Diversity of trematode genetic clones within amphipods and the timing of same-clone infections

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Abstract

The genetic diversity of trematodes within second intermediate hosts has important implications for the evolution of trematode populations as these hosts are utilized after the parasites reproduce asexually within first intermediate hosts and before sexual reproduction within definitive hosts. We characterised the genetic clonal diversity of the marine trematode Maritrema novaezealandensis within amphipod (Paracalliope novizealandiae) second intermediate hosts using four to six microsatellite loci to determine if multiple copies of identical trematode clones existed within naturally infected amphipods. To determine the relative timing of infections by identical clones within hosts, trematode metacercariae were assigned to six developmental stages and the stages of identical clones were compared. The genotypes of 306 trematodes were determined from 44 amphipods each containing more than one trematode. Six pairs of identical trematode clones were recovered in total (representing five amphipods: 11% of amphipods with greater than one trematode) and all pairs of clones belonged to the same developmental stage. This suggests that identical clone infections are effectively synchronous. A general decrease in the number of metacercariae recovered, prevalence, and mean intensity of infection for each subsequent developmental stage coupled with large numbers of metacercariae (>9) only being recovered from recent infections, supports the occurrence of post-infection amphipod mortality and/or within-host trematode mortality. Taken together, our results indicate that natural infections are characterised by high genetic diversity, but that amphipods also periodically encounter “batches” of genetically identical clones, potentially setting the stage for interactions within and between clonal groups inside the host.

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1. Introduction

Many parasite life cycles have evolved to incorporate multiple host species (Poulin, 1998; Parker et al., 2003a). Digenetic trematodes (Class Trematoda: Subclass Digenaea), for instance, typically utilize three hosts and multiple developmental stages to complete their life cycles (Kearn, 1998; Poulin and Cribb, 2002; Cribb et al., 2003). These digenecas infect second intermediate hosts after undergoing asexual reproduction within molluscan first intermediate hosts and before sexual reproduction within definitive hosts. Within the second intermediate host, trematode cercariae shed their tail, increase in size to varying degrees (Poulin and Latham, 2003), and encyst to form mature metacercariae until consumption of the second intermediate host by the definitive host. Since the metacercarial stage serves as an important bridge between asexual and sexual reproduction and multiple conspecific metacercariae often exist within the same second intermediate host, the genetic diversity of the metacercariae within hosts may be extremely important to the evolution of trematode populations.

Identifying the genetic diversity of trematodes within second intermediate hosts is essential to understanding the processes driving the evolution of trematode populations. The genetic diversity of trematodes within second intermediate hosts is predicted to reflect the number of first intermediate hosts providing cercariae as well as the genetic diversity of parasites within each first intermediate host. Variance in the number of cercariae received from each
first intermediate host will affect trematode diversity in the second intermediate host and can be influenced by host mobility and the physical environment (terrestrial vs. aquatic) during transmission (Criscione and Blouin, 2006; Keeney et al., 2006a). Differences in the infection success of trematode clones can lead to variation in reproductive success among trematode clones in the definitive host, lowering trematode effective population sizes (Prugnolle et al., 2005a,b; Criscione and Blouin, 2006) and influencing how inbreeding, genetic drift, natural selection and gene flow affect trematode populations (Criscione and Blouin, 2005, 2006; Rauch et al., 2005). The presence of identical clones within hosts may also influence the evolution of trematode life history strategies in terms of kin selection (including their ability to manipulate hosts) and within-host competition (density-dependent effects and optimal growth strategies). Many parasites are able to alter their intermediate host’s phenotype and/or behaviour to facilitate transmission to definitive hosts via predation (Poulin and Thomas, 1999; Poulin, 2000), including trematodes within amphipods (Helluy, 1984; McCurdy et al., 2000), and theoretical models predict that host manipulation will increase with parasite relatedness (Brown, 1999). Also, density-dependent reductions in metacercarial growth have been detected within intermediate hosts (Sandland and Goater, 2000; Brown et al., 2003) and a mathematical model predicts that these effects will be a function of the relatedness of parasites sharing the host (Parker et al., 2003b). Although empirical evidence is lacking to examine the effects of relatedness on trematode growth strategies, studies have demonstrated that intraspecific competition can develop among different laboratory strains of Schistosoma mansoni within Biomphalaria glabrata snail hosts (Davies et al., 2002; Gower and Webster, 2005).

