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Does a facultative precocious life cycle predispose the marine trematode *Proctoeces* cf. *lintoni* to inbreeding and genetic differentiation among host species?

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ABSTRACT

Intraspecific variability in parasite life cycle complexity (number of hosts and species of hosts in the life cycle) may have an impact how parasite genetic variation is partitioned among individual parasites, host individuals or host species within a given area. Among digenean trematodes, a three-host life cycle is common. However, a few species are precocious and may reach sexual maturity in what is typically regarded as the second intermediate host. The objective of this study was to determine whether a precocious life cycle predisposes digeneans to possible inbreeding or genetic subdivision among host species. As a study system, we used the digenean *Proctoeces* cf. *lintoni* whose metacercariae precociously mature (facultative) without a cyst wall in the gonads of multiple sympatric species of keyhole limpets (*Fissurella* spp.), typically regarded as the second intermediate hosts. Genotyped parasites were collected from four species of limpets and the clingfish *Sicyases sanguineus*, the third and final host where sexual maturity occurs. We found very high microsatellite diversity, Hardy–Weinberg equilibrium over all genotyped individuals, and little to no genetic structuring among parasites collected from the different host species. The fact that metacercariae do not encyst in the keyhole limpets, coupled with the high mixing potential of an aquatic environment, likely promote panmixia in local populations of *P.* cf. *lintoni*.

1. Introduction

Among metazoan parasite species there is extensive variation in life history characteristics such as modes of reproduction, life cycle patterns, use of multiple host species and population sizes. A thorough understanding of parasite evolution requires examination of how life history variability among parasite species might impact the partitioning of genetic variation on local scales (Criscione, 2008; Gorton et al., 2012). Local scale processes are important in parasitic organisms because their populations are not only potentially subdivided among geographic locations, but they can be subdivided among individual hosts within a locality or even among different host species at a given stage in the life cycle (Gorton et al., 2012). Thus, genetic subdivision on a local scale could occur through extrinsic (e.g., ecological separation of life cycles) or intrinsic (e.g., host species-induced selection against hybrid parasites) mechanisms (McCoy, 2003). Furthermore, mating systems (self-mating in hermaphroditic species or bi-parental inbreeding) can have a major impact on the partitioning of genetic variation among individuals and subpopulations by increasing homozygosity and reducing overall levels of genetic diversity (Charlesworth, 2003).

The transmission process has been hypothesised to be a major factor influencing the partitioning of metazoan parasite genetic variation on local scales (Nadler, 1995; Criscione et al., 2005; Gorton et al., 2012). Most metazoan parasites of animals sexually reproduce in or on their definitive host (DH) and release offspring into the external environment. If there is lots of mixing of parasite offspring before recruitment into the DH, there will be greater chances for 'panmixia' among all parasites in the host population. In contrast, genetic subdivision and greater chances for bi-parental inbreeding are likely to occur if there is clumped transmission of sibling parasites (Cornell et al., 2003; Prugnolle et al., 2005; Criscione and Blouin, 2006). One parasite transmission characteristic that has not been extensively studied on a local scale for its

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potential influence on the partitioning of parasite genetic variation is within-species life cycle variation (i.e., the number of hosts needed to complete a life cycle) (Gorton et al., 2012).

Among digenean trematodes a typical life cycle involves three hosts. Generally, a vertebrate is the DH wherein there occurs parasite maturity and obligate sexual reproduction. Fluke eggs are passed with host feces into the external environment. An egg is ingested by, or a hatched free-living miracidium penetrates, a first intermediate host (FIH), which is typically a mollusc. In the FIH, there are several rounds of asexual reproduction that ultimately produce clonal larvae called cercariae. The cercariae leave the FIH and enter the environment where they search for the second intermediate host (SIH), which may be another invertebrate or even a vertebrate. Within the SIH, a cercaria will encyst as a metacercaria (a juvenile stage that is not yet sexually mature). The life cycle is completed when the DH consumes an infected SIH. However, there are variations on the three-host life cycle. Of particular interest are species that can precociously produce eggs in what is typically regarded as the SIH. The most recent review on precocious development documents only 79 precocious digenean species (also called progenetic species) that are spread across 50 genera and 24 families (Lefebvre and Poulin, 2005). Approximately 26% of these species have obligate sexual reproduction in the second host with no known third host. The others are facultative such that eggs can be produced while still in the SIH, but the flukes are still infective to and can reproduce in a vertebrate definitive host.

