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Accumulation of diverse parasite genotypes within the bivalve second intermediate host of the digenean *Gymnophallus* sp.

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ABSTRACT

The complex life cycle of digenean trematodes with alternating stages of asexual multiplication and sexual reproduction can generate interesting within-host population genetic patterns. Metacercarial stages found in the second intermediate host are generally accumulated from the environment. Highly mobile second intermediate hosts can sample a broad range of cercarial genotypes and accumulate genetically diverse packets of metacercariae, but it is unclear whether the same would occur in systems where the second intermediate host is relatively immobile and cercarial dispersal is the sole mechanism that can maintain genetic homogeneity at the population level. Here, using polymorphic microsatellite markers, we addressed this issue by genotyping metacercariae of the trematode *Gymnophallus* sp. from the New Zealand cockle *Austrovenus stutchburyi*. Despite the relatively sessile nature of the second intermediate host of *Gymnophallus*, very high genotypic diversity of metacercariae was found within cockles, with only two cockles harbouring multiple copies of a single clonal lineage. There was no evidence of population structuring at the scale of our study, suggesting the existence of a well-mixed population. Our results indicate that (i) even relatively sessile second intermediate hosts can accumulate a high diversity of genotypes and (ii) the dispersal ability of cercariae, whether passive or not, is much greater than expected for such small and short-lived organisms. The results also support the role of the second intermediate host as an accumulator of genetic diversity in the trematode life cycle.

1. Introduction

The population genetics of parasites with complex life cycles can differ substantially from patterns observed in organisms with simpler life histories (Prugnolle et al., 2005). For instance, trematodes are characterised by a complex life cycle consisting of alternating phases of sexual reproduction in the definitive host, asexual multiplication in the first intermediate host, and accumulation in a second intermediate host en route to the definitive host (Kearn, 1998). The completion of the trematode life cycle is reliant, first, on the ability of short-lived free-swimming larval stages in the environment to reach the appropriate intermediate host on two separate occasions (miracidia and cercaria) and, second, on achieving trophic transmission to the definitive host. Natural selection may have favoured and maintained complex life cycles in trematodes because they confer advantages, including greater channelling toward the definitive host and enhanced growth and fecundity in the latter (Choisy et al., 2003; Poulin, 2007). Trophic transmission has also been suggested as a way to ensure that parasites can find conspecifics and cross-fertilise (Brown et al., 2001). The predator host can concentrate many isolated parasite genotypes from the environment and provide a place for cross-fertilisation (Brown et al., 2001). Therefore, the mixing of diverse genotypes might be a key advantage of complex life cycles. This depends, however, on the way genetic variability among parasites sharing the same host varies from one host to the next in the life cycle.

Rauch et al. (2005) found that for the trematode *Diplostomum pseudospathaceum*, while most of the individual snails that act as first intermediate host are parasitised by only one clone, in contrast the second intermediate hosts, three-spined sticklebacks, *Gasterosteus aculeatus*, harbour a diverse array of unique genotypes. This contrast in genotypic variation between the different hosts was attributed to the mobility of the fish host which allows it to sample cercariae issued from a number of different snails, resulting in the accumulation of a variety of clones (Rauch et al., 2005). This was echoed by the findings of Keeney et al. (2007a) who quantified the clonal diversity of *Maritrema novaezealandensis* in its first intermediate host, the snail *Zeacumantus subcarinatus*, and one of its second intermediate hosts, the crab *Macrophthalmus hirtipes*. They found that while the snail can harbour up to five trematode genotypes, only about half of the snails were actually infected by multiple clonal lineages. Meanwhile, the genetic diversity in individual crabs was markedly higher, with all metacercariae sampled in a crab representing different genotypes in the

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majority of crabs (Keeney et al., 2007a). A follow-up study looking at the genotypic diversity of *M. novaezealandensis* metacercariae in an alternate second intermediate host, the amphipod *Paracalliope novizealandiae*, also found that there were almost as many different genotypes as there were individual metacercariae in a host (Keeney et al., 2007b). However, the few metacercariae with identical genotypes in the same host were at the same developmental stage, suggesting that second intermediate hosts can become infected simultaneously by multiple individuals of a single clonal lineage if they are exposed to a batch of cercariae released from a single, nearby first intermediate host.