A fundamental question in trematode ecology concerns the relatedness of conspecific parasites sharing second intermediate hosts. As recipients of the products of asexual reproduction, it is possible that numerous identical trematode genetic clones are passed on to second intermediate hosts in “batches”. However, the extent of identical clone sharing may vary among different trematode populations and environments (Criscione and Blouin, 2006). It is necessary to empirically determine the frequency of identical conspecific trematodes sharing hosts within a population before the importance of genetic relatedness on trematode life history strategies can be examined. To date, only two studies have investigated the clonal diversity of trematode metacercariae within second intermediate hosts (Rauch et al., 2005; Keeney et al., 2006a). Rauch et al. (2005) detected few identical clones within fish second intermediate hosts in their lacustrine system and proposed that the mobility of fish enabled them to sample a diversity of trematodes from numerous first intermediate hosts. Keeney et al. (2006a) detected two contrasting patterns in a coastal system with crab second intermediate hosts. While the vast majority of crabs contained few identical clones (although only a subset of metacercariae where analysed per crab), one crab contained 10 identical trematode clones out of 25 analysed. Keeney et al. (2006a) hypothesised that the high density of infected snails coupled with tidal currents dispersing cercariae produced the diversity of trematode clones observed within crabs. Only occasionally do individual snails infect single crabs with large numbers of genetically identical cercariae, perhaps due to their proximity to a crab during cercarial emission.

The marine trematode Maritrema novaezealandensis (Microphallidae) inhabits coastal ecosystems on the South Island of New Zealand. It uses the snail Zeacumantus subcarinatus as a first intermediate host, several crustaceans as second intermediate hosts (including the crab Macrophthalmus hirtipes and amphipod Paracalliope novizealandiae), and shorebirds as definitive hosts (Martorelli et al., 2004). Parasite transmission patterns may differ between these two alternate second intermediate hosts. During low tides, high concentrations of both Z. subcarinatus and P. novizealandiae can be found in shallow tide pools. Within these shallow tide pools, warm temperatures can induce cercarial shedding from Z. subcarinatus (Fredensborg et al., 2004) and cercariae may potentially infect amphipods sharing tide pools in bursts, with many identical cercariae infecting an amphipod in a short period. Infected Z. subcarinatus in this area possess an average of two M. novaezealandensis genotypes and approximately half of the infected snails possess only one genotype (Keeney et al., 2006a). Thus, the genetic diversity of M. novaezealandensis within second intermediate hosts approximately reflects the number of snails from which cercariae were received. Keeney et al. (2006a) detected few identical clones within the crab alternative second intermediate host, M. hirtipes, which retreats to burrows in muddy sediment or their vicinity during low tides (Nye, 1974; Williams et al., 1985). Cercariae may be dispersed by tidal currents before infection, leading to few identical clones within crabs. Cercarial mixing may not occur in relatively still tide pools where warm temperatures favour cercarial shedding, allowing amphipods to become infected by numerous identical trematode clones. Therefore, identical M. novaezealandensis clones may be more common within amphipods versus crabs. In the previous study of Keeney et al. (2006a), all metacercariae found within the relatively long-lived M. hirtipes were mature and encysted, making it impossible to determine if identical clones infected these second intermediate hosts at the same time. Whether this was caused by crabs being predominantly infected as juveniles or by immature metacercariae being difficult to detect within the hosts cannot be determined. In the relatively small, short-lived amphipods, many individual M. novaezealandensis are often found at several stages of development, allowing a test of whether or not identical clones arrive at second intermediate stages in batches.

The goal of the current study was to characterise the genetic clonal diversity of M. novaezealandensis metacercariae within amphipod (P. novizealandiae) second intermediate hosts and determine if identical trematode clones...
coexist within hosts. It is likely that any identical trematode clones found within an amphipod would have infected the host at approximately the same time. To test for synchronicity of infections, we compared the genetic identity of each trematode with others at the same and different developmental stages within the same host. To the best of our knowledge, this work represents the first time genetic and developmental data have been utilized together to investigate natural transmission strategies in a trematode.

2. Materials and methods

2.1. Sample collection

Approximately 200 amphipods were collected from Lower Portobello Bay, Otago Harbor, South Island, New Zealand (45°52’ S, 107°42’ E) in March 2006. The total length (telson to rostrum) of each amphipod was measured to the nearest 0.1 mm using a compound microscope with an ocular micrometer. Amphipods were then dissected and all parasites were removed. Measurements and counts are reported as the range followed by the mean ± SD in parentheses. Only amphipods possessing parasites were included in this study and genotypes and sizes were only determined for parasites from multiple parasite infections (amphipods with single parasite infections \( n = 13 \) were treated as having single parasite genotypes for all analyses). Parasite length and width were measured to the nearest micrometer using a compound microscope with an ocular micrometer. Parasite metacercariae were then classified to one of six age cohort classes based on their development (early immature, immature, late immature, early cyst, mature cyst and melanized cyst; Fig. 1). This classification scheme was not intended to provide a definitive description of parasite development, but rather a means of identifying parasites that entered the host at approximately the same time. It takes 4–5 weeks for *M. novaezealandensis* to develop into mature metacercariae within amphipods during experimental infections (Martorelli et al., 2004). Encapsulation and subsequent cellular melanization of intruding parasites is relatively common in crustaceans (Johansson and Söderhäll, 1989; Thomas et al., 2000) and we cannot estimate the approximate host entry time for this distinct developmental class.

