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Clonal diversity of the marine trematode *Maritrema novaezealandensis* within intermediate hosts: the molecular ecology of parasite life cycles

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Abstract

We quantified the clonal diversity of the New Zealand marine trematode *Maritrema novaezealandensis* (*n* = 1250) within *Zeacumantus subcarinatus* snail (*n* = 25) and *Macrophthalmus hirtipes* crab (*n* = 25) intermediate hosts using four to six microsatellite loci, and investigated the potential biological and physical factors responsible for the observed genetic patterns. Individual snails harboured one to five trematode genotypes and 48% of snails were infected by multiple parasite genotypes. Overall, the number of parasite genotypes did not increase with snail size, but was highest in intermediate-sized snails. Significantly larger numbers of parasite genotypes were detected in crabs (relative to snails; $P < 0.001$), with 16–25 genotypes recovered from individual crabs. Although crabs are typically infected by small numbers of cercariae sourced from many snails, they are occasionally infected by large numbers of cercariae sourced from single snails. The latter cases explain the significant genetic differentiation of trematode populations detected among their crab hosts ($F_{ST} = 0.009$, $P < 0.001$). Our results suggest that the timing of infection and/or intraspecific competition among parasite clones within snails determine(s) the diversity of parasite clones that snails harbour. The presence of a large number of infected snails and tidal mixing of cercariae prior to infection results in crabs potentially harbouring hundreds of parasite genotypes despite the crabs' territorial behaviour.

Keywords: clonal diversity, *Macrophthalmus*, *Maritrema novaezealandensis*, microsatellites, trematode, *Zeacumantus*

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Introduction

Genetic analysis represents a powerful method for studying the evolutionary ecology of host-parasite systems. Unlike the subjects of most population genetic analyses, parasite populations are further fragmented into distinct infrapopulations within hosts, with individual hosts harbouring a portion of the genetic diversity of the parasite population. An infrapopulation consists of all the conspecific parasites within an individual host at a particular time (Bush *et al*. 1997). Parasites interact intimately with conspecifics within the host, and their relatedness may influence their behaviour (Brown 1999; Parker *et al*. 2003). The distribution of parasite genetic clones within hosts can also have a major influence on the effective population size of parasite populations (Prugnolle *et al*. 2005a, b). Determining the genetic clonal diversity of parasites within and among hosts is therefore essential to understanding the forces driving parasite evolution. However, studies investigating the clonal diversity of parasite populations are greatly under-represented in the literature compared to studies involving free-living organisms (Criscione *et al*. 2005).

The subclass Digenea (flukes) contains more than 18 000 nominal trematode species that are found throughout marine, freshwater and terrestrial environments and parasitize an extremely wide range of invertebrate and vertebrate hosts (Gibson & Bray 1994; Olson *et al*. 2003). Due to their ubiquity and often large biomass, trematodes can greatly influence their ecosystems at the individual, population, and community level (Thomas *et al*. 1997; Poulin 1999; Mouritsen & Poulin 2002). Digenetic trematodes are characterized by

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complex life cycles involving one to four hosts, sexual and asexual reproduction and usually include free-living stages (Cribb *et al*. 2003). Asexual reproduction within first intermediate hosts often produces large numbers of genetically identical clones that subsequently infect second intermediate and/or definitive hosts. Recent theoretical studies (Prugnolle *et al*. 2005a, b) have demonstrated that clonal reproduction can create variance in the reproductive success of individual parasite genotypes that greatly affects the distribution of genetic variability within parasite populations. Despite the widespread distribution and importance to ecosystems of trematodes, and the influence clonal diversity can have on trematode evolution, few empirical studies have addressed the clonal diversity of trematodes within individual hosts. Previous studies have focused almost exclusively on medically important schistosomes within molluscan intermediate hosts and definitive hosts (see Jarne & Theron 2001 and references therein; Brouwer *et al*. 2001; Sire *et al*. 2001; Curtis *et al*. 2002; Theron *et al*. 2004; Shrivastava *et al*. 2005). Additional studies examined the diversity of the cervid parasite *Fascioloides magna* (Mulvey *et al*. 1991), eel parasite *Lecithochirium fusiforme* (Vilas *et al*. 2003), and salmon parasite *Plagioporus shawi* (Criscione & Blouin 2006) within and among definitive hosts. Although digenean life cycles are extremely diverse, trematodes commonly utilize two intermediate hosts and are intestinal parasites as adults (Cribb *et al*. 2003). To date, only one study has investigated trematode clonal diversity in first (snail) and second (fish) intermediate hosts (Rauch *et al*. 2005). This study was conducted with a freshwater trematode and information is currently lacking on marine trematodes.