The general trend emerging from the few existing studies is that the metacercariae found in the second intermediate host are usually the result of continuous recruitment from the environment over time. As a result, the second intermediate host accumulates a large number of different genotypes. However, all available studies on the genotypic variability of metacercariae found in the second intermediate host involve hosts that are relatively mobile (sticklebacks, crabs, amphipods). It is unclear how much of this genetic variability is due to the parasite's own dispersal abilities within the aquatic habitat; only comparable data from a system with comparatively immobile hosts would allow an evaluation of the role of cercarial dispersal per se in generating high genetic diversity within-hosts.

The New Zealand cockle (*Austrovenus stutchburyi*) is a suspension-feeding bivalve common on New Zealand intertidal mudflats. Cockles are usually found buried just 1–3 cm under the surface of the sediment, and they represent relatively immobile second intermediate hosts for a number of different trematode metacercariae (Poulin et al., 2000; Leung and Poulin, 2007a), one of which is a species belonging to the genus *Gymnophallus*. The complete life cycle of this undescribed species of *Gymnophallus* has not yet been elucidated, but it follows that of other Gymnophallidae: the sporocyst stages use a bivalve first intermediate host, the metacercariae accumulate in a different bivalve second intermediate host (*A. stutchburyi*), and the adults live in the intestine of a bird (Bower et al., 1996). In this system, the relative immobility of both the first and second intermediate hosts can potentially result in a situation where the transmission of cercariae and the accumulation of different genotypes in cockles depend solely on cercarial mobility. Do cockles in close physical proximity acquire only cercariae of a few genotypes streaming from the few first intermediate hosts nearby? We might expect a small-scale spatial pattern in the genetic relatedness of the metacercariae, whereby cockles situated next to each other would each harbour multiple copies of the same clones, and also acquire the same genotype(s), while cockles that are situated a few metres away would collect completely different genotypes.

As in many other gymnophallids, the metacercariae of *Gymnophallus* sp. occur unencysted in the extrapallial space, on the mantle epithelium of the cockle where they often induce a host tissue reaction (Campbell, 1985; Cremonte and Ituarte, 2003). While most metacercariae are usually found in a dense aggregation close to the hinge of the cockle's shell, they also occasionally occur in other isolated clumps on other parts of the mantle epithelium. These clumps range from a single individual, to aggregations of up to 20 metacercariae. Could these clumps represent separate infection events by different genetic clones, with different clumps consisting of different genotypes? Our study uses microsatellite markers to answer the questions raised above, and to elucidate the small-scale genetic diversity and genetic structure of metacercarial infections in this system. We determined the co-occurrence of clones, i.e. metacercariae with identical genotype issued from the same original egg, at four hierarchical levels (within clumps, within the same cockle, among adjacent cockles, and among cockles separated by several metres), to assess the role of cercarial mobility and behaviour in creating genetic diversity.

2. Materials and methods

Sampling of cockles was carried out at Company Bay, Otago Harbour, South Island, New Zealand on 6th February, 2007. Cockles were collected at the low tidal mark along a 10 m-long transect. Three collection points were established along the transect, each 5 m apart, and five cockles located within a circular area 15 cm in diameter were haphazardly selected from each point. In the laboratory, individual metacercariae were dissected from the surface of the cockle's mantle epithelium located in the extrapallial space. Whenever possible, we aimed to collect 30 metacercariae from each cockle, keeping the metacercariae found in the aggregation near the hinge separate from those obtained from other isolated clumps elsewhere on the mantle epithelium. Each individual metacercaria was carefully isolated from the host tissue using fine dissection needles and transferred into a Petri dish containing 0.22 μm-filtered water using a 200-μL pipette. They were then transferred into another Petri dish containing filtered water using the pipette as before. This procedure served to rinse away any residual host material on the metacercaria. The metacercaria was then placed into a 1.5-mL Eppendorf tube for DNA extraction. DNA was extracted in 500 μL of 5% chelex containing 0.1 mg/mL proteinase K, incubated at 60 °C for 4 h and boiled at 100 °C for 8 min.

