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Host specificity and molecular phylogeny of larval Digenea isolated from New Zealand and Australian topshells (Gastropoda: Trochidae)☆

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Abstract

The maintenance of strict host specificity by parasites when several closely related host species live in sympatry is poorly understood. Species of intertidal trochid snails in the genera *Diloma*, *Melagraphia* and *Austrocochlea* often occur together and are parasitised by a single digenean morphotype (Platyhelminthes, Trematoda), tentatively placed in Opecoelidae. Of the 10 trochid species (6 from New Zealand, 4 from Australia) we examined, six were found to be infected, and the prevalence of infection was as high as 17.5%. We obtained molecular data (mitochondrial 16S and nuclear rDNA internal transcriber spacer 2 sequences, representing 774 bp), to infer phylogenetic relationships amongst these Digenea. Our phylogeny separated the single morphotype into three clearly defined clades (which are almost certainly separate biological species): (i) those infecting two species of *Austrocochlea* from Tasmania, (ii) those infecting *Diloma subrostrata* in Otago and Southland, New Zealand, and (iii) those infecting all the parasitised New Zealand topshells (*Melagraphia aethiops*, *D. subrostrata*, *Diloma nigerrima* and *Diloma arida*) throughout the country. This last group comprised two subclades, one infecting only *D. subrostrata* and one infecting the other three species. Two *D. subrostrata* populations were each found to be infected by genetically distinct parasites, yet sympatric populations of the other snails were not necessarily infected. This study is thus the first to reveal cryptic species of digeneans in a single population of a molluscan first intermediate host. We point out also that the degree of host specificity would have been grossly underestimated if, in the absence of our genetic analysis, we had only considered digenean morphology. Our results shed light on the conditions that may favour switching among intermediate hosts in digeneans, and on the presence/absence of host specificity in these parasites.

Keywords: 16S; Cercariae; Digenea; Host specificity; Internal transcriber spacer 2; Trochidae; Opecoelidae

1. Introduction

Despite the ubiquity and prevalence of digenean trematodes in intertidal and marine systems (see for example Cribb et al., 2001), the role parasites play in structuring these communities is still unresolved (Sousa, 1991; Mouritsen and Poulin, 2002), with traditional food web studies in intertidal communities often ignoring parasites (Marcogliese and Cone, 1997; Raffaelli, 2000). This gap in understanding may in part be due to their complex life cycle usually involving three host species, each harbouring parasites in morphologically distinct developmental stages. In the first intermediate host, which is usually a mollusc, larval sporocysts engage in asexual production of immature cercariae, which mature within the densely packed sporocysts. When mature, the motile cercariae emerge from the host to penetrate the second intermediate host, where they encyst as metacercariae. Amongst marine organisms, a wide variety of animals, including gastropods, bivalves, crustaceans, brachiopods annelids and fish, can act as the second intermediate host (Rankin, 1946; Bowers and James, 1967). Metacercariae remain encysted until the second intermediate host is ingested by a suitable definitive host, most probably a fish or shorebird. Once in the definitive host they develop into sexually mature adults.

☆ Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession numbers AY494876–AY494939.

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The digenean life cycle is completed when parasite eggs are shed in the faeces of the definitive host and hatch into the free-swimming miracidial stage, which infect the first intermediate host.

The ecological importance of such parasitism is underlined by the way in which the first intermediate hosts' physiology, behaviour and ultimately fitness may all be affected by the presence of a sporocyst infection (Moore and Gotelli, 1990; Poulin, 1998). Under normal environmental conditions the adverse effect on fitness may be insignificant, but when conditions approach the limit to which the host is adapted, parasitised animals often display reduced survivorship (Mouritsen and Poulin, 2002). Sporocyst infected populations of the periwinkle *Littorina littorea*, for example, exhibited a lower survival rate than non-parasitised populations upon exposure to high temperatures (McDaniel, 1969). More importantly, gastropods parasitised by cercariae almost invariably exhibit lower fecundity. For example, the trochid snail, *Diloma subrostrata*, may be effectively castrated by trematode parasites (Miller and Poulin, 2001); this phenomenon has been reported in numerous other systems (see Mouritsen and Poulin, 2002, for review). Consequently, despite often being overlooked in studies of intertidal ecosystems (Sousa, 1991), digenean parasites are likely to play an important role in intertidal ecosystem dynamics, due to their ubiquitous world-wide distribution and ability to infect and have an adverse affect on several host species.

An understanding of the ecological effects of digenean infections in marine ecosystems is additionally hampered by our poor knowledge of such basic aspects of digenean biology as the number of species in a particular ecosystem, their host specificity and the infection rates of the host populations. This study answers such questions, focusing on digenean sporocyst infections (speculatively placed in the Opecoelidae; Clark, 1958) previously reported from two intertidal neozelanic snails, the trochids *Melagraphia aethiops* and *D. subrostrata* (Clark, 1958; Miller and Poulin, 2001). In New Zealand, these snail species and up to four others in the genus *Diloma* can be found sympatrically (e.g. at Purakanui Bay, Otago, six species occur within a 20 m radius). Similarly, at least three species of the closely related genus *Austrocochlea* may coexist on Australian coasts. This level of overlap means that many potential first intermediate host species are exposed to digenean eggs, and thus provides a strong test of the prevailing parasitological paradigm that each digenean species usually infects just a single first intermediate host species (Gibson and Bray, 1994; Nunez and De Jong-Brink, 1997), with both host ecology and host–parasite physiological compatibility driving this specificity (Adamson and Caira, 1994; Sapp and Loker, 2000a,b). We surveyed 20 New Zealand and 18 Australian localities, examining large samples of snails in the family Trochidae for digenean infection. We then used phylogenetic analysis of digenean mitochondrial and nuclear DNA sequences to delimit species boundaries for each of the infections and thus we were able to determine the degree of host specificity. Taxonomic identification of parasites has previously been facilitated by molecular techniques (e.g. Luton et al., 1992; Després et al., 1995; MacManus and Bowles, 1996; Jousson et al., 1999; Schulenburg et al., 1999). This study goes beyond molecular identification, to examine patterns of host use by related digeneans and as a result allows us to address questions concerning the evolution of host specificity.