Maritrema novaezealandensis (Microphallidae) is a marine trematode found in coastal waters of New Zealand. It utilizes shorebirds, such as the red-billed gull *Larus novaehollandiae scopulinus*, as definitive hosts (Martorelli *et al*. 2004). Sexual reproduction occurs within the definitive host's intestine and fertilized eggs are passed into the environment with the bird's faeces. Eggs are ingested by mud snail (*Zeacumantus subcarinatus*) first intermediate hosts while grazing on algae and diatoms in the sediment. Once ingested, the trematode migrates to the snail gonad where asexual reproduction produces hundreds of sporocysts within the snail, castrating the host in the process. Inside the sporocysts, free-swimming larval cercariae develop and are shed from snails. The cercariae infect crustaceans such as crabs (*Hemigrapsus crenulatus*, *Macrophthalmus hirtipes* and *Halicarcinus whitei*) and amphipods (*Paracalliope novizealandiae*). Within these second intermediate hosts, the cercaria sheds its tail, encysts and develops into a metacercaria. Several hundred metacercariae can exist in a single crab (Martorelli *et al*. 2004). The importance of *M. novaezealandensis* within its environment is well documented. This trematode is a cause of mortality in amphipod hosts (Fredensborg *et al*. 2004), and also castrates the first intermediate hosts, producing geographical variation in snail biomass and life history traits (Fredensborg *et al*. 2005; Fredensborg & Poulin 2006). *Maritrema novaezealandensis* is highly abundant in Lower Portobello Bay, South Island, New Zealand, reaching 73% prevalence in mature *Z. subcarinatus* and 100% prevalence in *M. hirtipes* (Fredensborg, unpublished).

The diversity of organisms potentially available as hosts, the influence of tidal cycles, and the abundance of *M. novaezealandensis* within Lower Portobello Bay provides an ideal environment to address several novel questions related to intraspecific parasite diversity within intermediate hosts. The prevalence of *M. novaezealandensis* at Lower Portobello Bay likely reflects high densities of snails coupled with an ongoing supply of trematode eggs from bird faeces (Fredensborg *et al*. 2006). Therefore, we hypothesize that the chances of snails encountering eggs of multiple parasite genotypes should be high. If snails accumulate parasite infections via grazing, a roughly linear increase in the number of parasite genotypes should occur with increasing snail size as older snails have consumed more parasite eggs. Although snail length does not scale linearly with age, *Z. subcarinatus* grow in length as they age (Fredensborg & Poulin 2006). However, since the parasites utilize a limited resource (snail gonadal tissue) for their reproduction, parasite genotype numbers may asymptote as snails become older.

Crab second intermediate hosts are predicted to possess a larger number of parasite clones (metacercariae) than snails (sporocysts), as crabs can be infected by cercariae from multiple snails. Two different patterns of infection are possible within crabs. Crabs may commonly be infected by large numbers of cercariae issued from the same snails as snails are slow moving and able to shed thousands of cercariae daily. Alternatively, tidal currents may help to mix cercariae populations from large numbers of snails prior to infection, resulting in crabs rarely being infected by multiple cercariae from the same snail. If crabs are commonly infected by large numbers of cercariae from the same snails, the clonal diversity of metacercariae should increase with crab size, as higher numbers of clones are infecting larger crabs. Numerous identical or related clones would produce strong genetic structure among crab hosts and increase the likelihood of parasite inbreeding within definitive hosts. If crabs are rarely infected by multiple cercariae from the same snail, clonal diversity should be high in all crabs with few identical parasite clones recovered within single crabs. Mixing of clones within crabs would decrease the degree of inbreeding among parasites during sexual reproduction within definitive hosts (Rauch *et al*. 2005).