The genotype of each metacercaria was determined at six microsatellite loci (G3A4, GYM6b, GYM8, GYM11, GYM12, GYM14) following the protocol described in Leung et al. (2007). The loci were selected on the basis of their level of polymorphism and their statistical power at identifying true genetic clones. PCR products were resolved in 9% non-denaturing polyacrylamide gels using cloned fragments as size standards with a 10 bp DNA ladder and visualised by staining gels in 0.1× SYBR Green I (Invitrogen). A total of 406 metacercariae from 15 cockles were genotyped.

The genotypic data were analysed with GENALEX 6 (Peakall and Smouse, 2006) to identify multilocus matches. The probabilities of observing at least as many identical genotypes by chance based on the loci used were estimated using GENCLONE version 2.0 (Arnaud-Haond and Belkhir, 2007). GENCLONE can take into account any departure from Hardy–Weinberg equilibrium (HWE) when calculating the probability that identical multilocus genotypes were produced via sexual reproduction versus being true genetic clones. To test for deviations from HWE, Weir and Cockerham's (1984) f estimator of F_{is} was calculated for each locus and significance values determined with 6000 randomisations using FSTAT version 2.9.3 (Goudet, 1995).

Because significant deviation from HWE was detected for all but two of the loci, the sample was checked for the possibility that this resulted from the presence of null alleles, cryptic species or population structuring of the parasites at the site.

To check for the presence of null alleles, Brookfield's (1996) null estimator 1 was calculated for each locus using MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004), which calculated probabilities and confidence intervals for homozygote frequencies based on the observed data. The analysis was conducted using Monte Carlo bootstrap simulations with 1000 randomisations at 95% confidence interval.

The 16S gene was sequenced for a subset of 23 metacercariae, consisting of individuals that were collected from the aggregation near the hinge and from other isolated clumps, which were haphazardly chosen to verify that the population consisted of a single species. This was done using platyhelminth-specific 16S primers platy.16Sar [5′atctgttt(a/c)t(c/t)aaaaacat3′] and platy.16Sbr [5′ccaatcttaactcaactcatat3′] designed by Donald et al. (2004). The optimum cycling parameters for these primers included an initial denaturation step of 95 °C (2 min), followed by 40 cycles of 95 °C (30 s), 48 °C (40 s), and 72 °C (1 min), followed by a final extension phase at 72 °C (10 min). The PCR products

were separated from excess primers and oligonucleotide using a Purelink™ PCR Purification kit (Invitrogen), were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and resolved with an ABI PRISM 3730 Genetic Analyser (Applied Biosystems). Sequences were aligned using ClustalW in MEGA version 3.1 genetic analysis programme (Kumar et al., 2004). Pairwise comparisons of the sequences were conducted using a Kimura's two parameter model (Kimura, 1980) also in MEGA.

Genetic differentiation of potential populations was analysed using two methods. Firstly, the individual parasites were clustered into populations based on their microsatellite genotypes using the Bayesian approach of STRUCTURE version 2.2 (Pritchard et al., 2000). The number of populations (K) was allowed to vary from 1 to 3 and 10 replications were run for each K. The admixture-ancestry model with α inferred and correlated allele-frequency model with $\lambda = 1$ were used for all simulations. Each simulation was run with a burn-in of 100,000 iterations and a Markov chain Monte Carlo of 100,000 iterations. Second, the parasites were classified into three populations – based on the sampling points from which cockles were collected along the field transect. Weir and Cockerham's (1984) θ estimator of F_{st} was calculated over all loci and 95% confidence intervals were obtained by bootstrapping over loci with FSTAT version 2.9.3 (Goudet, 1995). Genetic differentiation among sampling points and among hosts was examined using the G-based test (Goudet et al., 1996) in FSTAT with 10,000 permutations of genotypes among sampling points. Pairwise θ values were also calculated between all pairs of sampling points with FSTAT. Alpha significance values were corrected for multiple pairwise comparisons using the Bonferroni approach (Rice, 1989).