2. Materials and methods

2.1. Sample collection

For 10 species of trochid snails, between 32 and 300 individuals per sample, were collected at low tide from beaches in New Zealand and Australia (see Tables 1 and 2, and Fig. 1 for sampling locations and numbers). Sample size was determined by the abundance of snails at each sampling site. As the distribution of parasitised animals in the intertidal zone may be patchy (Miller and Poulin, 2001), snails were collected over a large area of the beach to maximise the chances of collecting infected animals. Live snails were placed in seawater and returned to the laboratory. Within 72 h of collection, snails were crushed and their soft tissue examined under a binocular microscope for evidence of larval digenean infections. Sporocysts, containing cercariae, were carefully separated from host tissue, preserved in 70% ethanol and stored at 4 °C.

2.2. DNA extraction and PCR amplification

Between 30 and 100 sporocysts, dissected from an individual host, were placed in a 5% Chelex 100 (Walsh et al., 1991) solution, which was incubated overnight at 65 °C. Following brief vortexing, the solution was boiled for 10 min and then centrifuged at 15,000*g*. The DNA in the supernatant was used in subsequent PCR amplifications.

Regions of the mitochondrial gene 16S and the nuclear rDNA internal transcriber spacer region 2 (ITS2) were PCR-amplified from the trematode DNA. Universal 16S primers could not be used as they invariably amplified gastropod 16S DNA sequence. Consequently, platyhelminth-specific mtDNA 16S gene primers were designed using sequence entries in the EMBL database and software at the Human Genome Mapping Project Resource Centre (HGMP-RC), Cambridge, UK. 16S primers were designed from conserved regions of aligned 16S sequences from three platyhelminths: two digenean trematodes (*Fasciola hepatica* and *Paragonimus westermani*) and one cestode (*Hymenolepis diminuta*).

Platyhelminth-specific 16S primers:

platy.16Sar 5′atctgttt(a/c)t(c/t)aaaaacat3′
platy.16Sbr 5′ccaatcttaactcaactcatat3′

Table 1
Sampling sites and numbers for New Zealand topshell populations

Sampling site (sampling site code)	Map reference	*Diloma* and *Melagraphia* species collected (topshell sampling code)	Number collected	Presence of trematode infection
Shoal Bay, Auckland (AUK)	36°35′S 174°42′E	*D. subrostrata* (AUK-DS)	32	Yes
		M. aethiops (AUK-MA)	98	No
Governors Bay, Christchurch (GB)	43°37′S 172°39′E	*D. subrostrata* (GB-DS)	200	No
		M. aethiops (GB-MA)	300	Yes
Heathcote Estuary, Christchurch (HE)	43°33′S 172°45′E	*D. subrostrata* (HE-DS)	200	Yes
Diamond Harbour, Christchurch (DH)	43°37′S 172°43′E	*M. aethiops* (DH-MA)	300	Yes
		D. zelandica (DH-DZ)	300	No
Little Akaloa, Banks Peninsula (LA)	43°40′S 172°59′E	*D. nigerrima* (LA-DN)	290	Yes
		D. arida (LA-DA)	300	Yes
		D. zelandica (LA-DZ)	300	No
		D. bicanaliculata (LA-DB)	111	No
Akaroa, Banks Peninsula (AK)	43°48′S 172°58′E	*D. subrostrata* (AK-DS)	40	No
		M.aethiops (AK-MA)	100	Yes
Robinsons Bay, Banks Peninsula (RB)	43°45′S 172°57′E	*D. subrostrata* (RB-DS)	60	No
		M. aethiops (RB-MA)	100	Yes
Menzies Bay, Banks Peninsula (MZ)	43°64′S 172°97′E	*D. nigerrima* (MZ-DN)	300	No
		D. arida (MZ-DA)	300	No
		D. zelandica (MZ-DZ)	300	No
		D. bicanaliculata (MZ-DB)	100	No
All Day Bay, Otago (AD)	45°12′S 170°53′E	*M. aethiops* (AD-MA)	100	Yes
Moeraki Beach, Otago (MB)	45°21′S 170°50′E	*D. nigerrima* (MB-DN)	262	Yes
		D. zelandica (MB-DZ)	300	No
		D. bicanaliculata (MB-DB)	100	No
Karitane, Otago (KA)	45°39′S 170°39′E	*D. subrostrata* (KA-DS)	150	Yes
		D. zelandica (KA-DZ)	300	No
Purakaunui Bay, Otago (PB)	45°44′S 170°35′E	*M. aethiops* (PB-MA)	300	Yes
		D. arida (PB-DA)	300	No
		D. subrostrata (PB-DS)	80	Yes
Warrington Rocks, Otago (WR)	45°43′S 170°08′E	*D. zelandica* (WR-DZ)	275	No
		D. bicanaliculata (WR-DB)	260	No
Te Ngaru (Aramoana), Otago Peninsula (TN)	45°47′S 170°42′E	*D. arida* (TN-DA)	300	No
		D. zelandica (TN-DZ)	300	No
Company Bay, Otago Peninsula (CB)	45°51′S 170°35′E	*D. subrostrata* (CB-DS)	200	Yes
		M. aethiops (CB-MA)	300	No
St Clair, Dunedin (SC)	45°55′S 170°29′E	*D. nigerrima* (SC-DN)	300	No
		D. arida (SC-DA)	211	No
Pounawea, Southland (PO)	46°28′S 169°41′E	*D. subrostrata* (PO-DS)	250	Yes
Waikawa Harbour, Southland (WA)	46°38′S 169°08′E	*D. subrostrata* (WA-DS)	59	Yes
Bluff Harbour, Southland (BH)	46°37′S 168°18′E	*D. nigerrima* (BH-DN)	300	No
		D. zelandica (BH-DZ)	300	No
		D. bicanaliculata (BH-DB)	136	No
Omaui Beach, Bluff, Southland (OB)	46°32′S 168°15′E	*D. arida* (OB-DA)	300	No

The optimum cycling parameters for these 16S platyhelminth specific primers included an initial denaturation step of 95 °C (2 min), followed by 37 cycles of 95 °C (30 s), 48 °C (40 s) and 72 °C (1 min). A final extension phase at 72 °C (10 min) was followed by storage at 4 °C.

