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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