Broad geographic analyses reveal varying patterns of genetic diversity and host specificity among echinostome trematodes in New Zealand snails

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(*Received 7 May 2014; revised 16 June 2014; accepted 17 June 2014; first published online 26 August 2014*)

SUMMARY

Host specificity is a fundamental component of a parasite's life history. However, accurate assessments of host specificity, and the factors influencing it, can be obscured by parasite cryptic species complexes. We surveyed two congeneric species of intertidal snail intermediate hosts, *Zeacumantus subcarinatus* and *Zeacumantus lutulentus*, throughout New Zealand to identify the number of genetically distinct echinostome trematodes infecting them and determine the levels of snail host specificity among echinostomes. Two major echinostome clades were identified: a clade consisting of an unidentified species of the subfamily Himasthlinae and a clade consisting of five species of the genus *Acanthoparyphium*. All five *Acanthoparyphium* species were only found in a single snail species, four in *Z. subcarinatus* and one in *Z. lutulentus*. In contrast, the Himasthlinae gen. sp. was found in both hosts, but was more prevalent in *Z. lutulentus* (97 infections) than *Z. subcarinatus* (10 infections). At least two of the *Acanthoparyphium* spp. and the Himasthlinae gen. sp. are widespread throughout New Zealand, and can therefore encounter both snail species. Our results suggest that host specificity is determined by host–parasite incompatibilities, not geographic separation, and that it can evolve in different ways in closely related parasite lineages.

Key words: host specificity, trematode, echinostome, Zeacumantus, Acanthoparyphium, COI, ITS1.

INTRODUCTION

Host specificity is an essential component of parasite life histories and can incorporate the number and phylogenetic diversity of hosts that a parasite uses at specific life stages, as well as geographic differences in hosts used (Poulin and Keeney, 2008; Poulin *et al*. 2011). The extent of host specificity has direct bearing on parasite evolution and transmission dynamics as adding hosts can provide additional resources and colonization opportunities to the parasite while decreasing risks of extinction (Poulin and Keeney, 2008; Dunn *et al*. 2009; Poulin *et al*. 2011). The downside to relaxing host specificity is that a parasite can be less able to adapt to each individual host species and is exposed to increased competition from other parasite species (Poulin, 2007; Hayward, 2010). The ability of a parasite to exploit new hosts can be limited by physiological incompatibilities between parasites and potential hosts (Sapp and Loker, 2000; Locke *et al*. 2010), possibly resulting from evolved parasite specialization (Antonovics *et al*. 2013), and/or ecological/geographic separation decreasing the chances of parasites encountering hosts (Combes, 2001).

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The use of molecular data to reveal previously undescribed cryptic species of parasites has allowed for a more accurate determination of host specificity in many parasite taxa, as exemplified by the digenetic trematodes (Platyhelminthes: Trematoda) (Blasco-Costa *et al*. 2010; Detwiler *et al*. 2012; Curran *et al*. 2013). Digeneans typically utilize three hosts, including a molluscan first intermediate host and vertebrate definitive host, and morphologically distinct stages to complete their life cycles (Gibson and Bray, 1994; Cribb *et al*. 2003). Larval trematodes typically possess few morphological traits for identification, making it difficult to accurately assess the number and identity of parasite species infecting intermediate hosts (Poulin, 2011). Investigations into the genetic diversity of larval trematodes within intermediate hosts commonly reveal large numbers of previously undescribed species, altering interpretations of the number of parasite species a host is infected by and patterns of host specificity of the parasites (Donald *et al*. 2004; Miura *et al*. 2005; Leung *et al*. 2009*b*; Detwiler *et al*. 2010; Locke *et al*. 2010). The number of trematode genetic lineages within intermediate hosts must therefore be determined before assessments of host specificity can be made.

Our goals were to investigate the genetic diversity and host specificity of digeneans exploiting two congeneric gastropod hosts. *Zeacumantus subcarinatus* is a marine mud snail that is common in intertidal

Parasitology (2015), **142**, 406–415.
doi:10.1017/S0031182014001279

bays, rock pools and other coastal habitats throughout most of New Zealand (Morton and Miller, 1968; Morton, 2004). This species has been the subject of numerous parasitological investigations, and at least nine species of trematodes use *Z. subcarinatus* as a first intermediate host, including four 'cryptic' species of *Acanthoparyphium* echinostomes revealed using molecular data (Leung *et al*. 2009*a*, *b*). Echinostomes (Echinostomatidae) are a diverse group of digenetic trematodes characterized by a circumoral head collar consisting of one or two crowns of spines (Kostadinova, 2005). Many species are morphologically similar, and some are associated with animal diseases (Detwiler *et al*. 2010) and host manipulation (Leung *et al*. 2009*b*). The *Acanthoparyphium* echinostomes were identified from the Otago region of the South Island of New Zealand and their geographic distribution throughout New Zealand is currently not known. *Zeacumantus lutulentus* is a second species of mud snail that is also relatively common in New Zealand. *Zeacumantus lutulentus* is found on mid-tidal mud flats throughout the North Island and northern portion of the South Island of New Zealand, and can be found co-distributed or upshore from *Z. subcarinatus* in the same locations (Morton, 2004; Keeney *et al*. 2013). In contrast to *Z. subcarinatus*, the trematode assemblage utilizing this species as a first intermediate host is unknown. Since the trematodes utilizing *Z. lutulentus* have not been examined, the degree to which these two congeneric, and often sympatric, snails share trematode species, including echinostomes, is not known. As both species can be found in the same bays, they are likely exposed to similar trematodes in these areas, potentially increasing the likelihood that these phylogenetically related snails will share trematodes.