In this study we use multiple microsatellite loci to identify genetic clones of *M. novaezealandensis* within

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Z. subcarinatus first intermediate hosts and *M. hirtipes* second intermediate hosts and utilize the parasite clonal diversity present within hosts to address the following questions: (i) Are multiclone infections within slow-moving snail intermediate hosts common given the high prevalence of *M. novaezealandensis* in the study area? (ii) Are larger (and therefore older) snails more likely to possess multiple parasite genotypes than smaller snails? (iii) Do crabs possess significantly higher numbers of parasite genotypes than snails? (iv) Do crabs contain multiple copies of the same genotype from relatively few snails or large numbers of parasite genotypes from many snails with few duplicate genotypes? (v) Do larger crabs possess more diverse assemblages of parasite genotypes than smaller crabs?

Materials and methods

Host collection and screening for Maritrema novaezealandensis

Approximately 300 *Zeacumantus subcarinatus* (snails) and 50 *Macrophthalmus hirtipes* (crabs) were haphazardly collected during low tides at Lower Portobello Bay, Otago Harbor, South Island, New Zealand (45°52′S, 107°42′E) from July to November 2005. Snails representing the range of collected sizes were screened for the presence of larval *Maritrema novaezealandensis* by incubating individuals in 60 mm Petri dishes containing seawater at 25 °C for 1–6 h under constant illumination. Snails shedding *M. novaezealandensis* cercariae were identified under a stereomicroscope and kept isolated for approximately 1 week before being dissected for genetic analysis. Only snails thought to possess *M. novaezealandensis* single-species infections were retained for this study. After genotyping, two snails were identified as possessing *M. novaezealandensis* and an additional morphologically cryptic microphallid species. These snails possessed one and five genotypes (the smallest and largest number of clones from snails possessing *M. novaezealandensis* single-species infections) and were retained in the study (additional sporocysts were collected from all dissected snails which allowed us to analyse 25 sporocysts from these two snails) as no obvious effect on *M. novaezealandensis* genotype diversity was observed. The maximum shell lengths of snails used for genetic analysis were measured with Vernier calipers to the nearest 0.1 mm (mean values are followed by standard deviation) and snails were dissected and placed in 60-mm Petri dishes containing 0.22-μm filtered fresh water (water). Snails of varying sizes were intentionally selected to investigate the effect of snail size on *M. novaezealandensis* clonal diversity. Using a stereomicroscope, individual sporocysts were chosen haphazardly and isolated with dissecting pins, rinsed twice in 60-mm Petri dishes containing water using a 200-μL pipette and placed into 1.5-mL tubes for DNA extraction.

Crab maximum carapace widths were measured using Vernier calipers, crab carapaces were removed and crabs were placed into 100-mm Petri dishes containing water. Using a stereomicroscope, individual metacercariae were isolated with dissecting pins, rinsed twice in water (as described for sporocysts) and placed into 1.5-mL tubes for DNA extraction. Numerous metacercariae spilled out of the crabs' body cavities during dissections and were collected loose from the Petri dish. In addition, metacercariae were occasionally removed from gill tissue: in one case, 22 metacercariae were sampled from the gills of a single crab.

DNA extraction and PCR amplification

DNA was extracted from larval *M. novaezealandensis* by placing individual parasites in 400 μL of 5% chelex containing 0.1 mg/mL proteinase K, incubating at 60 °C for 2 h and boiling at 100 °C for 8 min. The genotypes of four microsatellite loci (Mno-1, Mno-28, Mno-30 and Mno-47) were determined for 25 individual *M. novaezealandensis* sporocysts collected from each of 25 snails and 25 individual *M. novaezealandensis* metacercariae collected from each of 25 crabs (total of 1250 larval *M. novaezealandensis*) as described in Keeney *et al*. (2006) with the following changes: loci Mno-30 and Mno-47 were multiplexed in the same reaction with an annealing temperature of 62 °C and 40 amplification cycles were used for metacercariae with locus Mno-1. These loci were selected based on their levels of polymorphism and/or ability to be combined in multiplex reactions. All crab metacercariae with identical genotypes were genotyped at two additional loci (Mno-2 and Mno-45) as described in Keeney *et al*. (2006) after probabilities of clones resulting from sexual reproduction were calculated (see Data Analyses).