3. Results

The shell length of the cockles collected for this study ranged from 25.8 to 32.0 mm, with a mean of 28.8 ± 1.9 mm, and no difference in shell sizes among cockles from the three collection points. The cockles were found to be infected with 23–126 *Gymnophallus* metacercariae, with a geometric mean of 46 metacercariae per cockle. The cockles were also found to be infected with 96–807 echinostome metacercariae that were encysted in their foot, with a geometric mean of 387.5 metacercariae per cockle.

Of the 15 cockles dissected and 406 *Gymnophallus* metacercariae genotyped at six microsatellite loci, only two of the cockles contained genetically-identical metacercariae, both of which were infected with four clonal metacercariae. Thus a total of 400 unique genotypes were found. Metacercariae of the same clone were never found in different cockles. Analysis by GENCLONE indicated that pairs of metacercariae with identical multilocus genotypes found in the same host can be reliably considered as true genetic clones (all $p_{sex} < 0.0005$). In one cockle, two of the clones were found in one clump, and the other two were in another clump; in the other cockle with clonal metacercariae, three were in one clump and the fourth was alone in a different clump.

The number of alleles and observed heterozygosity ranged from 9 to 31 and 0.663 to 0.855, respectively (see Table 1). All loci except GYM11 were found to nominally deviate significantly from Hardy–Weinberg equilibrium (HWE) (see Table 1), however, after Bonferroni adjustment of alpha ($\alpha = 0.0083$), only four of the loci were found to deviate significantly from HWE (G3A4, GYM6b, GYM8, GYM14), of which GYM8 was found to be only weakly significant. The population was found overall to be significantly out of HWE.

Analysis with MICRO-CHECKER found that all loci showed evidence of the presence of null alleles, thus the deviation from expected HWE can possibly be attributed to their presence. Even though the presence of null alleles among the loci can affect the outcome of the genotyping of individual metacercariae, apart from the eight individuals identified to be clones, the genotypes of the rest of the metacercariae differed from each other by at least two of the loci, and at least one of the loci at which they differed was either a pair of different heterozygote alleles, or heterozygotes with alleles different from those found in a homozygous individual. Thus the presence of null alleles did not affect the capacity of the loci to distinguish between genetic clones.

Table 1
Summary of microsatellite data for each locus

Locus	G3A4	GYM6b	GYM8	GYM11	GYM12	GYM14
N_a	10	14	9	13	21	31
H_o	0.729	0.663	0.675	0.667	0.771	0.855
H_e	0.788	0.765	0.723	0.707	0.822	0.943
F_{is}	0.0008[a]	0.000[a]	0.0082[a]	0.0710	0.0137	0.0002[a]

N_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, inbreeding coefficient (positive values = heterozygote deficiency).
[a] Denotes values deviating significantly from Hardy–Weinberg expectations.

PCR amplification of 16S gave a product of approximately 500 bp; subsequent sequencing of this product yielded 402 bp of readable sequence. Pairwise comparison of the sequences obtained found divergence levels of 0.0–0.5% between individual sequences, well within the range of genetic divergence expected at an intraspecific level (Vilas et al., 2005).

Significant genetic differentiation was not detected among the nominated potential populations. No significant genetic differentiation was detected among the three sampling points with F_{st} estimates obtained [$\theta = 0.001$ (95% confidence interval = −0.001–0.003), $P = 0.2569$]. Genetic differentiation between any pair of sampling points (adjusted $\alpha = 0.017$, $P = 0.053$, 0.370, 0.333) was also not found to be significant. This corroborated the results obtained by Bayesian clustering analyses performed with STRUCTURE. The probability for each K (number of populations) was similar for each replication, with $K = 1$ having overwhelmingly the highest probability (values are mean ± SD): Pr $(K = 1) = 1.00 \pm 0.0$ versus Pr $(K = 2) = 3.78 \times 10^{-54} \pm 1.20 \times 10^{-54}$ and Pr $(K = 3) = 1.89 \times 10^{-54} \pm 5.98 \times 10^{-54}$. The results from the analyses conducted with both FSTAT and STRUCTURE indicate that the sample is most likely to consist of just a single population.