The present study examines the genetic diversity of echinostome trematodes in *Z. subcarinatus* and *Z. lutulentus* throughout the majority of their ranges in New Zealand. Our goals are to (1) quantify the genetic diversity and geographic distribution of echinostome trematodes infecting *Z. subcarinatus* and *Z. lutulentus* throughout New Zealand, (2) determine the levels of host specificity of echinostomes between these two snail hosts and (3) provide initial data to determine if geographic separation or host–parasite incompatibilities are responsible for observed host specificity patterns.

MATERIALS AND METHODS

A total of 45 sites were visited throughout New Zealand and 5590 *Z. subcarinatus* and 3298 *Z. lutulentus* were collected and examined for echinostomes between December 2006 and November 2007. Two to 849 (mean = 169 ± 179 S.D.) *Z. subcarinatus* and 9–311 (mean = 143 ± 73 S.D.) *Z. lutulentus* were examined from sites where they were recovered. Snails were kept in 2 L containers with water from the collection site until dissected. The maximum shell length of each snail was measured with vernier calipers to the nearest 0·1 mm (length data not used in this study), snails were dissected, and echinostome rediae were placed into 95% ethanol. We were not able to distinguish echinostome species prior to DNA sequencing. DNA was extracted from a single redia from each snail in 400 μL of 5% chelex containing 0·1 mg mL^{-1} proteinase K, incubated at 60 °C between 2 and 10 h, and heated to 100 °C for 8 min. An approximately 800 bp region of the COI gene was amplified using the primers JB3: 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ (Bowles *et al*. 1995) and CO1-R trema: 5′-CAACAAATCATGATGCAAAAGG-3′ (Miura *et al*. 2005). Polymerase chain reaction (PCR) reactions (50 μL) contained 1·5 μL of DNA extraction, 200 μM each dNTP, 1·5 mM $MgCl_2$, 0·5 μM each primer, 1 × Taq buffer and 1·25 units GoTaq DNA polymerase (Promega). PCR amplification consisted of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C and a final extension for 8 min at 72 °C. The ribosomal internal transcribed spacer region 1 (ITS1) was additionally amplified from a subset of trematodes representing all major COI clades. PCR conditions for the ITS1 region were as described for COI using 40 cycles with the primers BD1 5′-GTCGTAACAAGGTTTCCGTA-3′ and 4S 5′-TCTAGATGCGTTCGAARTGTCGATG-3′ (Bowles and McManus, 1993). COI and ITS1 PCR products were purified using QIAquick gel extraction and PCR purification kits (Qiagen), respectively. Purified PCR products were sequenced using PCR primers at the SUNY Upstate Medical University DNA core facility and the DNA Analysis Facility at Yale University.

DNA sequences were aligned using Clustal W (Thompson *et al*. 1994) as implemented in MEGA5 (Tamura *et al*. 2011). Bayesian phylogenetic analyses were conducted for COI and ITS1 separately using MrBayes 3·2 (Ronquist *et al*. 2012). Both analyses sampled across GTR model space during the Markov chain Monte Carlo (MCMC) analysis by setting nst = mixed and rates = gamma. MCMC searches utilized three heated chains and were run for 5 000 000 (COI) and 1 000 000 (ITS1) generations with sampling every 100 generations. Maximum likelihood analyses were also conducted for COI and ITS1 separately using Garli v.2.01 (Zwickl, 2006) with initial parameters set to run under the TPM2uf + I + G and TPM1uf models of sequence evolution for COI and ITS1, respectively, as selected by the Akaike information criterion (AIC) of jModelTest (Posada, 2008). For each gene, 1000 bootstrap replicates were conducted and a consensus tree was produced using SumTrees (Sukumaran and Holder, 2010). Phylogenetic trees were visualized using FigTree v1.3.1 (Rambaut, 2009). Outgroup sequences were *Fasciola hepatica* (GenBank accession

https://doi.org/10.1017/S0031182014001279 Published online by Cambridge University Press

AF216697.1) and *Fascioloides magna* (GenBank accession # EF534998.1) for COI and *F. hepatica* (GenBank accession # JF708029.1) and *Fasciola gigantica* (GenBank accession # KC476171.1) for ITS1. Sequences from known *Acanthoparyphium* species recovered from *Z. subcarinatus* were included in phylogenetic analyses for species identification. These included *Acanthoparyphium* sp. A (GenBank accession #s FJ765457.1 for COI and FJ396155.1 for ITS1), sp. B (GenBank accession #s FJ765461.1 for COI and FJ396146.1 for ITS1), sp. C (GenBank accession #s FJ765463.1 for COI and FJ396153.1 for ITS1) and sp. D (GenBank accession #s FJ765466.1 for COI and FJ396157.1 for ITS1). Uncorrected *p* distances were calculated within and among major clades using MEGA5 with all insertion/deletions treated as single nucleotide differences for ITS1.

Genetic population structure of *Acanthoparyphium* sp. A and Himasthlinae gen. sp. (see Results) COI haplotypes among all sample sites and between pairs of sample sites was examined using analysis of molecular variance (AMOVA) with Arlequin 3·5 (Excoffier *et al*. 2005). Insufficient sample sizes for the other *Acanthoparyphium* species prohibited useful inferences. AMOVA analyses for both species incorporated Tamura and Nei's (1993) model of sequence evolution and gamma-distributed substitution rate variation with shape parameter $\alpha = 0{\cdot}01$. This was the most appropriate model of sequence evolution available based on the AIC of jModelTest (Posada, 2008).