Data analyses

Microsatellite genotypes were determined manually. Identical multilocus genotypes were identified using the software package AGARST (Harley 2003). The probability that identical trematode genotypes occurred by chance via sexual reproduction and not through clonal reproduction was calculated using the program MLGSIM (Stenberg *et al*. 2003). The number of alleles per locus, expected and observed heterozygosities, deviations from Hardy–Weinberg expectations for each locus and across all loci, and tests of genotypic disequilibrium between all pairs of loci were calculated with GDA 1.1 (Lewis & Zaykin 2001). Alpha significance levels were corrected for multiple simultaneous pairwise comparisons using the sequential Bonferroni approach (Rice 1989). Only unique genotypes were used for Hardy–Weinberg and disequilibrium analyses as

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identical genotypes are the result of asexual reproduction within snail intermediate hosts. To examine parasite genetic heterogeneity among crab hosts, we calculated F_{ST} values (Weir & Cockerham 1984) among crabs for all genotypes and only unique genotypes using GENEPOP version 3.3 (Raymond & Rousset 1995a). Significance of genetic differentiation was calculated using the exact test of Raymond & Rousset (1995b) implemented in GENEPOP. Significance values were calculated by permuting both alleles (genic) to determine if allelic distributions differed among crabs and genotypes (genotypic) to determine if genotypic distributions differed among crabs. We also calculated F_{ST} between pooled unique genotypes from crabs vs. snails to see if genetic data supported hosts being infected by the same parasite population.

Additional statistical analyses were performed using the statistical package SPSS 13.0 for Windows. The strength of the relationship between number of parasite genotypes and host size was examined for snails and crabs using Spearman's rank-order correlation. The mean number of parasite genotypes from snails and crabs were compared using the Mann–Whitney U-test. In addition to the Spearman's test, we applied the statistical test developed by Mitchell-Olds & Shaw (1987) (MOS test) posthoc to determine if the relationship between the number of parasite genotypes and snail size was significantly unimodal. This test determines whether a model possessing an intermediate maximum is a better fit to the data than a model with a maximum value at higher or lower snail sizes (a model hypothesizing that intermediate snails possess more genotypes than small and/or large snails). This test specifically addresses intraspecific competition and therefore the two snails possessing multispecies infections were not included in this analysis. The MOS test was carried out using a program written in S-PLUS by David Mouillot (Université de Montpellier, France).

Results

Parasite clonal diversity within snails

Snail host-size ranged from 5.5 to 14.5 mm (mean = 11.1 ± 2.3). The genotypes of 625 *Maritrema novaezealandensis* sporocysts were determined for four loci (25 sporocysts from each of 25 snails). Total numbers of alleles and observed heterozygosities for each locus ranged from 6 to 21 and from 0.67 to 0.75, respectively (Table 1). Significant deviations from Hardy–Weinberg expectations were not detected at any loci ($P = 0.173$) and genotypic disequilibrium was not detected between any locus pairs ($P = 0.381$). No two snails possessed genetically identical trematodes, and nearly all (96%) the distinct genotypes were distinguishable at multiple loci. The probability that sporocysts with identical genotypes within snails were the product of sexual reproduction and not true genetic clones was low ($P < 0.002$ for any pair of sporocysts, $P < 0.0001$ for any three sporocysts, etc.). Individual snails were found to harbour between one and five of the 48 identified parasite genotypes (mean = 1.92 ± 1.26) (Table 2, Fig. 1). Twelve snails (48%) harboured more than one parasite genotype. There was no temporal element to the frequency of mixed-clone snail infections as all collections contained approximately 50% of snails with mixed-clone infections

Table 1 Summary of microsatellite data for each host species and the entire data set

		Microsatellite loci				
Host	Parameter	Mno-1	Mno-28	Mno-30	Mno-47	All loci
Snails	Alleles	21	9	6	8	44
	H_O	0.69	0.75	0.67	0.67	0.69
	H_E	0.76	0.78	0.68	0.67	0.72
	F_{IS}	0.10	0.03	0.03	0.01	0.04
Crabs	Alleles	44	12	10	11	77
	H_O	0.78	0.75	0.65	0.61	0.70
	H_E	0.81	0.76	0.68	0.61	0.71
	F_{IS}	0.04	0.02	0.04	0.00	0.02
Total	Alleles	44	13	10	11	78
	H_O	0.77	0.75	0.65	0.61	0.70
	H_E	0.81	0.76	0.68	0.62	0.72
	F_{IS}	0.04	0.02	0.04	0.00	0.03

Parameters are as follows: alleles, number of microsatellite alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient (positive values, heterozygote deficiency; no values deviate significantly from Hardy–Weinberg expectations).