4. Discussion

The co-occurrence of multiple genotypes of the same parasite species within a host can have various ecological and evolutionary consequences, with potential impact on virulence, intraspecific competition and/or parasite life history traits (Bell et al., 2006; Koskella et al., 2006; Lagrue et al., 2007; Poulin, 2007; Wargo et al. 2007). Our study provided optimal conditions for identical parasite genotypes to reach the same host: both the source host and the target host are relatively immobile, which should result in the latter being exposed repeatedly to only the few clones produced by the closest infected first intermediate hosts. Yet our results indicate extensive mixing of cercariae, even on the smallest spatial scale, to produce genetically diverse infections in cockles and a lack of parasite population structure.

The significant deviation from HWE found for multiple loci of *Gymnophallus* sp. is an unusual phenomenon but certainly not unprecedented amongst trematodes. For instance, very significant deviations from HWE have been found in three species of trematodes from the genus *Lecithochirium* (Vilas et al., 2004). Significant heterozygote deficiency was also found in a natural population of *Schistosoma mansoni* from Brazil, which was attributed to population sub-structuring and the possible presence of null alleles (Stohler et al., 2004); the latter is the likely cause of deviations from HWE here as well. The biological explanation behind the presence of these multiple null alleles remains unknown.

Our analyses suggest that all the individuals collected from the study site belong not only to the same species (based on 16S gene sequences), but also to a single interbreeding population. Bird definitive hosts are efficient dispersal agents over large geographic scales, let alone within a single bay (Criscione and Blouin, 2004; Keeney et al., 2008). For *Gymnophallus* sp., the mobility of the definitive host would serve to homogenise the allele frequencies of the parasites that infect the first intermediate hosts. The lack of genetic structure among metacercariae in cockles suggests that cercariae are well-mixed following their emission from first intermediate hosts, probably passively by water movements, and accumulate as genetically diverse groups within cockles.

In a population genetics model examining the implications of the complex trematode life cycle, Prugnolle et al. (2005) concluded that migration rate at various stages of the life cycle can have different implications for the genetic diversity of trematodes within their hosts, and suggested that a lack of genetic diversity can be compensated by processes throughout the life cycle. While genetic diversity is low during clonal amplification in the first intermediate host, if the rate at which the clonally produced cercariae infect the second intermediate host is high, then overall high genetic diversity can be regained despite clonal amplification in the first intermediate host or selfing by the adult worms in the definitive host (Prugnolle et al., 2005). Empirical studies also show the importance of the mobility of the definitive host (Criscione and Blouin, 2004; Theron et al., 2004) or the presence of paratenic hosts (Vilas et al., 2003) in maintaining genotypic diversity of trematodes.

The New Zealand cockle *A. stuchburyi* appears to play a crucial role in maintaining genetic diversity of *Gymnophallus* sp. The powerful filtering capacity of cockles compensates for their relative immobility in terms of accumulating cercariae that are emitted from different first intermediate hosts in the environment. While the filtering capacity of *A. stuchburyi* is unknown, Mouritsen et al. (2003) suggested that it is likely to be within the range of the similarly-sized bivalve *Cerastoderma echinatum*. Based on data available for *C. echinatum*, Mouritsen et al. (2003) calculated that the cockle population of Company Bay (the same location as in this study) at the low tidal mark would be able to filter the water column 8–19 times during each submersion period. Perhaps cockles, although relatively immobile, expose themselves to genetically diverse cercariae by filtering large water volumes, especially if cercariae are well-mixed by physical processes. Because of their small size, one would expect that water currents play a substantial role in the distribution of cercariae. De Montaudouin et al. (1998) noted that water flow velocity can have a significant effect on the infection success of *Himasthla elongata* cercariae in the cockle *Cerastoderma edule*. However, it is unknown how that might contribute to the mixing of cercariae from heterogeneous sources in the environment. In addition, there is evidence that some cercariae have the ability to overcome the influence of water movement despite their small size (Fingerut et al., 2003). No such empirical behavioural study has been conducted on the cercariae of *Gymnophallus* sp. and it is unknown whether they can overcome water currents. It seems more likely that the swimming behaviour of cercariae and abiotic factors work synergistically to produce the genotypically diverse groups of *Gymnophallus* sp. metacercariae found in each cockle. Bartoli and Prévot (1986) reported that the cercariae of *Gymnophallus fossarum* tend to stay in suspension, which allows them to be drawn into the filtration current of their cockle host. While this apparently passive infection mode might conceivably lead to the host becoming infected with multiple clonal cercariae (as found by this study), Bartoli and Prévot (1986) consider the infection success of gymnophallid cercariae to be quite low, which means the host will instead mostly accumulate metacercariae from being exposed to successive waves of genetically heterogeneous cercariae, resulting in a genetically diverse infrapopulation within the cockle.