The platyhelminth-specific primers LC1 (5′GCACTATCGATGAAGAACGC3′) and HC2 (5′ATATGCTTAAGTTCAGCGGG3′) described by Després et al. (1992) were used to amplify ITS2. HC2 was defined from the 5′ end of digenean trematode *Schistosoma mansoni* 28S sequence (Qu et al., 1988). LC1 was defined from nematode *Caenorhabditis elegans* 5.8S sequence (Ellis et al., 1986). The optimum cycling parameters for these ITS2 primers have been described by Després et al. (1992) and consisted of 30 cycles of 91 °C (30 s), 52 °C (1 min) and 71 °C (1 min). In this study, we included an initial denaturation step of 91 °C (2 min) and following cycling, a final extension phase at 72 °C (10 min). To confirm that the amplifications were successful, 5 μl aliquots of PCR amplifications were visualised by agarose gel electrophoresis.

The PCR products were separated from excess primers and oligonucleotides in High Pure PCR Purification Columns (Roche). Purified DNA was quantified using agarose gel electrophoresis and subsequently sent to the Allan Wilson Centre Genome Sequencing Service at

Table 2
Sampling sites and numbers for Australian topshell populations

Sampling site (sampling site code)	Map reference	*Austrocochlea* species collected (topshell sampling code)	Number collected	Presence of trematode infection
Hallet Cove, Adelaide (HA)	35°08′S 138°50′E	*A. concamerata* (HA-ACC)	250	No
		A. constricta (HA-ACS)	250	No
		A. porcata (HA-AP)	250	No
Marino, Adelaide (MA)	35°04′S 138°52′E	*A. concamerata* (MA-ACC)	250	No
		A. constricta (MA-ACS)	300	No
		A. porcata (MA-AP)	250	No
Onkaparinga, Adelaide (OK)	35°16′S 138°47′E	*A. constricta* (OK-ACS)	300	No
		A. porcata (OK-AP)	300	No
Devonport, Tasmania (DV)	41°18′S 146°36′E	*A. concamerata* (DV-ACC)	300	No
		A. constricta (DV-ACS)	250	No
		A. porcata (DV-AP)	250	No
Taranna, Tasmania (TA)	43°07′S 147°85′E	*A. concamerata* (TA-ACC)	250	No
		A. constricta (TA-ACS)	250	No
Swansea, Tasmania (SW)	42°13′S 148°07′E	*A. constricta* (SW-ACS)	100	No
		A. porcata (SW-AP)	50	No
Pitt Water, Tasmania (PI)	42°79′S 147°56′E	*A. porcata* (PI-AP)	150	No
		A. brevis (PI-AB)	150	No
Porpoise Hole, Tasmania (PH)	42°50′S 147°51′E	*A. constricta* (PH-ACS)	100	Yes
		A. porcata (PH-AP)	100	Yes
		A. brevis (PHAB)	50	No
Camp Cove, Sydney (CC)	33°85′S 151°28′E	*A. porcata* (CC-AP)	200	No
Manly, Sydney (MN)	33°80′S 151°28′E	*A. porcata* (MN-AP)	200	No
Little Bay, Sydney (LB)	33°98′S 151°24′E	*A. porcata* (LB-AP)	200	No
Nielson Park, Sydney (NP)	33°86′S 151°27′E	*A. porcata* (NP-AP)	200	No
Forty Baskets Beach, Sydney (FB)	33°84′S 151°26′E	*A. porcata* (FB-AP)	200	No
Bondi Beach, Sydney (BO)	33°89′S 151°27′E	*A. porcata* (BO-AP)	200	No
Maroubra, Sydney (MR)	33°95′S 151°24′E	*A. porcata* (MRAP)	200	No
Harbord, Sydney (HB)	33°78′S 151°29′E	*A. porcata* (HR-AP)	200	No
Cape Banks, Sydney (CB)	34°00′S 151°15′E	*A. porcata* (CB-AP)	200	No
Taronga Zoo, Sydney (TA)	33°84′S 151°24′E	*A. porcata* (TA-AP)	200	No

Massey University for sequencing in an ABI3730 automated sequencer. Sequencing reactions used the original PCR primers, pl.16Sbr and LC1, diluted to a final concentration of 0.213 μM, as sequencing primers for 16S and ITS2, respectively. To check that only trematode DNA had been amplified, Blast searches (Altschul et al., 1990) were performed to compare amplified sequences with all sequences stored in the EMBL database.

2.3. Phylogenetic analyses

Sequences were aligned using the ClustalX alignment program (Thompson et al., 1997). Phylogenetic analyses were performed with PAUP* version 4b10 (Swofford, D.L., 2002. PAUP* 4.0b10, Sinauer Associates, Sunderland, MA) for all neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) searches and bootstrap values (Felsenstein, 1985, 1988). MrBayes (Huelsenbeck and Ronquist, 2001) was used for Markov-chain Monte-Carlo Bayesian posterior probabilities, and Spectrum (Charleston, 1998) for spectral analysis (Hendy and Penny, 1993). After initially analysing the two genes separately we used the partition-homogeneity test (Swofford, D.L., 2002. PAUP* 4.0b10, Sinauer Associates, Sunderland, MA; the ILD of Farris et al., 1995) to test whether the 16S and ITS2 sequences contain the same signal and could thus be analysed as a single data set (to allow this test to be performed the 'MulTrees' option was not in effect). For visualisation purposes, the digenean trematodes *F. hepatica* and *P. westermani* were defined as outgroup taxa as both 16S and ITS2 sequence data are available for these organisms in the EMBL database. The NJ trees for each gene and both genes combined were constructed using GTR corrected distances. For the ML tree estimation, NJ was used to obtain the starting trees for branch-swapping (TBR). The HKY + G nucleotide substitution model was selected for the ML analysis using the hierarchical likelihood ratio test of Modeltest (Posada and Crandall, 1998). The transition/transversion ratio, nucleotide frequencies, and gamma shape parameter were all estimated by maximum-likelihood. The NJ bootstrap analyses consisted of 10,000 replicates. The MP bootstrap analyses consisted of 10,000 replicates with the 'MulTrees' option not in effect. For the likelihood bootstrap analysis, 1000 replicates with a fast heuristic search were used (with the estimated parameters from the ML search). Bayesian analysis was performed using MrBayes with the following settings. The ML model employed two substitution types