Table 2 Frequencies of *Maritrema novaezealandensis* sporocysts belonging to the different clones recovered from *Zeacumantus subcarinatus* harbouring mixed-clone infections

Snail	Size (mm)	# Clones	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
1	9.2	2	13	12	—	—	—
2	10.9	2	17	8	—	—	—
3	11.9	2	17	8	—	—	—
4	12.5	2	13	12	—	—	—
5	12.6	2	14	11	—	—	—
6	13.1	2	23	2	—	—	—
7	10.1	3	15	8	2	—	—
8	10.6	3	22	2	1	—	—
9	12.4	3	10	8	7	—	—
10	12.1	4	10	8	5	2	—
11	11.1	5	18	3	2	1	1
12	13.3	5	15	6	2	1	1

Clones are listed in order of decreasing frequency within each snail.

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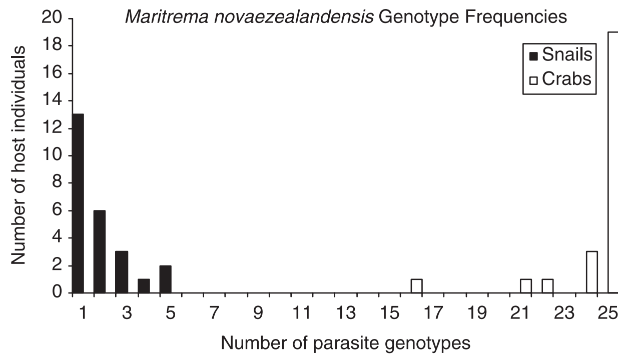

Fig. 1 Frequency of *Maritrema novaezealandensis* genotype counts found within *Zeacumantus subcarinatus* (snails) and *Macrophthalmus hirtipes* (crabs).

(data not shown). Single-clone infections were found at all snail sizes and included the five smallest snails and four largest, whereas multiclone infections were only found in intermediate-sized snails (Fig. 2). Within individual snails, the 25 sporocysts genotyped were not distributed equally among clones; multiclone infections often possessed relatively rare clones (Table 2). No significant linear relationship was detected between snail size and number of parasite genotypes ($r_S = 0.191$, $P = 0.361$). A unimodal model with an intermediate maximum gave a significantly better fit to our data than a model with a monotonic increase or decrease [MOS test: $t_{21} = 2.173$, $P < 0.05$ (Fig. 2)].

Parasite clonal diversity within crabs

Crab host size ranged from 15.5 to 22.0 mm (mean = 18.8 ± 1.6). The genotypes of 625 metacerariae were determined for four loci (25 metacercariae from each of 25 crabs). Number of alleles and observed heterozygosity for each locus ranged from 10 to 44 and 0.61–0.78, respectively (Table 1). Significant deviations from Hardy–Weinberg expectations were not detected at any locus ($P = 0.158$) and genotypic disequilibrium between loci Mno-28 and Mno-30 was nominally significant ($P = 0.036$), but not significant after sequential Bonferroni adjustment of alpha (initial $\alpha = 0.008$). Pooling genotypic data from both snail and crab infections yielded 10–44 alleles per locus and observed heterozygosities of 0.61–0.77 (Table 1). No significant deviations from Hardy–Weinberg expectations were detected ($P = 0.260$) and genotypic disequilibrium was not detected between any locus pairs ($P = 0.061$ for Mno-28 and Mno-30). Several metacercariae had high probabilities ($P > 0.05$) that their identical genotypes were the chance product of sexual reproduction and that they were not true genetic clones. After determining the genotypes for all genetically identical metacercariae at two additional loci, the probability that metacercariae with identical genotypes were not true genetic clones was low ($P < 0.001$). Six hundred and seven