Fig. 1. Representative parasite developmental stages. 1 = early immature, 2 = late immature, 3 = early cyst, 4 = mature cyst. Scale bars are 100 \( \mu \)M.
2.2. Trematode DNA extraction and genotyping

Individual parasites were placed into 1.5 ml tubes for DNA extraction. DNA extractions consisted of placing individual parasites in 400 μl of 5% chlex 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 all parasites following the protocols outlined in Keeney et al. (2006a,b). Any identical parasite genotypes were genotyped at two additional loci (Mno-2 and Mno-45) as described in Keeney et al. (2006a,b) after probabilities of ‘false’ clones resulting from sexual reproduction were calculated (see statistical analyses).

2.3. Statistical analyses

Microsatellite genotypes were determined manually and identical multilocus genotypes were identified using GENALEX 6 (Peakall and Smouse, 2006). The probabilities of observing at least as many identical trematode genotypes by chance via sexual reproduction (P_{sex}) were calculated using the program MLGsim (Stenberg et al., 2003). A corresponding critical P_{sex} value was generated with 1 × 10^6 simulations and trematodes possessing identical genotypes with P_{sex} values below this were considered true clones. The number of alleles per locus, expected and observed heterozygosities, deviations from Hardy–Weinberg expectations for each locus, and across all loci, were calculated with GDA 1.1 using unique genotypes observed from the amphipods of this study and from snails and crabs from the study of Keeney et al. (2006a) using Genepop version 3.3 (Raymond and Rousset, 1995a) to test the assumption that hosts were infected by the same parasite population. Significance of genetic differentiation (F_{ST}) was calculated among pooled amphipod genotypes from this study and from snails and crabs from the study of Keeney et al. (2006a) using Geenepop version 3.3 (Raymond and Rousset, 1995a) to test the assumption that hosts were infected by the same parasite population. Significance of genetic differentiation was calculated using the exact test of Raymond and Rousset (1995b) implemented in Genepop and interchanging alleles to determine if allelic distributions differed among hosts. The following statistical analyses were performed with SPSS 13.0 for Windows. Data were analysed for extreme outliers that may be biasing any results by examining Boxplots. SPSS 13.0 defines an extreme outlier as a data point extending greater than three box-lengths from the edge of a Boxplot. Differences in size, number of trematodes, and number of trematode genotypes were compared between male and female amphipods using Mann–Whitney U tests. To determine if male and female amphipods of the same size possessed different mean numbers of trematodes and/or trematode genotypes, amphipods were divided into five size classes of 0.5 mm intervals and Mann–Whitney U tests were performed for each size class as described in Fredensborg et al. (2004).

Relationships between amphipod length and the number of trematodes, trematode genotypes and trematode cohorts within amphipods, were examined using Spearman’s rank order correlation.

3. Results

A total of 319 larval M. novaezelandensis were collected from 25 male and 32 female amphipods and the genotypes of 306 of these (from 18 male and 26 female amphipods) were determined (the other 13 metacercariae and hosts were single infections). The number of alleles and observed heterozygosities for each locus ranged from seven to 35 and 0.61 to 0.75, respectively (Table 1). Locus Mno-30 deviated from Hardy–Weinberg expectations nominally (P = 0.021), but not significantly after Bonferroni adjustment of alpha (initial α = 0.013) and genotypic disequilibrium between loci Mno-30 and Mno-47 was nominally significant (P = 0.031), but not significant after Bonferroni adjustment of alpha (initial α = 0.017). Initial MLGsim results indicated that several metacercariae with identical genotypes could not be definitively classified as clones (P_{sex}-values >3.5 × 10^{-9} critical value). After determining the genotypes for all genetically identical metacercariae at two additional loci, the probabilities that any metacercariae with identical genotypes were not true genetic clones were below the simulated critical P_{sex} value. Six pairs of identical genetic clones were recovered from five amphipods (one amphipod had two pairs of identical clones), resulting in a total of 300 different trematode genotypes. All pairs of clones belonged to the same developmental cohort: three pairs were very immature, two pairs were immature, and one pair consisted of melanized cysts. No identical clones were recovered from different amphipods.

