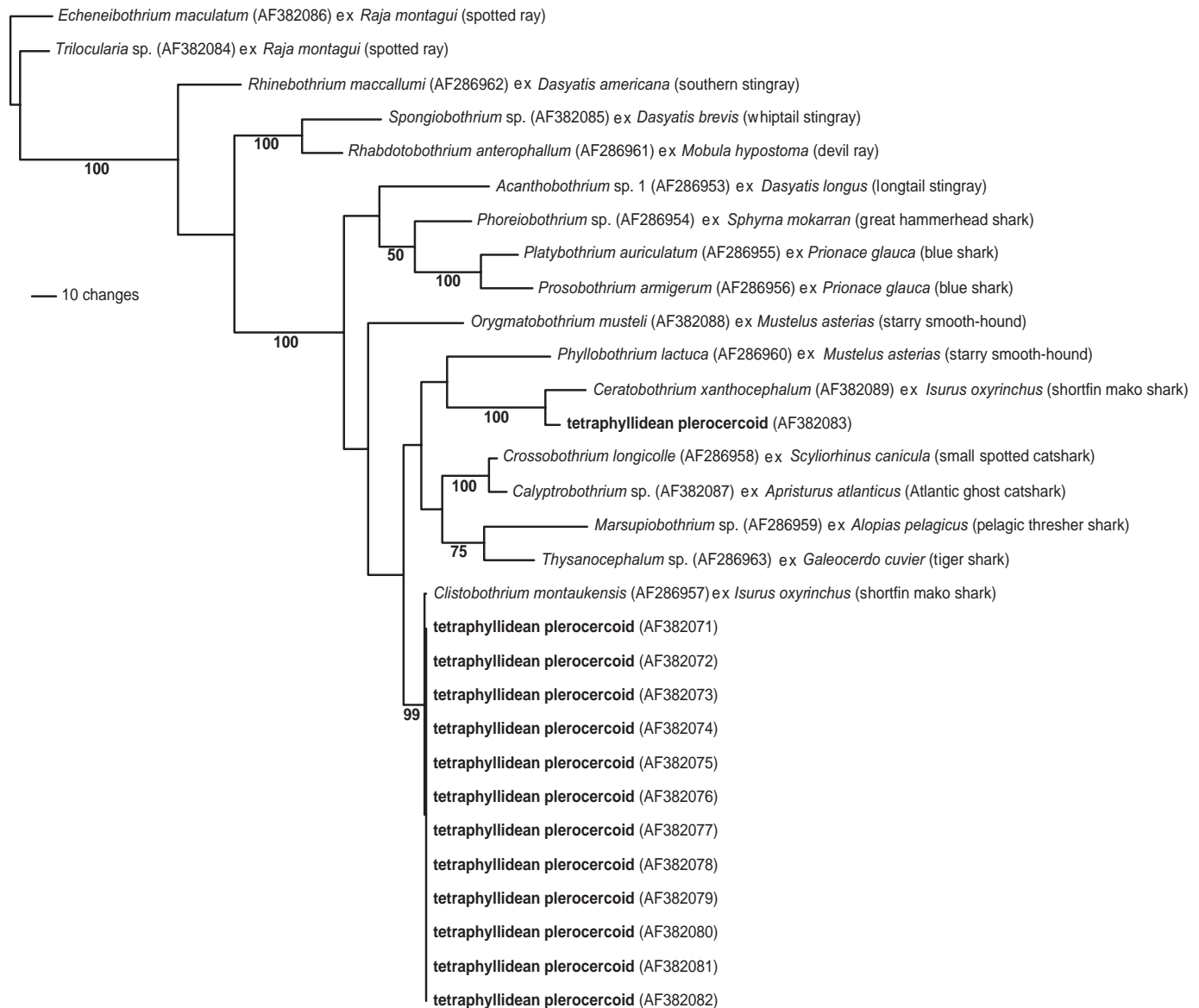
Derogenes varicus has been reported (Reimer 1974, 1975; Overstreet and Hochberg 1975) from the cuttlefish *Sepia officinalis*, but has not been reported from *Loligo* spp. As it only occurred once during this study, it was likely to have been an accidental infection. *Derogenes varicus* is considered by some to be the most widely distributed of all marine parasites (Hochberg 1990). Køie (1979) reviewed the life cycle of *D. varicus* and reported that it uses the gastropod *Natica* sp. as its first intermediate host in the northern hemisphere. It then uses copepods, larger crustaceans, and chaetognaths as further intermediate hosts. Mature worms develop in molluscs (cuttlefish) and a variety of fish hosts.