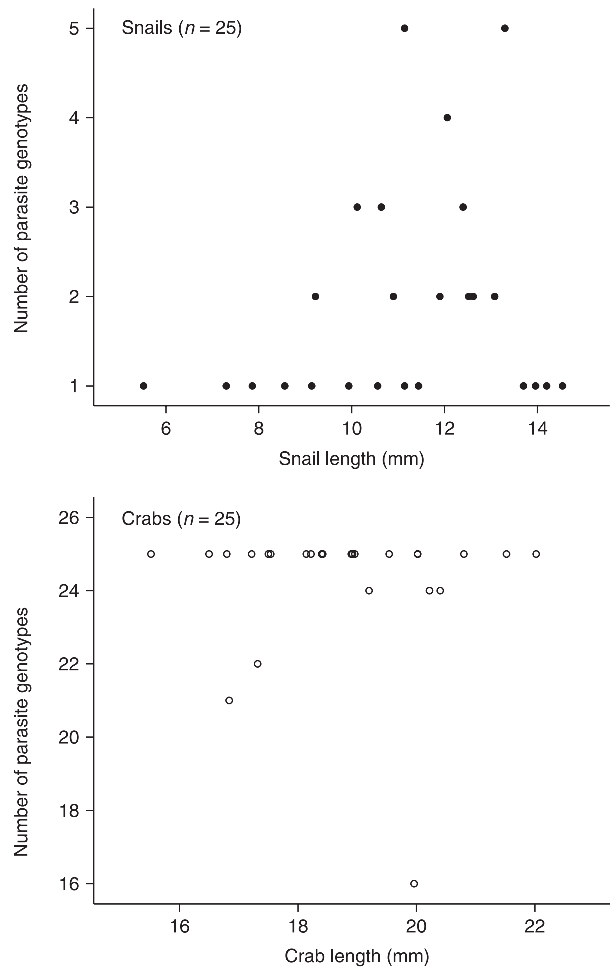

Fig. 2 Number of *Maritrema novaezealandensis* genotypes found within individual hosts vs. host size for *Zeacumantus subcarinatus* (snails) and *Macrophthalmus hirtipes* (crabs).

M. novaezealandensis genotypes were identified within crabs, and each crab contained between 16 and 25 parasite genotypes (mean = 24.2 ± 2.0) (Fig. 1). The mean number of parasite genotypes recovered from crabs was significantly higher than the number recovered from snails (Mann–Whitney *U*-test, $P < 0.001$). Six (24%) of the crabs possessed multiple copies of particular genotypes [three possessed a single replicate, one possessed four pairs, one possessed one pair and three of the same genotypes, and one crab possessed 10 copies of the same genotype (all from gill infections)]. There was no temporal element to the frequency of identical parasite genotypes within crabs as all collections contained crabs with predominately 25 genotypes (data not shown). No two crabs possessed genetically identical trematodes, and no identical trematode genotypes were recovered from both snails and crabs. No significant relationship was detected between crab size and number of parasite genotypes ($r_S = -0.080$, $P = 0.703$) (Fig. 2).

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Parasite genetic structure between hosts and among crabs

Genetic structure was not detected among unique genotypes pooled from crabs vs. snails ($F_{ST} < 0.001$, genic $P = 0.218$, genotypic $P = 0.275$), indicating parasite sampling from different intermediate hosts was from the same parasite gene pool. Genetic structure was not significant among unique genotypes from different crabs ($F_{ST} = 0.004$, genic $P = 0.069$, genotypic $P = 0.278$), reflecting that different crabs are not commonly recruiting different groups of related parasites. When all identical trematode genotypes are included, parasite genetic structure exists among crabs due to infections of multiple genetically identical cercariae from individual snails [$F_{ST} = 0.009$, genic $P < 0.001$, genotypic $P < 0.001$ ($F_{ST} = 0.005$, genic $P = 0.003$, genotypic $P = 0.114$ when the crab containing 10 copies of the same genotype was removed as it was likely to inflate overall estimates of genetic differentiation by being genetically distinct from all other crabs)].