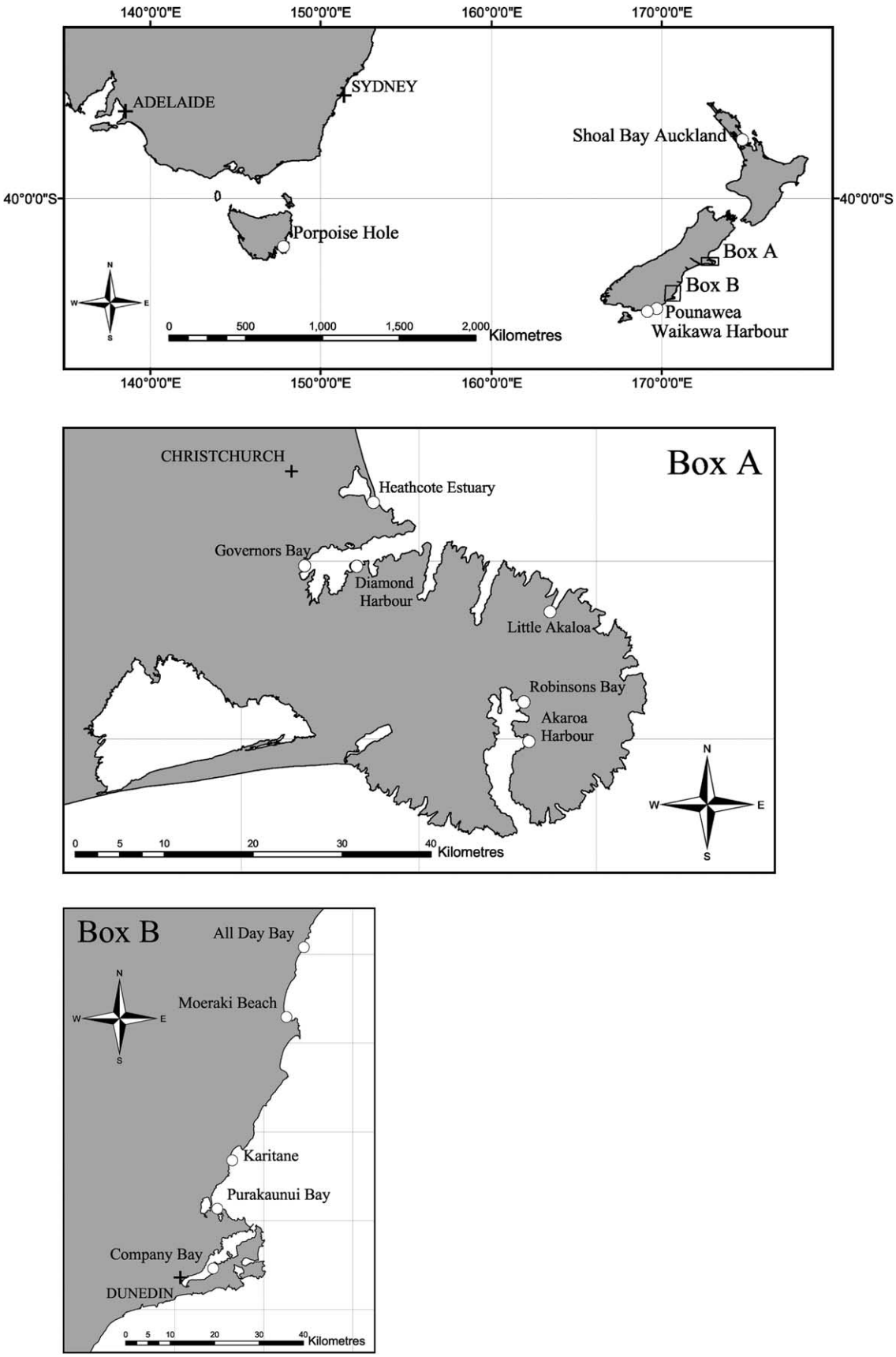

Fig. 1. Locations of digenean infected topshell populations. Main map shows sampling sites in New Zealand and Australia; Box A, collection sites in Canterbury; Box B, collection sites in Otago.

('nst = 2'). Rate variation across sites was modelled using a gamma distribution. The Markov-chain Monte-Carlo search was run with four chains for 500,000 generations, with trees being sampled every 100 generations (the first 1000 trees, i.e. 100,000 generations, were discarded as 'burnin').

3. Results

3.1. Occurrence of trematode infected topshells

Of the 15,114 snails collected (representing three genera and 10 species), 103 individuals were infected with larval digeneans. The number of parasitised snails varied greatly between both species and sampling locations (see Table 3). We were only able to distinguish one morphotype of digenean in all infected snail populations: digeneans dissected from different New Zealand snail species exhibited almost identical body length, body width and diameter of oral and ventral suckers (A. Miller, personal observation). In all cases, the cercariae were indistinguishable from those described by Clark (1958) from *M. aethiops*; being characterised by a stumpy tail forming a terminal sucker, as commonly seen in members of the family Opecoelidae. Of all the populations sampled, two New Zealand topshell species (*Diloma bicanaliculata* and *Diloma zelandica*) and two Australian topshell species (*Austrocochlea concamerata* and *Austrocochlea brevis*) were never found to be infected. Of the remaining six species, the level of infection in individual populations ranged from 0 to 17.5%. Populations of the New Zealand species, *D. subrostrata* and *M. aethiops*, were most consistently infected by larval digeneans, with 70 and 75%, respectively, of populations sampled exhibiting some level of infection. The highest levels of infection (17.5 and 15%) were observed in *D. subrostrata* populations sampled at Purakaunui Bay and Company Bay. At three locations larval digenean infestations occurred in two host species (*D. subrostrata* and *M. aethiops* at Purakaunui Bay, *Diloma nigerrima* and *Diloma arida* at Little Akaloa and *Austrocochlea constricta* and *Austrocochlea porcata* at Porpoise Hole). At all other sampling locations, despite different snail species co-existing, parasites were only recorded in one species of topshell host. For example, despite *D. subrostrata* at Company Bay exhibiting an infection rate of 17.5%, parasites were not detected in any of the 300 *M. aethiops* sampled there. Tasmania was the only place in Australia where infected topshells were sampled; no infected *Austrocochlea* populations were found on mainland shores near Adelaide or Sydney.