Anisakis spp. have been found frequently in the tissues of myopsid and oegopsid cephalopods, some species of which are known to prey on euphausiids (Clarke 1966). Adult *Anisakis* spp. are parasites of the stomach and intestine of pinnipeds and cetaceans (Anderson 2000). Tetraphyllidean procercoids use copepods and euphausiids as intermediate hosts and cephalopods are thought to pick up postlarval stages by feeding on these crustaceans or on small fish (Hochberg 1990). Both groups of parasites infect *L. gahi* through its diet and the squid act as paratenic hosts.

Characterization of species via molecular analysis does indeed add another layer of subjectivity to the delineation of Linnean ranks and to concepts of morphological species. Relatively few tetraphyllidean taxa, for example, have been characterized genetically, and with lineage-specific differences in rates of evolution it may not be possible to generalize the levels of divergence corresponding to species, genera, and higher taxonomic ranks, even within restricted clades of taxa (e.g., order Tetraphyllidea). Nevertheless, it is reasonable to expect that minor variation such as that found herein between larval worms and adult exemplar taxa originating from disparate geographic regions may be well within the genetic variation of a "morphological" genus, if not a species. Thus, 12 of the 13 tetraphyllidean plerocercoids represent species of the genus *Clistobothrium* (with the remaining plerocercoid more closely allied with *Ceratobothrium*), and the sole trypanorhynch plerocercoid represents a species of the genus *Grillotia*.

Grillotia erinaceus is a common parasite of skates (Rajidae) in the Atlantic Ocean (Schmidt 1986; Bates 1990). These elasmobranch hosts are generally bottom feeders; it is therefore not surprising that plerocercoids of *G. erinaceus* were uncommon among *L. gahi*. Adult *G. erinaceus* in the Falklands are no doubt to be found in the local species of *Raja*, the most common elasmobranchs in the Falkland Islands (P. Brickle, personal observation).

Fig. 4. Unrooted phylogram based on parsimony analysis of lsrDNA-D2 sequences (626 bp) with branch lengths proportional to the number of inferred steps. The topology shown is one of two equally parsimonious trees found; the other topology differed primarily in the more basal placement of *Orygmatobothrium musteli*. Numbers in boldface type are bootstrap values ≥50%. GenBank sequence accession numbers are shown parenthetically. Common names of hosts follow FishBase (Froese and Pauly 2001).

The plerocercoids provisionally identified as *Phyllobothrium* sp. were, in fact, representatives of tetraphyllideans (*Clistobothrium* sp., and *Ceratobothrium* sp.) found in pelagic sharks. The latter finding suggests that previous reports of plerocercoids of *Phyllobothrium* spp. in squid may have been in error (e.g., Stunkard 1977; Nigmatullin 1989), identifications being further complicated by the historical use of the genus *Phyllobothrium* as a "catch-all" for non-hooked tetraphyllidean worms with "leaf-like", marginally crenulated bothridia.

According to Ruhnke (1993), reliable host reports indicate that species of *Clistobothrium* are restricted to sharks of the family Lamnidae (mackerel sharks), having been reported to date (Dailey and Vogelbein 1990; Ruhnke 1993) only from the great white shark, *Carcharodon carcharias*, and shortfin mako shark, *Isurus oxyrinchus*. The porbeagle shark, *Lamna*

nasus (Bonnaterre 1788), is the only lamnid found in Falkland Islands waters (P. Brickle, personal observation) and it is likely that this shark is the normal definitive host of the *Clistobothrium* species found to infect *L. gahi* in the area.

The remaining tetraphyllidean plerocercoid sequenced showed a closer affinity to *C. xanthocephalum* than to any other species in the phylogenetic analysis (Fig. 4). However, the degree of divergence between *C. xanthocephalum* and the plerocercoid sequence (3.7%) and the lack of representative tetraphyllidean sequences for comparison are reasons to cast doubt on the possibility that the plerocercoid is congeneric with *Ceratobothrium* spp. Nevertheless, like *Clistobothrium* spp., *C. xanthocephalum* has been reported only from lamnid sharks (Schmidt 1986; Euzet 1994), including the genera *Lamna* and *Isurus*, and the adult form of the unidentified plerocercoid is also likely to be found in the porbeagle sharks

that are endemic to the Falkland Islands. Future work aims to examine specimens of the latter species from the Falkland Islands to confirm the identities of the larval forms found in *L. gahi*.

This technique of using molecular tools to confirm the identities of larval parasites, especially where morphology proves to be inadequate for distinguishing closely related species or genera, may prove extremely useful in the elucidation of life cycles and oceanic food webs.

Acknowledgements

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