RESULTS

Genetic diversity of echinostomes within snails

Snails infected with echinostomes were successfully collected from 30 sites, including 17 sites with infected *Z. subcarinatus* and 16 sites with infected *Z. lutulentus* (Table 1; Fig. 1). A total of 3863 *Z. subcarinatus* and 2361 *Z. lutulentus* were examined from these sites. Total echinostome prevalence within sites was 0·0–25% in *Z. subcarinatus* and 0·0–21·5% in *Z. lutulentus* (Table 1). As all echinostomes were not sequenced and the analysis of a single redia from each host precluded identification of co-infections, individual species prevalences could not be determined accurately. A 778 bp fragment of the COI gene was sequenced from 204 echinostomes, producing 124 different haplotypes (GenBank accession #s KJ956252–KJ956375).

Bayesian analysis utilizing the COI gene produced two highly diverged clades; a relatively shallow unidentified echinostome clade and a clade consisting of *Acanthoparyphium* species (Fig. 2). The unidentified echinostome's COI sequences were 18·9–22·1% divergent from those of the *Acanthoparyphium* species (Table 2). Based on cercariae, we have relatively few morphological characters to classify this species, but spine count (31) in the collar around the oral sucker associates it with the marine genera *Curtuteria* and *Himasthla*, both of which belong to the subfamily Himasthlinae (Yamaguti, 1975). The cercariae most closely resemble *Himasthla* spp. in having two pairs of five angle spines, and possessing cystogenous cells with cytoplasm filled with bacilliform granules. COI sequences of this species differ from *Himasthla* spp. by 19·0–19·5% (Osamu Miura, unpublished data). NCBI BLASTN searches (Altschul *et al*. 1997) for COI returned a *Curtuteria australis* sequence as the most similar match with 82% maximum identity, but numerous species had maximum identities between 80 and 81%, overlapping with additional *C. australis* haplotypes. ITS1 comparisons were also inconclusive as the present species most closely matched several *C. australis* (ex: GenBank accession # FJ396159.1) with 92% maximum identity, several *Acanthoparyphium* species (ex: GenBank accession # FJ396157.1) with 88–89% maximum identity, and an Echinostomatidae sp. (GenBank accession # KC527820.1) with 88% maximum identity. Given the lack of both adult specimens and genetic similarities among existing echinostome sequences, it is prudent to maintain this species as an unclassified member of the subfamily Himasthlinae, and we refer to it throughout the manuscript as Himasthlinae gen. sp.

Members of the Himasthlinae gen. sp. clade ($n = 107$ trematodes) were recovered from both host species, but were more common in *Z. lutulentus* ($n = 97$ trematodes) than *Z. subcarinatus* ($n = 10$ trematodes). There was no evidence of lineage partitioning between host species and two identical haplotypes were recovered from both hosts. Within the *Acanthoparyphium* clade ($n = 97$ trematodes), five well-supported clades, representing the four previously identified species of *Acanthoparyphium* (spp. A–D), and an additional fifth species, referred to as species 'E' throughout the remainder of this manuscript, were recovered. None of the four species previously identified from *Z. subcarinatus* were recovered from *Z. lutulentus* while *Acanthoparyphium* species E was only recovered from *Z. lutulentus*. Relationships among the five *Acanthoparyphyium* species were not well resolved, with the exception of sp. C and D forming a clade (Fig. 2). With the exception of *Acanthoparyphium* species A and E forming a clade (poorly supported in the Bayesian tree), maximum likelihood analysis yielded a similar topology with slightly lower support for some of the relationships within the *Acanthoparyphium* clade and only Bayesian trees are presented with both Bayesian support and maximum likelihood bootstrap values (Fig. 2).

COI *p* distances were 0–2·3% within *Acanthoparyphium* species and 0–1·5% within the Himasthlinae gen. sp. In contrast, COI *p* distances were 9·3–14·8% between different *Acanthoparyphium*

https://doi.org/10.1017/S0031182014001279 Published online by Cambridge University Press

Table 1. Sample sites where echinostomes were recovered with total prevalence and species identified. Data presented are the number of snails dissected from each site (*n*), the total prevalence of echinostomes and the echinostome species identified using COI. The number of each species recovered from individual sites is listed in parentheses after the species name. The sums of species identified within sites do not match total prevalences as not all echinostomes were sequenced for identification