Trematode genetic differentiation was not detected among the amphipod hosts and the previous snail and crab hosts examined (F_{ST} < 0.001, P = 0.909), indicating all host species were infected from the same parasite gene pool.

Summary statistics for each parasite developmental cohort including the total number, mean number, prevalence, mean intensity of infection, length, and width are provided in Table 2. Both the prevalence and the number of metacercariae per host decreased from the earliest immature stage to the mature cyst stage (Table 2). Male amphipods...

Table 1

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<th>Parameter</th>
<th>Microsatellite loci</th>
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<td>Mno-1</td>
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<td>Alleles</td>
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<td>H_e</td>
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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)
pods were significantly larger than female amphipods (male mean length = 4.08 ± 0.43 mm, female mean length = 3.31 ± 0.29 mm; Mann–Whitney U test, \( P < 0.001 \)). Males possessed 1–35 (6.80 ± 7.74) trematodes and 1–34 (6.76 ± 7.59) trematode genotypes, and females possessed 1–15 (4.75 ± 3.57) trematodes and 1–14 (4.59 ± 3.39) trematode genotypes (overall amphipods were 2.60–4.70 (3.65 ± 0.52) mm long and possessed 1–35 (5.65 ± 5.81) trematodes and 1–34 (5.54 ± 5.68) trematode genotypes). Male trematode counts were biased by an outlier infected by 35 trematodes. Without this individual, males possessed 1–20 (5.63 ± 5.15) trematodes and trematode genotypes. The mean number of trematodes and trematode genotypes did not differ between males and females if the male outlier was included (Mann–Whitney U test, \( P \geq 0.603 \)) or excluded (Mann–Whitney U test, \( P \geq 0.789 \)). The mean number of trematodes and trematode genotypes did not differ between males and females of the same size class (Mann–Whitney U test, \( P \geq 0.134 \)) and data for both sexes were pooled for length relationship analyses. A significant positive relationship was detected between amphipod length and number of trematodes \( (r_S = 0.285, P = 0.032) \) and trematode genotypes \( (r_S = 0.308, P = 0.020) \) (Fig. 2), but not between amphipod length and number of trematode cohorts \( (r_S = 0.164, P = 0.223) \). The significant positive relationship was still detected between amphipod length and number of trematode genotypes when the outlier amphipod with 35 trematodes was removed from the analysis \( (r_S = 0.285, P = 0.033) \), but not between amphipod size and number of trematodes \( (r_S = 0.261, P = 0.052) \).

### 4. Discussion

An increase in the number of trematode genotypes was detected with increasing amphipod length. This reflects the accumulation of trematodes during an amphipod’s development that has been identified previously in this system (Fredensborg et al., 2004). Smaller amphipods have typically been exposed to fewer cercariae, resulting in a tendency for fewer trematodes and trematode genotypes to be found within them. There was a general decrease in the number of metacercariae recovered, prevalence and mean intensity of infection for each trematode developmental stage as development progressed (melanized cysts did not follow this trend, but we cannot estimate their relative age of infection versus other stages). One reason for this could be parasite mortality during development, with few individuals surviving to the cyst stage infective to bird definitive hosts. This may also reflect higher mortality in amphipods once they are infected. Indeed, infection has been shown to cause mortality in this system when large numbers of cercariae penetrate a host within a short time period (Fredensborg et al., 2004). In the current study, the “early immature” trematodes were the only cohort represented by more than nine metacercariae within a single amphipod. This suggests that amphipods may not commonly live long enough for significant trematode development to occur after infections by large numbers of cercariae.

Our study demonstrates that multiple copies of the same genetic clone exist in a substantial proportion of amphipod second intermediate hosts (11% of amphipods harboring...
more than one trematode possessed multiple copies of the same parasite clone). We suggest, therefore, that the relatedness of parasites within a host may influence their behaviour and/or life history strategies. Fredensborg and Poulin (2005) detected density-dependent effects on *M. novaezelandensis* within *M. hirtipes* crabs. Metacercariae from large infrapopulations within crabs typically had smaller volumes and produced smaller adult worms and fewer eggs in vitro. Density-dependent effects may be particularly important for *M. novaezelandensis* which, unlike many trematodes, increase almost 200 times in volume within second intermediate hosts (Poulin and Latham, 2003; Fredensborg et al., 2004). Furthermore, identical trematode clones may utilize host resources more cooperatively versus non-related trematodes (Parker et al., 2003b), resulting in less variation among the size of metacercariae that infect the host at the same time. Although these predictions must be tested using experimental infections, our data indicate multiple copies of the same parasite genotype do infect wild amphipod hosts within relatively short time periods.