3.2. Molecular data

PCR amplification of 16S gave a product of approximately 400 bp; subsequent sequencing of this product routinely yielded at least 368 bp of readable sequence. Blast searches performed on these sequences demonstrated highest matching with other digenean trematode sequences. Our 16S sequences and the EMBL 16S sequences from the digenean trematodes *F. hepatica* (AF216697) and *P. westermani* (AY190071) were most similar (*E* value

Table 3
Prevalence of digeneans in New Zealand and Australian topshell populations

Host species	No. of populations sampled (range of n; mean (x) sampled)	No. of infected populations	Sites of infected populations (and population code)	Habitat type	Percent infected
Diloma subrostrata	10 (32–200; $x = 115$)	7	Shoal Bay, Auckland (AUK-DS)	Mudflat	3.1
			Heathcote Estuary (HE-DS)	Sand and rocks	0.7
			Karitane (KA-DS)	Sandy	0.7
			Purakaunui Bay (PB-MA)	Sand and rocks	17.5
			Company Bay (CB-DS)	Mudflat	15.0
			Pounawea (PO-DS)	Sandy	4.0
			Waikawa Harbour (WA-DS)	Mudflat	3.4
Melagraphia aethiops	8 (98–300; $x = 200$)	6	Diamond Harbour (DH-MA)	Rocky	6.0
			Governers Bay (GB-MA)	Rocky	1.3
			Robinsons Bay (RB-MA)	Sand and rocks	2.0
			Akaroa Harbour (AK-MA)	Sand and rocks	7.0
			All Day Bay (AD-MA)	Rocky	3.0
			Purakaunui Bay (PB-MA)	Sand and rocks	3.3
Diloma nigerrima	5 (262–300; $x = 290$)	2	Little Akaloa (LA-DN)	Rocky	0.3
			Moeraki Beach (MB-DN)	Rocky	0.3
Diloma arida	6 (211–300; $x = 285$)	1	Little Akaloa (LA-DA)	Rocky	0.3
Diloma zelandica	8 (275–300; $x = 297$)	0	–		–
Diloma bicanaliculata	5 (100–160; $x = 121$)	0	–		–
Austrocochlea constricta	8 (100–300; $x = 212$)	1	Porpoise Hole (PH-ACS)	Mudflat	2.0
Austrocochlea concamerata	4 (250–300; $x = 263$)	0	–		–
Austrocochlea porcata	16 (100–300; $x = 200$)	1	Porpoise Hole (PH-AP)	Mudflat	3.0
Austrocochlea brevis	2 (50–150, $x = 100$)	0	–		–

$<2 \times 10^{-17}$). PCR amplification of ITS2 yielded a product of 445 bp, sequencing of this product routinely gave at least 407 bp of readable sequence. All ITS2 sequences generated in this study showed highest similarity (*E* value $<10^{-100}$) with ITS2 sequence data from two adult opecoelids (*Macvicaria alacris* (AJ241801) and *Podocotyle scorpaenae* (AJ241794)) isolated from Mediterranean fish and an unnamed larval opecoelid (AJ241809) isolated from the Mediterranean topshell, *Clanculus jussieui* (Jousson et al., 1999). These BLAST searches indicated that the correct digenean gene had been sequenced and confirmed speculation, based on morphological data, that these cercariae belong to the Opecoelidae. Our 16S sequences, therefore, are the first to be derived from an opecoelid. The aligned partial 16S and ITS2 nucleotide sequence data matrices and resultant phylogenetic trees have been submitted to TreeBASE (www.treebase.org).

3.3. Molecular phylogeny

The 16S phylogenetic analysis was based on a 368 character alignment, of which 163 were variable and 86 were parsimony informative (within the ingroup taxa, 69 sites were variable and 63 were parsimony informative). This analysis used *F. hepatica* (AF216697), *P. westermani* (AY190071) and *Rudolphiella szidati* (AJ388617) 16S sequences as outgroups. For the ITS2 analysis the alignment produced a 407 character data set, of which 203 were variable and 98 were parsimony informative (within the ingroup taxa, 46 sites were variable and 39 were parsimony informative). Six outgroup ITS2 sequences were obtained from EMBL: *F. hepatica* (AJ272053), *P. westermani* (AF333278), *Cainocreadium labracis* (AJ241795), *M. alacris* (AJ241801), *P. scorpaenae* (AJ241794) and Opecoelidae sp. (AJ241809). Neighbour-joining analyses of the 16S and ITS2 sequences gave two very similar trees (Fig. 2), with three major clades within the ingroup taxa. Not surprisingly 16S, the mitochondrial gene (which evolves more quickly than nuclear genes), provided more structure and support within the three ingroup clades than ITS2 (Table 4). This congruence between trees based on nuclear and mitochondrial sequences implies that the clades in the trees represent real biological