		Zeacumantus subcarinatus			*Zeacumantus lutulentus*		
Site	#	Snails (*n*)	Echino %Prevalence	Echino species (#)	Snails (*n*)	Echino %Prevalence	Echino species (#)
Takapuna Beach	1	80	3·8	A(3)	0	–	–
Waihi	2	0	–	–	126	4·8	Hsp(5)
Ohiwa	3	46	0·0	–	254	0·4	E(1)
Napier	4	105	0·0	–	185	1·6	Hsp(3)
Porirua	5	120	1·7	D(2)	173	0·0	–
Raglan	6	19	0·0	–	257	2·3	Hsp(6)
Port Motueka	7	73	1·4	Hsp(1)	0	–	–
Kina Beach	8	83	6·0	A(3), Hsp(1)	124	20·2	Hsp(16)
Marahau Inlet	9	0	–	–	141	11·3	E(3), Hsp(8)
Kaiteriteri Inlet	10	0	–	–	101	16·8	E(6), Hsp(6)
Tapu Bay	11	2	0·0	–	76	1·3	Hsp(1)
Mapua	12	0	–	–	148	13·5	Hsp(7)
Cable Bay	13	0	–	–	49	18·4	Hsp(8)
Maori Pa Beach	14	20	5·0	A(1)	108	15·7	Hsp(9)
Okiwi Bay	15	0	–	–	124	7·3	Hsp(9)
Ohauparuparu Bay	16	0	–	–	151	3·3	E(1), Hsp(4)
Havelock	17	0	–	–	110	0·9	Hsp(1)
Ngakuta Bay	18	8	25·0	Hsp(2)	130	21·5	Hsp(9)
Whatamango Bay	19	0	–	–	95	5·3	Hsp(5)
Waikawa Bay	20	9	22·2	A(2)	9	0·0	–
Christchurch	21	167	21·0	A(3), C(3), D(2), Hsp(4)	0	–	–
Blueskin Bay	22	220	9·1	A(6), Hsp(1)	0	–	–
Deborah Bay	23	471	6·6	A(6)	0	–	–
Sawyers Bay	24	332	5·4	A(8)	0	–	–
Andy Bay	25	228	2·2	A(4)	0	–	–
Company Bay	26	403	4·2	A(8)	0	–	–
Lower Portobello	27	151	6·6	A(7), C(1)	0	–	–
Otakau	28	849	0·8	A(7)	0	–	–
Papanui Inlet	29	331	9·1	A(2), B(1), C(8)	0	–	–
Bluff Harbour	30	146	13·0	A(7), D(2), Hsp(1)	0	–	–

species and 18·9–22·1% between *Acanthoparyphium* species and Himasthlinae gen. sp. (Table 2).

An approximately (depending on insertion/deletions) 541 bp sequence including portions of the ITS1 region and 5·8S rRNA was analysed from 46 trematodes representing the major clades identified with COI, producing 11 different sequences (GenBank accession #s KJ956376–KJ956386). Overall, Bayesian results were consistent with COI results. Trematodes from the Himasthlinae gen. sp. COI clade ($n = 16$) all possessed an identical ITS1 sequence that was divergent from the remaining sequences (Fig. 3). The remaining sequences formed a well-supported *Acanthoparyphium* clade. All previous COI species identifications were supported with ITS1. Two different sequences from *Acanthoparyphium* species E found in *Z. lutulentus* ($n = 6$ trematodes) formed a clade with sp. A (0·87/86 support), and did not form their own clade due to the minimal sequence divergence of ITS1 between species A and E. Maximum likelihood analysis produced a similar topology, but did not recover individual clades for haplotypes comprising species C and D, which were not well supported in the Bayesian tree (Fig. 3).

ITS1 *p* distances were 0–1·1% within *Acanthoparyphium* species and 0% within Himasthlinae gen. sp. In contrast, ITS1 *p* distances were 0·4–2·6% between different *Acanthoparyphium* species and 9·1–10·2% between *Acanthoparyphium* species and Himasthlinae gen. sp. (Table 2).

Significant genetic differentiation was not detected over all populations ($\Phi_{ST} = 0{\cdot}012$, $P = 0{\cdot}422$) or between any population pairs ($P > 0{\cdot}050$) for *Acanthoparyphium* sp. A. For Himasthlinae gen. sp., genetic differentiation was not detected over all populations ($\Phi_{ST} = 0{\cdot}022$, $P = 0{\cdot}296$), but was detected between 15 out of 171 population pairs ($P < 0{\cdot}050$), all but one of which included site 2 (Waihi; 6 comparisons) or site 15 (Okiwi Bay; 9 comparisons) (Table 3). These results should be viewed as preliminary given that the small sample

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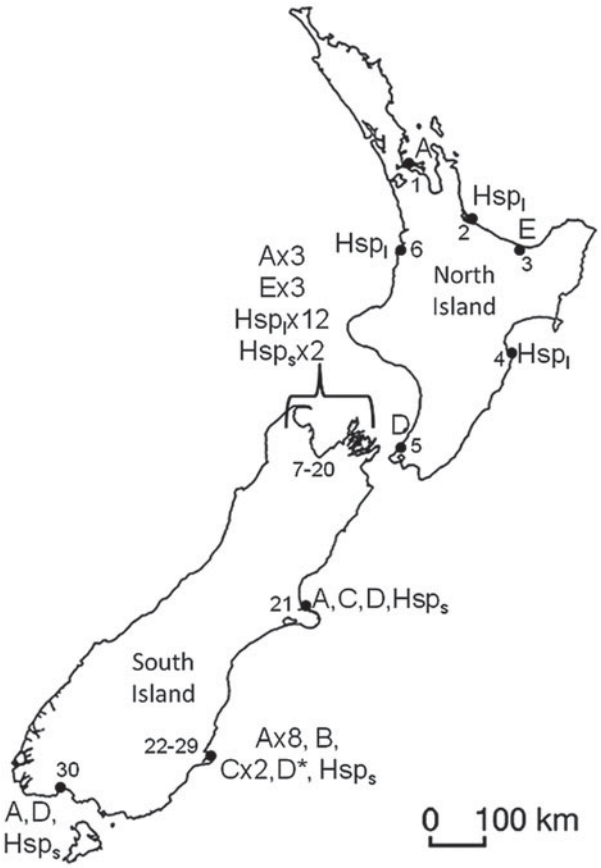

Fig. 1. Sample sites where echinostomes were recovered. Site numbers 1–30 follow Table 1. Species recovered from each site are identified as *Acanthoparyphium* sp. A–E (A–E) and Himasthlinae gen. sp. (Hsp). Subscripts for Hsp represent host species: l = *Zeacumantus lutulentus* and s = *Z. subcarinatus*. Numbers represent how many sites in that region the species was recovered from (e.g. x3 = 3 sites). * Denotes that the GenBank sequence was recovered from a site, but the species was not recovered there in the present study.

sizes in many sites for both species (Table 1) can decrease the likelihood of detecting existing genetic structure.