Discussion

Factors influencing parasite diversity in snail first intermediate hosts

Approximately half of the snails examined from Lower Portobello Bay contained mixed-clone infections. Our estimates of the clonal diversity of *Maritrema novaezealandensis* within snails likely reflect the true diversity as no new genotypes were detected within snails once 20 sporocysts had been sampled. However, we cannot rule out that low-copy parasite genotypes within snails are occasionally missed. In our sample, small snails (< 9.2 mm, $n = 5$) possessed a single *M. novaezealandensis* genotype. These snails may not have had the chance to ingest multiple parasite eggs and therefore only one parasite genotype has established. The largest snails (13.7–14.5 mm, $n = 4$) also possessed a single genotype indicating that snails do not necessarily continue to accumulate parasite infections throughout their lives. If snails are ingesting parasite eggs while grazing on sediments, a positive relationship would be expected between snail size (and therefore age) and number of parasite genotypes. This relationship was not detected in this study and multiclone infections were present solely in intermediate-sized snails. Although we did not examine a large number of snails, the lack of multiple clone infections in large and small snails indicates that multiple parasite genotypes are less frequent in these snail size classes.

Several nonexclusive scenarios could explain our results. First, there may be a limited time for additional *M. novaezealandensis* miracidia to infect a snail once an initial parasite has become established. *Maritrema novaezealandensis* utilizes snail gonadal tissue to produce sporocysts and castrates the snail host in the process. Once all or most of the gonadal tissue has been used, it may be difficult for other conspecific parasites to establish. Although we do not know the longevity of individual *M. novaezealandensis* infections and therefore cannot rule out that single-clone infections in larger snails are the results of recent infections, marine trematode infections can persist for many years (Sousa 1983, 1993; Curtis 2003) and multiple infections would be as likely at large snail sizes as intermediate sizes if new infections were common in larger snails.

Second, if two *M. novaezealandensis* genotypes enter the snail while host gonadal tissues are available, there may be resource competition with genotypes that either enter slightly earlier or are better at utilizing host tissues eventually excluding other genotypes from the host. Depending on the number of successful sporocysts each genotype is able to establish, low copies of one genotype may co-exist with the dominant genotype for a limited time, eventually leaving only one genotype. This could explain the disparity in the proportion of each clone within snails (Table 2). A mechanism for intraspecific competition is not clear-cut, such as the consumption of sporocysts by rediae in some interspecific scenarios (Esch *et al*. 2001), but indirect antagonism and chemicals have been proposed as mechanisms in competitive heterospecific infections involving sporocysts (see Sousa 1993 and references therein). The study of interspecific competition among co-occurring trematodes within snail hosts has a long history and its importance to infracommunity (all infrapopulations within a host) structure varies greatly among systems and scales of observation (Kuris & Lafferty 1994; Esch *et al*. 2001). Intraspecific competition among trematodes within first intermediate hosts is difficult to investigate empirically and has received relatively little attention. Intraspecific competition has been proposed as a regulatory mechanism allowing mixed-species infections to persist within hosts by limiting the numbers of one species in nematodes (Adamson & Noble 1993) and possibly larval trematodes within snail hosts (Hendrickson & Curtis 2002). Gower & Webster (2005) demonstrated intraspecific competition and differences in competitive success between laboratory strains of *Schistosoma mansoni*. The prevalence of *M. novaezealandensis* and percentage of mixed-clone infections at Lower Portobello Bay may make intraspecific competition more likely to develop within this species. Intraspecific density-dependent effects in second intermediate hosts have previously been shown to affect adult parasite size and egg-production in *M. novaezealandensis* (Fredensborg & Poulin 2005). Given the range of *M. novaezealandensis* prevalence among different sites within Otago Harbor (Fredensborg & Poulin 2006) high levels of gene flow throughout these areas would weaken the power of natural selection to favour competitive clones if mixed-clone infections are rare in other sites.

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A third possibility is that snails with multiclone infections either grow more slowly or have higher mortality than snails with single-clone infections, perhaps due to increased virulence of multiple parasite strains (Davies *et al*. 2002). Therefore, the larger snails with single-clone infections may represent the same cohort as smaller snails harbouring multiple clones and fewer snails harbouring multiple parasite clones may live to attain a large size. Although most classical models predict that the co-occurrence of multiple parasite genetic strains within a host will lead to increased virulence (Van Baalen & Sabelis 1995; Frank 1996), several studies suggest that virulence can increase or decrease with parasite relatedness based on the importance of individual vs. group parasite exploitation (Turner & Chao 1999; Brown *et al*. 2002). Little empirical information regarding virulence evolution in trematode-snail systems is currently available and data on natural infections are lacking. The only study to investigate the effects of trematode clonal diversity on host mortality involved experimental infections of laboratory strains of *S. mansoni* and its snail host *Biomphalaria glabrata* (Davies *et al*. 2002). In that study, multiple genotype infections were more virulent than single genotype infections as measured by host reproductive success and survival. However, Gower & Webster (2005) suggest that a high frequency of multiple infections (as is the case in our study) would favour the evolution of reduced virulence.