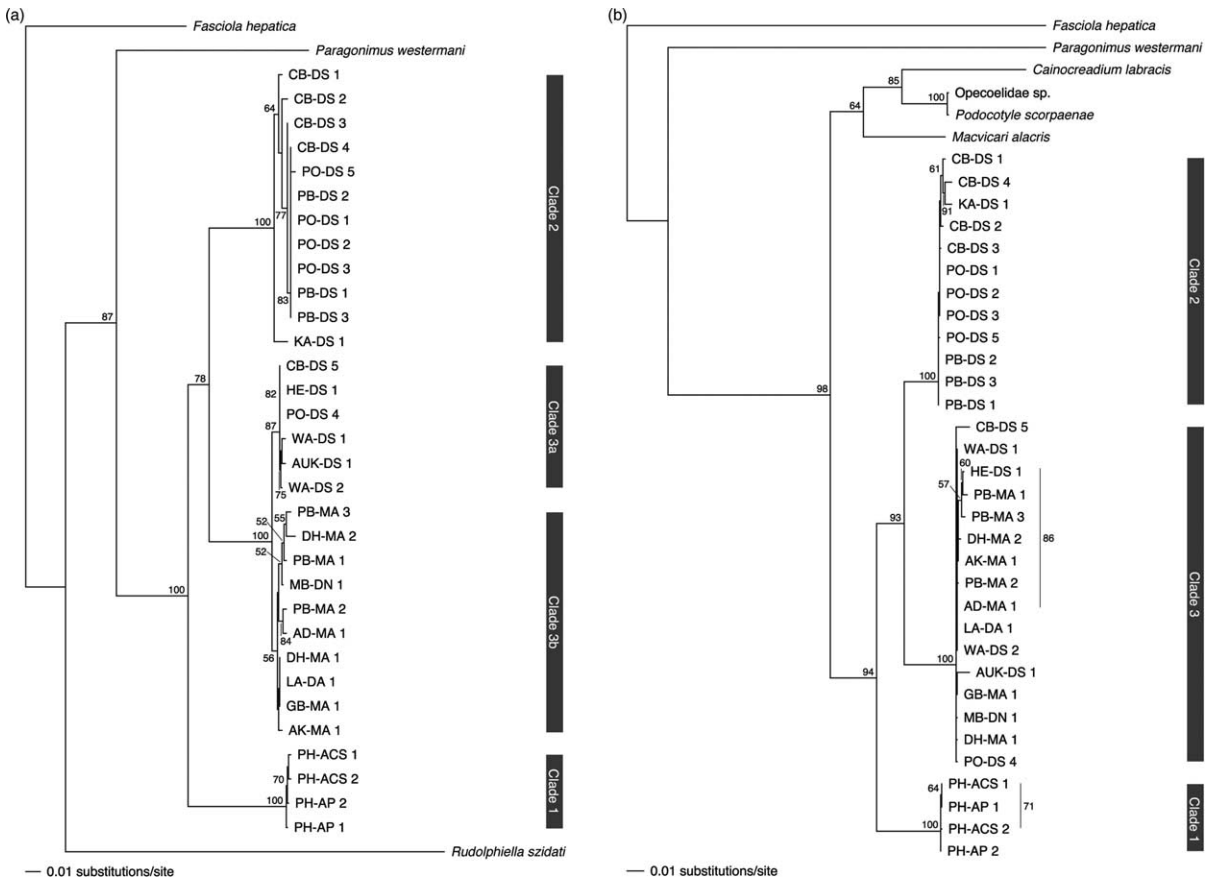

Fig. 2. (a) Phylogenetic relationships of cercariae isolated from six topshell species inferred by a neighbour joining analysis of the 16S mitochondrial DNA sequences. The key to infected trochid populations is recorded in Table 3. Numbers associated with the branches represent NJ bootstrap values (10,000 replicates). (b) Phylogenetic relationships of cercariae isolated from six topshell species inferred by a neighbour joining analysis of the internal transcriber spacer 2 nuclear rDNA sequences. The key to infected topshell populations is recorded in Table 3. Numbers associated with the branches represent NJ bootstrap values (10,000 replicates).

Table 4
Range of percentage sequence divergence (HKY + G nucleotide substitution model) within (diagonal) and among (off-diagonal) digenean clades

	16S			ITS2		
	Clade 1	Clade 2	Clade 3	Clade 1	Clade 2	Clade 3
Clade 1	0.0–0.0	14.7–17.5	14.6–17.5	0.0–0.0	8.0–10.2	9.9–11.5
Clade 2		0.0–1.6	10.8–12.7		0.0–0.0	5.7–8.0
Clade 3			0.0–2.9			0.0–1.7

entities, rather than merely clusters of related genes. A partition-homogeneity test confirmed that there was no significant difference in the phylogenetic signal among these sequences (10,000 replicates, $P = 0.52$) and thus the two sequences were subsequently concatenated and analysed as a single dataset. The combined aligned dataset of 774 characters (367 bp for 16S and 407 bp for ITS2) included the two outgroup taxa for which both gene sequences were available (*F. hepatica* and *P. westermani*). The combined data set had 323 variable, and 142 parsimony informative characters.

The trees produced by NJ, MP, ML, and Bayesian analysis of the combined data set were all broadly concordant with the neighbour-joining trees found for the individual genes. The NJ and Bayesian trees from the combined data set with measures of branch support (bootstrap values and posterior probabilities) are shown in Fig. 3. The ML tree (not shown) for the combined data set did differ from those found by the other methods of analysis by grouping clades 1 and 2 rather than clades 2 and 3 as sister groups. While the optimal ML tree topology grouped clades 1 and 2, the ML bootstrap analysis grouped clades 2 and 3 as sister taxa in accordance with all the other analyses.

These phylogenetic trees all provided evidence that parasites from Australia and New Zealand are monophyletic compared to the outgroups, and can be divided into three well-supported clades; the first comprised solely the Tasmanian digeneans (clade 1), the second was made up of parasites extracted from four Otago and Southland populations of *D. subrostrata* (clade 2) and the final clade contained digeneans isolated from four New Zealand species of topshell, including *D. subrostrata* from all over New Zealand (clade 3). Bootstrap support for the monophyly of the three clades was 100% for both NJ and parsimony. The likelihood bootstrap support for these three clades ranged from 95 to 100% and Bayesian analysis gave posterior probabilities of 1.00 for all three clades.