Geographic distributions of species

Acanthoparyphium sp. A was found in *Z. subcarinatus* throughout the South Island and from site 1 on the North Island (Table 1, Fig. 1), sp. B was recovered from *Z. subcarinatus* at site 29 on the South Island, sp. C was found in *Z. subcarinatus* from three sites on the South Island, sp. D was found in *Z. subcarinatus* from sites 21 and 30 on the South Island and site 5 on the North Island, and sp. E was recovered from *Z. lutulentus* from sites 9, 10 and 16 on the Northern South Island and site 3 on the North Island (Table 1, Fig. 1). Himasthlinae gen. sp. was common throughout *Z. lutulentus*' range (12 sites on the Northern South Island and sites 2, 4 and 6 on the North Island) and was found in *Z. subcarinatus* from five sites on the South Island (sites 7, 8, 21, 22 and 30) (Table 1, Fig. 1). Within *Z. subcarinatus*, this species was recovered from a single snail in each site, except for site 21 (Table 1).

DISCUSSION

This study is the first to examine the genetic diversity and distribution of echinostomes in New Zealand *Zeacumantus* species outside of the Otago region and the first to provide information regarding the specific trematode species parasitizing *Z. lutulentus*. We have identified five species of *Acanthoparyphium* utilizing *Zeacumantus* snails as first intermediate hosts throughout New Zealand. Four species (A–D) were previously identified from *Z. subcarinatus* in the Otago region (Leung *et al*. 2009*b*) and sampling throughout New Zealand did not increase that number. COI and ITS1 sequence divergences within and among these species in the present study support the presence of four species (Vilas *et al*. 2005) initially identified with 16S and ITS1 (Leung *et al*. 2009*b*). We recovered all of these species, except species B, outside of this area, with species A and D found in widespread locales. Given the relatively limited innate dispersal capability of free-living *Acanthoparyphium* cercariae, which can live 24–36 h outside of a host (Martorelli *et al*. 2006), their dispersal is likely dictated by the vagility of their hosts (Blasco-Costa and Poulin, 2013). As these species utilize cockles and nereid polychaetes (species B) for second intermediate hosts, and oystercatchers and potentially other shore birds for definitive hosts (Martorelli *et al*. 2006; Leung *et al*. 2009*a*; Peoples *et al*. 2012), it is likely that their species integrity is maintained over widespread distributions by the movements of bird definitive hosts (Blasco-Costa and Poulin, 2013).

While only *Acanthoparyphium* sp. A was examined, the lack of detectable genetic structure among populations also supports the movement of *Acanthoparyphium* via definitive hosts. This pattern is in contrast to the strong levels of genetic structure observed among populations of *Zeacumantus* species throughout New Zealand (Keeney *et al*. 2013). Occasional dispersal of infected snails could also contribute to these geographic patterns as both *Zeacumantus* species have complex patterns of genetic structure throughout New Zealand, with evidence of occasional long-distance dispersal (Keeney *et al*. 2013). Given the small sample sizes for many sites, we do not want to overstate the lack of genetic structure observed in *Acanthoparyphium* sp. A. *Acanthoparyphium* species B appears to have the most restricted distribution of the four species infecting *Z. subcarinatus*. Given its low prevalence (we recovered one infected snail), it is possible that the geographic distribution of species B is more widespread than we documented. It is interesting to