All six pairs of identical trematode clones detected in this study consisted of metacercariae from the same developmental cohort, indicating that they infected the amphipod at approximately the same time. Although our developmental cohort classification does not allow us to accurately distinguish between truly synchronous infections and infections separated by a few days, our data provide no evidence of asynchronous infections involving clones. Indeed, given that tide pools are ephemeral with incoming tides enabling amphipods to move and aggregate in different tide pools, the likelihood of synchronous infections involving trematode clones from a particular snail would seem extremely small. Therefore, our results likely reflect synchronous infections of identical clones.

It should be emphasised that few identical trematode clones were recovered from amphipods, even when as many as 35 trematodes were found within a single amphipod. Despite the potential for large numbers of cercariae to be shed from individual snails, we did not detect genetic evidence for any of the 57 amphipods being infected by a large number of cercariae from an individual snail. Approximately half of the infected snails in this area contain single-clone trematode infections (Keeney et al., 2006a), supporting the rarity of multiple cercariae from the same snail successfully infecting the same amphipod host. It is possible that large numbers of cercariae simultaneously infecting an amphipod may cause mortality, making it less likely for these individual amphipods to be sampled when it does occur. Fredensborg et al. (2004) observed significantly higher short-term mortality in amphipods exposed to 25 or more *M. novaezelandensis* cercariae versus amphipods exposed to five or fewer. They also detected a lower variance-to-mean ratio in numbers of trematodes per amphipod in larger amphipod size classes (indicating heavily infected amphipods are removed from the population). In the current study, only two of 57 amphipods were infected by greater than 15 trematodes, consistent with heavy host mortality associated with large numbers of cercariae simultaneously infecting an amphipod. Although we cannot rule out the possibility that amphipods harboring many copies of the same trematode clone exist in low frequencies within this population, it is apparently not a common occurrence.

The low number of identical parasite genotypes recovered from amphipods indicates that mixing of trematode genotypes is occurring between snail first intermediate hosts (which contain large numbers of genetically identical larval *M. novaezelandensis*; Keeney et al., 2006a) and amphipod second intermediate hosts. This is similar to results from *M. hirtipes* crab second intermediate hosts in this system (Keeney et al., 2006a) and we believe it demonstrates for the first time that alternative second intermediate hosts can harbor similar patterns of within-host trematode clonal diversity despite differences in the biology of the hosts. This supports Criscione and Blouin’s (2006) prediction that multi-host aquatic trematodes will experience genotype mixing before entering definitive hosts. Although few empirical studies have been performed, our results are consistent with previous findings of high trematode genetic diversity in second intermediate hosts within freshwater (Rauch et al., 2005) and marine (Keeney et al., 2006a) systems.

The diversity of *M. novaezelandensis* genotypes within and among amphipods reflects the abundance of infected *Z. subcarinatus* (>70% prevalence in mature snails (Fredensborg, unpublished data)) which can serve as sources of cercariae in this area. Since numerous *Z. subcarinatus* can aggregate within tide pools, amphipods are exposed to cercariae from multiple snails during any particular low tide as well as during different low tides. It is also possible that amphipods are commonly encountering cercariae between tides as well as in tidal pools. There would likely be less of a chance of encountering multiple copies of the same parasite genotype between tides (tide pool conditions favour cercarial shedding and pools are relatively small enclosed areas), a pattern consistent with our data. Therefore, it is likely that different amphipods are exposed to numerous different infected snails during their lifespan, resulting in the diversity of parasite genotypes detected within and among amphipods. The availability of infected snails as sources of cercariae coupled with the potential for high mortality to occur when large numbers of cercariae simultaneously infect an amphipod could explain the observed parasite genetic diversity. The same high diversity of parasite genotypes within and among hosts was detected within *M. hirtipes* in this area (Keeney et al., 2006a).

The low number of identical clones within amphipods would decrease the chances of trematodes inbreeding within definitive hosts (Rauch et al., 2005) as well as decrease variation in reproductive success among individual trematode clones, enabling this population of trematodes to maintain a large effective population size (Prugnolle et al., 2005a,b). A large effective population size would enable this population of trematodes to avoid inbreeding.
depression and other problems potentially associated with rapid genetic drift (Hedrick, 2000). More generally, parasite populations need to adapt to their hosts and the maintenance of large effective population sizes may facilitate adaptation in parasite populations (Criscione and Blouin, 2005).

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