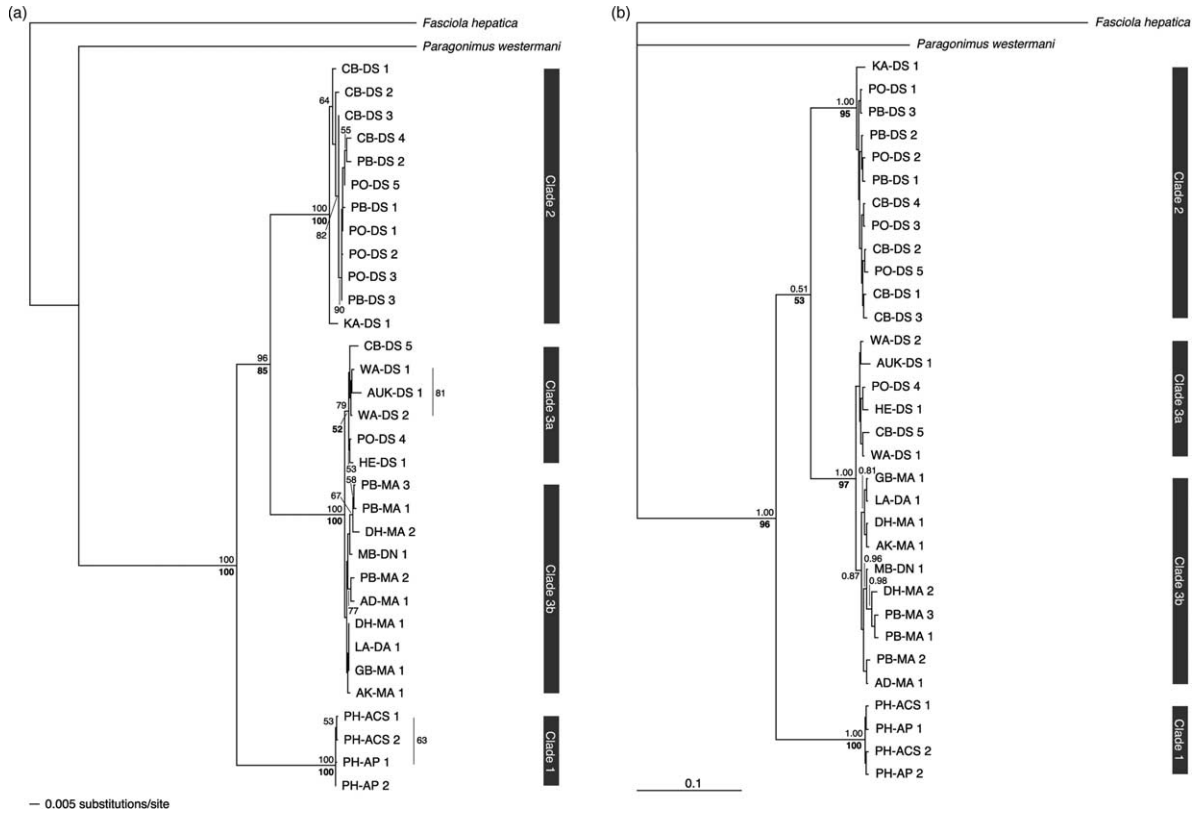

Fig. 3. (a) Phylogenetic relationships of cercariae isolated from six topshell species inferred by neighbour joining of the combined data set. The key to infected topshell populations is recorded in Table 3. The numbers associated with the branches represent NJ bootstrap values (10,000 replicates) and the bold numbers associated with the branches represent MP bootstrap values (10,000 replicates). (b) Phylogenetic relationships of cercariae isolated from six topshell species inferred by Bayesian analysis of the combined data set. The key to infected topshell populations is recorded in Table 3. The numbers associated with the branches represent Bayesian posterior probabilities and the bold numbers associated with the branches represent ML bootstrap values (1,000 replicates).

The level of sequence divergence between these clades was high, especially for the 16S sequence (ranging from 10.8 to 17.5%, see Table 4). Even the slower-evolving ITS2 sequence showed from 5.7 to 11.5% divergence between the three clades. These values greatly exceed that suggested by Jousson et al. (1999) as maximal for intraspecific variation in ITS sequence (1.5%). This level of variation, combined with the strong phylogenetic support, leads us to conclude that these three clades almost certainly correspond to three separate genetic species.

Digenea from the two Tasmanian topshells, *A. constricta* and *A. porcata*, (clade 1) could not be separated, and no genetic variability was found within this clade (see Table 4). Table 4 shows that clade 2 also displayed little sequence variation, whereas clade 3 showed substantially higher variability. This greater variability in clade 3 was due to the parasites isolated from *D. subrostrata* displaying several base pair differences from parasites making up the remainder of the clade, and they consequently form their own subgroup (clade 3a), which was reciprocally monophyletic with respect to the rest of clade 3 (clade 3b; see Figs. 2a and 3). For the combined data set, spectral analysis (spectral support, 0.0029, normalised conflict, 0.0000; for an example of the use of spectral analysis see Kennedy and Spencer, 2004), bootstrapping and posterior probabilities all support separating clades 3a and 3b. The level of divergence between clades 3a and 3b for the 16S sequences for example, ranged from 0.8 to 2.9%. Moreover, the chance that these two subclades would arise by chance is remote: the probability that 16 items, six of one type and 10 of another, would assort perfectly when randomly divided into two groups of sizes 6 and 10 is

$$1/\binom{16}{6} = 0.000125.$$

For all these reasons, we strongly suspect that clades 3a and 3b probably constitute separate genetic species, making four species in total.

4. Discussion

We have shown that a single morphospecies parasitises at least four snail species from New Zealand and two from Tasmania. There is significant variability in the infection rate among snail populations and species, with sympatric populations of different species often exhibiting the greatest differences (e.g. 17.5 vs 0%). This variation is in part due to the morphospecies consisting of several genetically distinct groups, which almost certainly comprise separate biological species. In some, but not all cases, these digenean species show strong host specificity. Without our genetic analysis of the digenean individuals, interpreting our field results would have been strikingly misleading.

The degree of host specificity we find is particularly interesting in light of the prevailing parasitological paradigm that digeneans at the sporocyst stage usually infect just a single host species (Gibson and Bray, 1994; Nunez and De Jong-Brink, 1997; Sapp and Loker, 2000a,b; but see Jousson et al., 1999; Blair et al., 2001), with the host's ecology driving this specificity (Adamson and Caira, 1994). Two Australian topshell species, *A. constricta* and *A. porcata*, clearly share one parasite species. Interestingly, these two topshells are ecologically and genetically very similar and can be found together on the same mudflats; indeed, until recently they were classified as morphological variants of a single species (Parsons and Ward, 1994). Nevertheless, a third Tasmanian species, *A. brevis*, which was also lumped with the other two until 1994 and lives sympatrically, was not found to be infected. Our preliminary phylogenetic analysis of these molluscs (Donald et al., in preparation) suggests that *A. brevis* is sister to the other two species. This pattern suggests that the phylogenetic relationships of the hosts may be more important than their ecology in determining host specificity. Nevertheless, at this site the *A. brevis* population was small, consequently only 50 individuals could be collected. If the *A. brevis* infection rate was similar to that encountered for *A. constricta* and *A. porcata* at this site (2–3%), then the chances of encountering an infected individual in a sample size of 50 would be fairly low. A sample size of several hundred would have allowed us to state with greater certainty that the *A. brevis* population was uninfected.

Similarly, digeneans from clade 3b appear to infect at least three New Zealand topshell species, *M. aethiops*, *D. nigerrima* and *D. arida*. Unlike the Australian instance above, however, these New Zealand host species are not particularly closely related (Donald et al., in preparation), they are obviously morphologically distinct, and their habitat preferences do not overlap to the same extent. *D. nigerrima*, for example, lives on decaying kelp cast high on exposed rocky shores, whereas *M. aethiops* occurs on mid-tidal rocks over a wide range of exposures, from harbours to open coasts. Nevertheless, this trematode is predominantly found in just one host species, *M. aethiops*: of the 47 snails found to be infected by parasites belonging to clade 3b, all but three were this species, and the overall infection rate for *M. aethiops* was 2.75% (= 44/1598) compared to 0.14% and 0.06 for *D. nigerrima* and *D. arida*, respectively.