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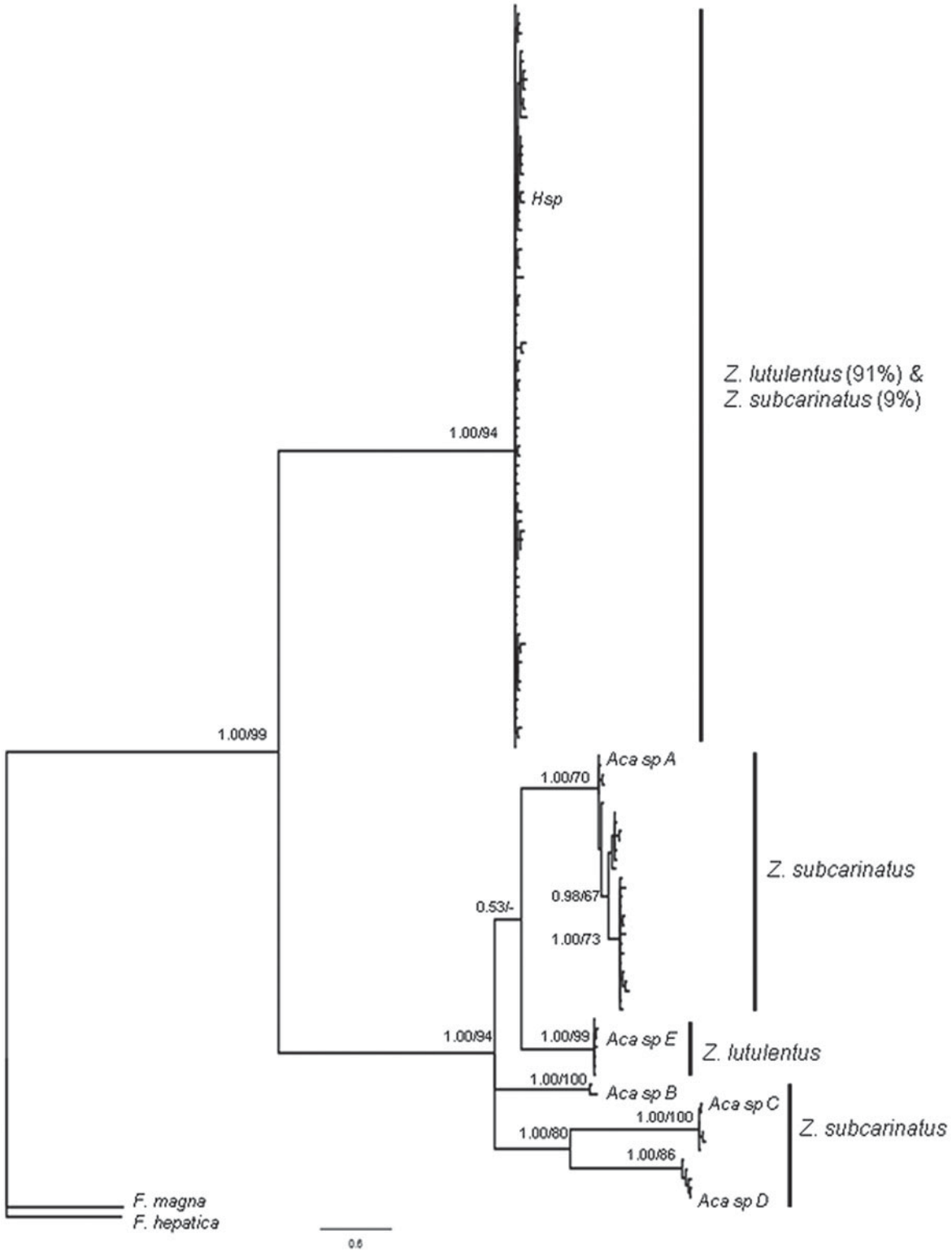

Fig. 2. Phylogenetic relationships of echinostomes based on COI sequences. Bayesian tree topology is shown with node support representing Bayesian support values/maximum likelihood bootstrap values. *Acanthoparyphium* species A–D GenBank sequences and representative sequences of *Acanthoparyphium* species E and Himasthlinae gen. sp. (Hsp) are labelled to provide species identification. The snail intermediate hosts from which major echinostome clades were recovered from are listed to the right of the tree.

note that this is the only species identified as using a nereid polychaete, *Perinereis* sp., as a second intermediate host, although this could be the result of accidental infections with bivalves being the typical hosts (Leung *et al*. 2009*a*; Peoples *et al*. 2012). How this could influence the species distribution is not clear if bird definitive hosts are also being utilized and polychaetes are widespread, but it could impact

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Table 2. Ranges of *p* distances within (diagonal) and between *Acanthoparyphium* (Aca) species A–E and Himasthlinae gen. sp. (Hsp) for COI and ITS1

COI	Aca sp. A	Aca sp. B	Aca sp. C	Aca sp. D	Aca sp. E	Hsp
Aca sp. A	0·0–2·3					
Aca sp. B	11·2–13·0	0·8				
Aca sp. C	13·7–14·8	13·8–14·4	0·0–0·7			
Aca sp. D	12·1–13·2	13·6–14·2	12·8–13·7	0·0–0·7		
Aca sp. E	9·3–10·4	10·9–11·4	12·6–13·6	12·8–13·7	0·0–0·5	
Hsp	18·9–21·0	20·3–22·1	20·5–21·5	20·3–21·4	19·7–21·0	0·0–1·5
ITS1	Aca sp A	Aca sp B	Aca sp C	Aca sp D	Aca sp E	Hsp
Aca sp. A	0·0–0·6					
Aca sp. B	0·7–1·3	0·2				
Aca sp. C	1·3–1·8	0·9–1·3	0·0–0·4			
Aca sp. D	1·3–2·6	0·9–2·0	0·4–1·5	0·0–1·1		
Aca sp. E	0·4–0·7	0·4–0·7	0·9–1·3	0·9–2·0	0·0–0·2	
Hsp	10·0–10·2	10·2	9·1–9·2	9·2–9·4	9·6–9·8	0·0