The subject of this study is the hermaphroditic digenean *Proctoeces* cf. *lintoni* (we follow Valdivia et al., 2010, who stated: "Due to the absence of genetic information on specimens originally described as *P.* cf. *lintoni* Siddiqi & Cable, 1960, we prefer to consider our material as *P.* cf. *lintoni*."), which has a facultative precocious life cycle and can be found along the coasts of Chile (Oliva et al., 2010; Valdivia et al., 2010). Its FIH is likely a marine mollusc, but the particular mollusc species is still unknown (Oliva et al., 2010). The cercariae can infect at least eight sympatric species of keyhole limpets of the genus *Fissurella* (George-Nascimento et al., 1998). However, unlike many digeneans, the cercariae of *P.* cf. *lintoni* do not form cysts upon infection in the SIH. This is an important change in life history because this means worms might be able to outcross in the SIH. In fact, metacercariae can move freely within the host gonads, mature to adults and sexually reproduce in the gonads of the keyhole limpets (Oliva and Alvarez, 2011). A third host can also be utilised. Infected keyhole limpets are eaten by the clingfish *Sicyases sanguineus* (DH), in which the parasite can also reach sexual maturity and produce eggs (Oliva and Zegers, 1988). It has been suggested that the precocious worms in the limpets allow an alternative pathway of development, i.e., reducing the life cycle to only two hosts, the FIH and SIH (George-Nascimento et al., 1998). Indeed, nearly all worms within limpet hosts are gravid and although there are slight differences in parasite fecundity among the different definitive hosts, parasite fecundity in some limpet species can be as high as that in clingfish (Oliva and Alvarez, 2011).

How might this facultative precocious life cycle influence local scale genetic structure? First, Rauch et al. (2005) put forth the hypothesis that second or subsequent intermediate hosts for digeneans serve as a mechanism to increase the intermixture of different clones originating from the FIH, thereby increasing the chances of outcrossing between genetically unique individuals when they meet in the DH. Thus, in a facultative precocious species that may mature in the SIH, there may be more chances to outcross with a clonemate (which is equal to self-mating) in the SIH as additional mixing at the level of the DH has not yet occurred. Such identical clone mating will increase homozygosity in the population relative to that expected under Hardy–Weinberg equilibrium. Second, the facultative use of two or three hosts in the life cycle potentially creates ecological separation (extrinsic mechanism; McCoy et al., 2003) or even differential host-selective pressures (intrinsic mechanism; McCoy et al., 2003) that may cause genetic subdivision between parasites with the different life cycle patterns. Moreover, by being able to reproduce in the SIH, there is also now potential for isolation to evolve among the different species of *Fissurella*, especially since these species often occupy different intertidal zones. Here, our goal was to test for possible local scale partitioning of genetic variation in the facultatively precocious *P.* cf. *lintoni* sampled from different SIH and DH species and from the same locality. In particular, two hypotheses were tested. First, it was tested whether there would be a high frequency of repeated clones within the SIH (following the arguments of Rauch et al. (2005)), and if so, whether this would lead to inbreeding. Thus, we tested the null hypothesis that there is no aggregation of identical clones being transmitted from the FIH to the SIH. Second, because *P.* cf. *lintoni* can sexually reproduce in multiple hosts, this sets the stage for extrinsic or intrinsic reproductive isolating mechanisms among parasites in the different host species (McCoy et al., 2003). Thus, the null hypothesis of no genetic differentiation among the different host species was tested.

2. Materials and methods

2.1. Species background and sample collection

Proctoeces cf. *lintoni* has high prevalence of infection (50–96%) in all limpet species and the mean number of worms in limpets ranges from 6 to 16 (Valdivia et al., 2010). The limpet species coexist in the rocky intertidal and shallow intertidal zone of the southeastern Pacific Ocean, and as in many gastropods, the adult phase is benthic and has low dispersal ability with a maximum distance from home of <1 m (Serra et al., 2001). The DH clingfish lives in the same habitat as the keyhole limpets but has a greater vagility than the SIH, which allows it to prey on different populations of keyhole limpet species present in an area. Nonetheless littoral fish such as clingfish tend to show a high fidelity to habitat as experimental data suggest that littoral fish do not move more than 1,600 m from their "home" (Gibson, 1967).

We studied a total of 179 *P.* cf. *lintoni* parasites, of which 153 were collected from the gonads of four species of *Fissurella* and 26 from *S. sanguineus*. All hosts were sampled in the locality of El Lagarto (23°22′S; 70°36′W) in northern Chile (Fig. 1). In this area,

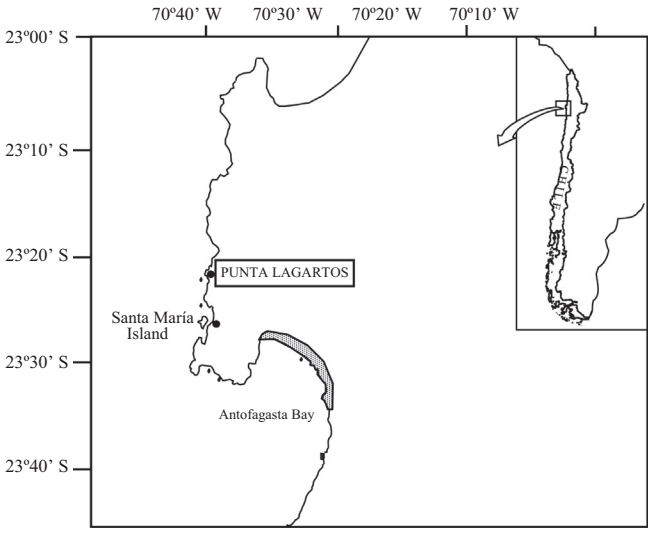

Fig. 1. Map of northern Chile, showing sampling locality.