It may even be that the trematode's presence in these last two species is accidental, and that it normally infects only *M. aethiops*. In support of this possibility we note that in two populations *M. aethiops* manifested a high infection rate, while sympatric populations of these *Diloma* species were not detectably infected. At Purakaunui Bay, 10% of *M. aethiops* were infected, but *D. arida* was apparently not infected even though 300 individuals were checked; in Diamond Harbour, *M. aethiops* exhibited an infection rate of 6%, whereas no infected *D. zelandica* were found, again out of 300 individuals. An alternate explanation is that this host sharing is genuine and the result of host-addition,

which occurs when a parasite increases the number of existing hosts (Blair et al., 2001). Upon host addition, parasites infecting a new host species may exhibit reduced fitness. Consequently, novel hosts are usually added in allopatry rather than sympatry, as sympatric hosts would be selected against whereas parasites in novel allopatric hosts could not be selected against and may undergo increased fitness and compatibility over time (Blair et al., 2001). Allopatric host addition results in the geographical range of the parasite being extended. Host addition being unsuccessful in sympatric snail species would also explain why no host sharing was observed at sites with sympatric *Melagraphia* and *Diloma* populations, such as Purakaunui Bay and Diamond Harbour.

In contrast to these cases of host sharing, the digeneans infecting *D. subrostrata* do appear to be host specific. Those trematodes from clades 2 and 3a infect only *D. subrostrata*, even though *M. aethiops* (and often other species) is also present in the same locations, and the infection rate of *D. subrostrata* can be fairly high (15% or more). Similarly, *D. subrostrata* is not infected by those trematodes that infect any of the other New Zealand trochids. Thus, in spite of the substantial degree of host microsympatry, the tremendous potential for first intermediate host sharing is not realised.

Our finding of morphological similarity masking substantial genetic differences among parasites calls into question the interpretation of studies that rely on parasite morphology to assign the degree of host specificity. For example, Stunkard (1983) found three common North American littorinid species (*L. littorea*, *Littorina obtusata* and *Littorina saxatilis*) could all be infected with *Cryptocotyle lingua* (family Heterophyidae). This parasite species was identified based on its morphology; further genetic analysis of *C. lingua* may reveal differences between populations infecting the three littorinid species. Without our genetic analysis, we would have concluded that one digenean species infected six snail species in two countries.

Our genetic analysis also shows unequivocally that *D. subrostrata* harbours two genetic species of morphologically cryptic digeneans, something previously unsuspected. Moreover, in at least two populations, those at Pounawea and Company Bay, both digenean taxa are present. Previous studies from various parts of the world (e.g. Lebour, 1911; Cable, 1956; Holliman, 1961; Abdul-Salam and Al-Khedery, 1992) have recorded several instances of different snail populations being infected by more than one morphological cercarial type, and Jousson et al. (2000) detected cryptic variation in adult digeneans in the definitive hosts, but our study is the first to discover cryptic cercarial variants in a single snail population.

The variation we found in cercarial infection rate among snail populations is fairly high (e.g. 0–17.5% for *D. subrostrata*), but the pattern of many populations being either uninfected or infected at low levels is typical. Over the past century several extensive surveys of the occurrence of larval digeneans in marine molluscs have concluded that the prevalence of infection is usually relatively low and can show great spatial heterogeneity (Mouritsen and Poulin, 2002; Poulin and Mouritsen, 2003). For example, out of a sample size of 250,000 *L. saxatilis* collected from the Welsh Coast, Popiel (as cited by Irwin, 1983) found only a single infected individual, whereas an infection rate of 9% was encountered for the same species collected in Ireland (Irwin, 1983). Our overall infection rate was low (<1%); this low rate concurs with other studies measuring cercarial infection rates in topshells (Trochidae). Abdul-Salam and Al-Khedery (1992), for instance, measured an overall infection rate of 0.2% in 1592 trochid snails, belonging to three species, collected in Kuwait Bay.

This low infection rate may mean that populations with low infection rates could be erroneously classified as uninfected. For example at Little Akaloa, four trochid species were sampled (approximately 300 *D. nigerrima*, *D. arida* and *D. zelandica* and 111 *D. bicanaliculata*). Of the more than 1000 individuals collected at this site, only a single individual of each of *D. nigerrima* and *D. arida* was found to be infected with cercariae. It would have been easy for us to miss either of these infections and, indeed, we may have failed to detect an actual infection in *D. zelandica* and *D. bicanaliculata*. Strictly, all that can be concluded from this site is that the parasites are capable of infecting more than one species of topshell.

Nevertheless, the level of larval digenean infestation is clearly affected by host ecology. In almost all cases, snail populations from softer shores were more likely to be infected. At Company Bay, a sheltered mudflat with more exposed rocks to one side, *D. subrostrata* inhabited the mudflat and were highly parasitised, whereas *M. aethiops* lived exclusively on the rocks and was not. At Purakaunui Bay, a sandy sheltered bay with a rock retaining wall built around the edge, *D. subrostrata* and *M. aethiops* were collected from the sheltered soft-sediment and were both parasitised (albeit with different parasites), whereas the infection-free *D. arida* lived further up the intertidal zone on seaweed attached to the rocks. Nevertheless, host ecology can only be part of the explanation: for instance, at Diamond Harbour, a sheltered rocky shore, there is no easily discernable difference in the habitats of *M. aethiops* (infected) and *D. zelandica* (uninfected), and yet the same digenean can potentially parasitise both species.

The observation that populations from soft-sediment beaches display a higher cercarial infection rate than those living on nearby rocky shores has previously been made by Abdul-Salam and Al-Khedery (1992). They argued that if soft-sediment beaches possess a richer flora and fauna than exposed rocky shores (Clayton, 1986), the definitive hosts would spend a greater proportion of their time in these areas, increasing the likelihood of the digeneans completing their life cycle. In a comparative study across several digenean–snail associations, Poulin and Mouritsen (2003) have found

that the higher prevalence of infection in soft-sediment intertidal habitats relative to rocky shores is a ubiquitous pattern.

In summary, we have shown that the trochid snails of New Zealand and Australia are infected by the cercarial stage of at least three (and probably four) morphologically cryptic but genetically separate digenean species. Two of these four parasite species appear to be host specific, both infecting *D. subrostrata*, and two are more general, infecting two and three snail species, respectively. Both the phylogenetic relatedness and habitat preference of the host species appear to affect the level of infection and the degree of parasite sharing. The results suggest that the usually narrow host specificity of digeneans for their first intermediate host can break down when ecological conditions favour host switches or host additions.

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