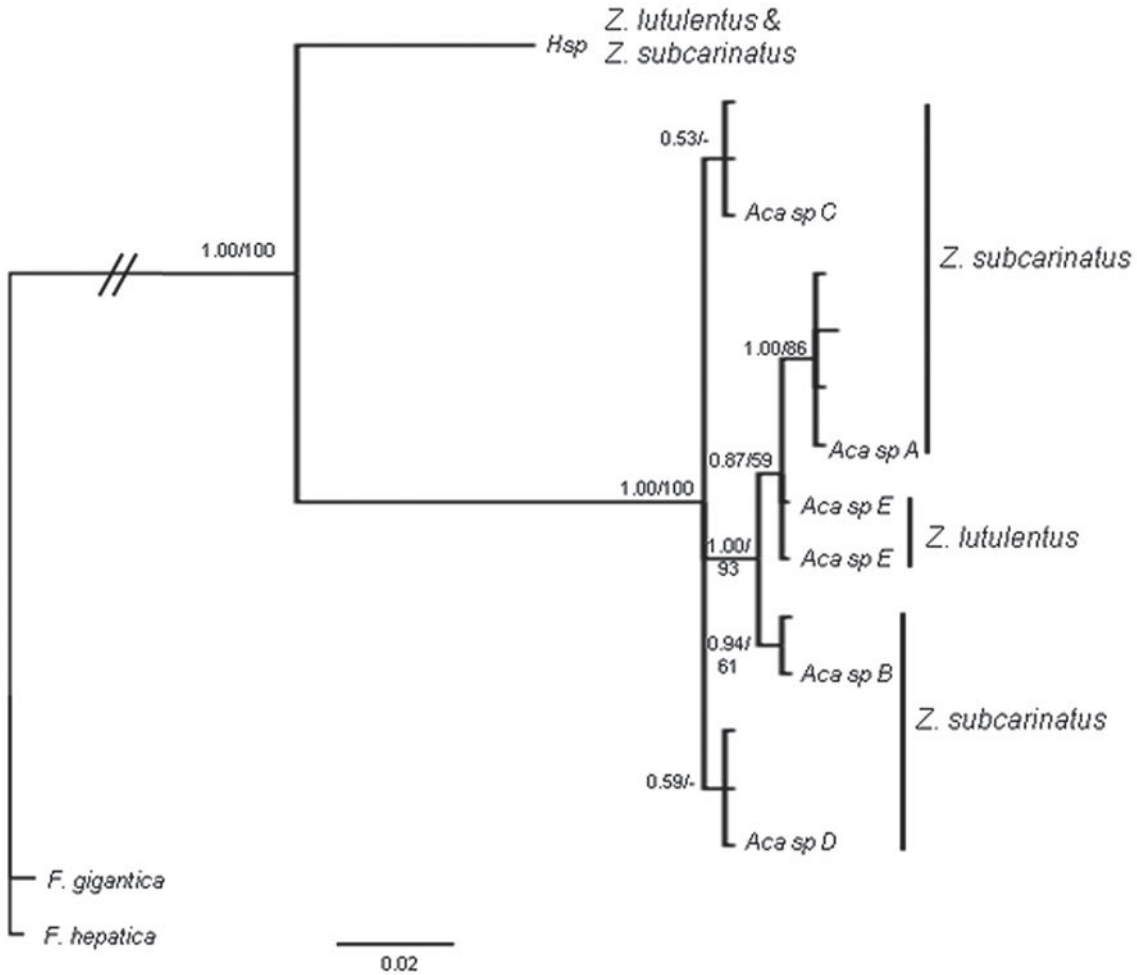

Fig. 3. Phylogenetic relationships of echinostomes based on ITS1 sequences. Bayesian tree topology is shown with node support representing Bayesian support values/maximum likelihood bootstrap values. *Acanthoparyphium* species A–D GenBank sequences and representative sequences of *Acanthoparyphium* species E and Himasthlinae gen. sp. (Hsp) are labelled to provide species identification. The snail intermediate hosts from which major echinostome clades were recovered from are listed to the right of the tree.

prevalence in snails if birds are more likely to be infected by species utilizing cockles as second intermediate hosts, such as species A, C and D (Leung *et al*. 2009*a*). A definitive identification of *Acanthoparyphium* species B's second intermediate host(s) is necessary before conclusive statements can be made regarding the mechanisms influencing its' distribution.

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Table 3. Himasthlinae gen. sp. pairwise Φ_{ST} values with $P < 0{\cdot}050$. Site numbers are the same as in Table 1

Sites compared	Φ_{ST}	*P* value
2 and 8	0·341	0·006
2 and 10	0·555	0·003
2 and 12	0·240	0·034
2 and 15	0·552	0·001
2 and 16	0·320	0·048
2 and 18	0·262	0·006
6 and 15	0·324	0·006
8 and 15	0·199	0·006
9 and 10	0·247	0·030
9 and 15	0·288	0·003
10 and 15	0·227	0·004
12 and 15	0·164	0·026
15 and 18	0·240	0·004
15 and 19	0·278	0·024
15 and 21	0·283	0·039

An additional species of *Acanthoparyphium* (species E) was only recovered from *Z. lutulentus*. Sequence divergences and phylogenetic analyses revealed that this species falls within the *Z. subcarinatus Acanthoparyphium* species complex. COI sequences for this species are least divergent from *Acanthoparyphium* species A (Table 2) and Bayesian phylogenetic analyses group these species together, although this relationship is not well supported given the levels of divergence among species (Figs. 2 and 3). Given its location within the *Z. subcarinatus Acanthoparyphium* species complex, this species may have arisen via speciation following successful host switching to *Z. lutulentus*. Host switching may occur when a species that is ecologically and physiologically similar to the host is encountered (Poulin, 2007). For speciation to occur, gene flow would have to have been interrupted between trematodes utilizing the new host and those utilizing *Z. subcarinatus*. While we can only speculate, this could involve alternative definitive hosts feeding primarily on *Z. lutulentus vs Z. subcarinatus*, resulting in reproduction primarily among *Acanthoparyphium* infecting *Z. lutulentus*. Also, these species may utilize different second intermediate hosts which would increase the potential for differential definitive host use. This could have initially arisen by the slightly different ecological conditions the two *Zeacumantus* species prefer in some areas, as *Z. lutulentus* can be found upshore in more silty environments (Morton, 2004) and *Z. subcarinatus* can often be found in sheltered rocky areas (Ozawa *et al*. 2009). Although thorough examinations have not been performed, different second intermediate hosts have been detected among *Acanthoparyphium* species A–D (Leung *et al*. 2009*b*). However it initially occurred, the strong host specificity for *Zeacumantus* observed among *Acanthoparyphium* species suggests that first intermediate host use could facilitate/maintain isolation with species evolving to infect *Z. lutulentus* potentially losing the ability to infect *Z. subcarinatus* (McCarthy, 1990; Detwiler *et al*. 2010). While experimental studies would be necessary to definitively document strict host specificity (Poulin and Keeney, 2008), the high number of each species of *Zeacumantus* examined provides support for the existence of strong host specificity for *Zeacumantus* hosts among New Zealand *Acanthoparyphium* species; not a single individual of *Acanthoparyphium* spp. A, C or D, was recovered from *Z. lutulentus* and no sp. E individuals were found in *Z. subcarinatus*.