Factors influencing parasite diversity in crab second intermediate hosts

Crab second intermediate hosts from Lower Portobello Bay possessed few identical parasite genotypes. Our sampling within crabs was not exhaustive as new genotypes were identified with almost every metacercariae examined regardless of crab size. Crabs are likely to contain hundreds of parasite genotypes with low numbers of identical genotypes. The large number of snails and high prevalence of *M. novaezealandensis* within snails produce a large pool of genotypes from which crabs can be infected. The low number of parasite genotypes per snail indicates that crabs are commonly sampling cercariae emitted from many (potentially hundreds) of snails. This likely begins when crabs are young, as small crabs possessed many parasite genotypes. Occasionally, crabs are infected with large numbers of the same parasite genotype (one crab possessed 10 identical genotypes out of 25 metacercariae sampled). This likely results from a snail shedding large numbers of cercariae next to a crab and the successful establishment of many of the parasites within the crab before currents can displace the cercariae. This occurred in the one crab with most metacercariae sampled from the gills and may indicate that the cercariae were drawn into the crab's branchial chamber by respiratory currents as described by Saville & Irwin (2005) for *Microphallus primas* within *Carcinus maenas*. Our data support the mixing of mobile cercariae within the water being common at this site and each crab is typically sampling parasites from hundreds of snails. This is the first investigation into parasite clonal diversity within intermediate hosts in a coastal system and tidal currents are likely playing a role in mixing clones as cercarial mobility alone might produce more crabs with higher numbers of identical parasite clones.

A significant increase in parasite genotypes occurs between snail first intermediate hosts and crab second intermediate hosts. The mixing of parasite genotypes within the second intermediate host is consistent with the findings of Rauch *et al*. (2005) and predictions of Criscione & Blouin (2006). In the lacustrine system studied by Rauch *et al*. (2005), the mobility of the fish second intermediate host ensured that numerous parasite genotypes would be sampled along the shoreline; in our system, tidal currents and the large number of infected snails are responsible for the mixing of many parasite genotypes, as the crab hosts are territorial and remain within the vicinity of their burrow (Williams *et al*. 1985). Criscione & Blouin (2006) predicted that trematodes primarily utilizing aquatic transmission routes and multiple intermediate hosts may experience more genotype mixing before entering definitive hosts than trematodes that use fewer hosts and terrestrial transmission. Our system satisfies their requirements for well-mixed trematode populations within intermediate hosts and our data reflect high levels of trematode clonal mixing prior to infection of definitive hosts. The large number of parasite clones and few identical clones within crabs would reduce the chances of parasite inbreeding within definitive hosts (shorebirds) as parasites possessing identical genotypes would rarely encounter each other in the definitive host's gut. Excess homozygosity within parasites (significant F_{IS} values) was not detected in this study, indicating that this population of *M. novaezealandensis* is not severely inbred. This increased clonal diversity could be one of the advantages of maintaining second intermediate hosts as proposed by Rauch *et al*. (2005). The small number of identical clones within crabs indicates there is relatively low variation in the success of individual parasite clones to infect second intermediate hosts. High variance in clonal reproductive success can decrease the genetic diversity and effective population size of a parasite population (Prugnolle *et al*. 2005a, b). Criscione & Blouin (2006) detected effectively no variance in clonal reproductive success within definitive hosts of the aquatic trematode *Plagioporus shawi*. Our study extends previous findings on freshwater systems to include second intermediate hosts in marine coastal environments. Some variation does exist within second intermediate hosts in our system as low (but statistically significant) levels of genetic structure were detected among crabs as a result of multiple infections

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from the same snail (largely due to one crab with 10 identical clones). A further level of clone mixing in definitive hosts would likely homogenize the already low genetic structure we detected and produce low levels of variance in reproductive success within definitive hosts similar to those detected by Criscione & Blouin (2006).

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