Results from a previous study suggest that the accumulation rate of *Gymnophallus* sp., metacercariae in *A. stutchburyi* is relatively low (Leung and Poulin, 2007b), and that the cockles are most likely infected by successive waves of cercariae over time. The presence of multiple clonal individuals within two of the cockles indicates that, occasionally, cockles do become infected by multiple cercariae being shed from a single first intermediate host, which probably occurs as a result of close proximity to a host that is releasing cercariae. However, judging from the diverse genotypes found in all cockles, it seems that most cercariae become thoroughly mixed and distributed in the environment. The present findings are quantitatively very similar to those of earlier studies on the genetic diversity of metacercariae sharing the same aquatic second intermediate hosts (Table 2), suggesting that whether this host happens to be mobile or not has no measurable influence on the observed patterns of genetic variation.

Like the findings of previous studies on the genotypic diversity of trematodes in their second intermediate host, we found that clonal individuals rarely co-occur. Due to the low likelihood of encountering clones, competition would be the more likely type of interaction between trematodes within the second intermediate host. Heavy infection of a second intermediate host can result in metacercariae achieving smaller sizes and lower infectivity (e.g. Fredensborg and Poulin, 2005). Unlike most trematode metacercariae, *Gymnophallus* sp., like other gymnophallids, do not encyst in their second intermediate host and the metacercariae may continue to have dynamic interactions such as feeding on host tissue (Cremonte and Ituarte, 2003). For instance, the metacercariae of *Gymnophallus rebecqui* have been found to feed on the hyperplastic tissue induced by the parasite's presence (Campbell, 1985). In a situation with high intensities of infection, where clones rarely co-occur in the same cockle, and in a species capable of feeding throughout its stay in the second intermediate host, intraspecific competition or other forms of antagonistic interactions are likely to take place.

The main result of this study supports previous findings regarding the evolutionarily important role played by second intermediate hosts in promoting trematode genetic diversity and maintaining large effective population sizes. However, we believe our study is the first to show that this is also true on very small spatial scales and in the absence of any host movements. While limited mobility may seem to constrain the ability of cockles to sample cercariae from a large number of different first intermediate hosts, this is compensated by their relatively long lifespan and ability to filtrate large volumes of water containing a pool of genotypically

Table 2
Summary of results from previous studies examining the genotypic diversity of trematodes in their second intermediate host as compared with this study

Parasite species	Host	No. of parasites analysed	No. of genotypes found	Parasite vs Genotype ratio	Source
Diplostomum pseudospathaceum	Stickleback	224	218	1.03:1	Rauch et al. (2005)
Maritrema novaezealandensis	Crab	625	607	1.03:1	Keeney et al. (2007a)
Maritrema novaezealandensis	Amphipod	306	300	1.02:1	Keeney et al. (2007b)
Gymnophallus sp.	Cockle	406	400	1.02:1	This study

diverse cercariae. In what may be a key benefit of complex life cycles (Brown et al., 2001; Rauch et al., 2005), using a second intermediate host thus ensures that the definitive host is infected by a packet of genetically diverse parasites each time it eats a single prey item.

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