A second species of echinostome, which we have referred to as Himasthlinae gen. sp. due to its morphological similarities to members of this subfamily and lack of genetic association to known genera, was common in *Z. lutulentus*. This species was found throughout *Z. lutulentus*' range on the northern portion of the South Island and the North Island and occurred at relatively high prevalences in sites where it was recovered. This species was also found in *Z. subcarinatus*, making it the only species recovered from both snails. Although it was found throughout *Z. subcarinatus*' range (Table 1, Fig. 1), it was less common in this host with 10 infections found in *Z. subcarinatus vs* 97 in *Z. lutulentus*. Genetically, all individuals are supported as a single species with maximum COI divergence of 1·5% and no differences detected among ITS1 sequences (Table 2) (Vilas *et al*. 2005). Two identical haplotypes were recovered from both snail species, revealing a lack of divergence between host species.

In general, digeneans exhibit the highest levels of host specificity for their molluscan first intermediate host (Pearson, 1972; Adamson and Caira, 1994), due to specific host-finding behaviours and/or immune response of non-host species (Sapp and Loker, 2000; Poulin, 2007). However, host specificity at this level can vary among even closely related echinostomes, revealing the importance of identifying cryptic species and host use before detailed studies of their biology and ecology can be conducted (Detwiler *et al*. 2010). We detected strong host specificity for snail first intermediate hosts among *Acanthoparyphium* species, some of which are widespread throughout New Zealand and were recovered from sites where both host species were present. Given that the relatively low prevalences of most species resulted in their recovery from only a few sites, we should not overstate that we have strong direct evidence that both hosts are exposed to the same echinostomes throughout New Zealand, as the majority of species A–D were recovered from sites without *Z. lutulentus* and the majority of species E were recovered from sites without *Z. subcarinatus* (Table 1). Species A provides the strongest evidence that host specificity persists despite exposure to both snail hosts. Therefore, host specificity appears to be driven by host–parasite incompatibilities. In addition, while

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Himasthlinae gen. sp. was rare within *Z. subcarinatus*, most specimens were recovered from sites without *Z. lutulentus*, further revealing that mobile and widespread avian definitive hosts are leading to exposure of both snails to similar trematodes.

As the only shared species, Himasthlinae gen. sp. provides a contrast to the pattern observed with *Acanthoparyphium*. Both clades are geographically widespread, with *Acanthoparyphium* species characterized by speciation and host specificity *vs* a lack of speciation and use of multiple hosts by Himasthlinae gen. sp. The utilization of multiple hosts at a particular parasite's life stage can occur when gene flow is maintained among parasite populations in both hosts following host speciation, host switching or the colonization of new host species (Brant and Orti, 2003; Poulin, 2007). While taxonomic uncertainty does not allow us to determine precisely why Himasthlinae gen. sp. differs in its host specificity compared to the *Acanthoparyphium* species, multiple mechanisms could be at work. Infections of *Z. subcarinatus* could be accidental, as only 10 infections were recovered. However, given that no 'accidental' *Acanthoparyphium* infections were found, this would still suggest decreased fidelity or incomplete evolution of host specificity compared to *Acanthoparyphium* spp. Although more common in allopatric species, utilization of *Z. subcarinatus* could represent a host addition to take advantage of a commonly encountered alternative host as parasites can display reduced fitness in the novel host (Blair *et al*. 2001; Donald *et al*. 2004). Given the relatedness and potential physiological similarities of the hosts, there may have been weaker selection against adding it when sympatric, or it may have initially occurred in regions lacking *Z. lutulentus*, such as the Southern South Island. While high levels of gene flow would reduce the ability of parasites to adapt locally, we have preliminary evidence that some populations of this species may be more isolated. Overall, our findings suggest different patterns of host specificity between two related clades of echinostome trematodes, and highlight the need for further detailed studies of model systems before any generalizations can be made about the evolution of specialization in parasites.

ACKNOWLEDGEMENTS

We are extremely grateful to Isa Blasco-Costa for her tremendous help with trematode identification, and thank Kim Bryan-Walker, Janet Koprivnikar, Deanna Clement and Tommy Leung for their assistance in collecting snails from the South Island, and Katherine, Jack and Henry Keeney for their help in collecting snails on the North Island, and Tania King for her assistance with molecular lab work. We are also grateful to Sean Locke, Osamu Miura and Ryan Hechinger for providing unpublished sequence data for comparison, and to Hilary McManus for assistance with Garli.

FINANCIAL SUPPORT

Funding was provided by a University of Otago Research Grant, the Royal Society of New Zealand's Marsden Fund, Le Moyne College's Student Research Committee and Le Moyne College's Research and Development Committee.

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https://doi.org/10.1017/S0031182014001279 Published online by Cambridge University Press

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