five of eight fissurelid species are known as SIH for *P.* cf. *lintoni* (Valdivia et al., 2010). Individuals of *Fissurella* spp. were collected at low tide following transects 10 m long by 2 m wide. Within the transect we established four sampling points according to the habitats occupied by the different species: upper and middle intertidal (*Fissurella crassa*), middle and lower intertidal (*Fissurella maxima*), exposed intertidal and subtidal (*Fissurella latimarginata* and *Fissurella cumingi*, respectively). The clingfish were collected by divers within a range of 2 km from the point of collection of the limpets. The host species were transported alive to the laboratory for analysis and searched for parasites. The gonads of each keyhole limpet and the intestine of each individual fish were examined using conventional parasitological techniques. Table 1 shows the number of sampled hosts of each host species and the range of genotyped worms per individual host.

2.2. Microsatellite genotyping

A total of 179 parasites were genotyped using nine microsatellite loci previously described by Austin et al. (2011). Genomic DNA was extracted using a tissue DNA kit (E.Z.N.A. Omega Biotek, USA). All loci were amplified using the Multiplex PCR Kit (QIAGEN, Germany). The multiplex PCRs were conducted in 10 μL reaction volumes containing the QIAGEN Multiplex PCR, using 5 μL of Master Mix (including HotStartTaq DNA polymerase, dNTPs and 6 mM of $MgCl_2$ pH 8.7), 2 μM of each primer, approximately 30 ng of genomic DNA and 3 μL of RNase-free water. The PCR protocol included an initial activation of 15 min at 95 °C with HotStartTaq polymerase, followed by 45 cycles with denaturation at 94 °C for 30 s, annealing at 56 °C for 90 s, extension at 72 °C for 90 s, and a final extension of 72 °C for 10 min. DNA fragments were run on an Applied Biosystems 3730 DNA Analyser. Size was determined by co-running a size standard (Genescan™-500 LIZ™, Applied Biosystems, USA) and fragments were scored manually with the aid of GeneMarker v1.8 Software (Soft Genetics, USA).

2.3. Data analysis

The quality of the multilocus data was evaluated using a locus-by-locus analysis in the software MICRO-CHECKER 2.2.3 (http://www.microchecker.hull.ac.uk/), searching for the presence of null alleles and/or technical artifacts such as stuttering. Because we sampled parasite stages after the clonal phase in the FIH, it was necessary to test the presence of individuals that may be clones. The probability that identical multilocus genotypes (MLGs) were the product of sexual reproduction (P_{sex}) was estimated using the program GENCLONE version 2.0 (http://www.ccmar.ualg.pt/maree/software.php?soft=genclon). If the P_{sex} of a multicopy MLG at $n = 2$ is significant ($P < 0.05$), then all copies of that MLG can be considered to be the product of clonal reproduction (Gregorius, 2005). Gene diversity (H_s), number of alleles per locus (N_{all}), and allelic richness (R_{all}, rarefied to the *S. sanguineus* sample size of 26) were calculated in Fstat version 2.9.3 (http://www.unil.chlizea/softwares/fstat.html). The Hardy–Weinberg equilibrium for each locus and the multilocus estimate of F_{IS} (Weir and Cockerham, 1984) with 10,000 randomisations (two-tailed tests) of alleles among individual parasites in SPAGEDI version 1.2 (http://ebe.ulb.ac.be/ebe/SPAGeDi.html) was tested. Hardy–Weinberg tests were conducted for all parasites within each host species or over all parasites from all host species. Genotypic disequilibrium for pairs of loci was tested in GENEPOP version 4.0 (http://genepop.curtin.edu.au/) within each host species sample. Significance was determined using the Markov chain method (10,000 dememorisations, 100 batches, 1,000 iterations per batch). The *P* value that was evaluated was the global test (Fisher's method) for each pair of loci across host species samples as implemented in GENEPOP.

To determine whether there was any cryptic structure without a priori delimiting population boundaries, two methods were used. First, the Bayesian approach was implemented in the program STRUCTURE version 2.3.4 (http://pritchardlab.stanford.edu/structure.html). We ran values of $K = 1$ to $K = 4$ with five replicates each (50,000 burn-in, 100,000 iterations) using the correlated alleles and admixture models. Second, the Principal Coordinates Analysis (PCoA), a multivariate method, was computed in GENALEX version 6.41 (http://biology.anu.edu.au/GenAlEx/Welcome.html).

Genetic structure was also analysed with hierarchical *F*-statistic analyses where we examined among parasites within hosts (F_{IS}), among host individuals within host species (F_{SC}) and among host species (F_{CT}). Two analyses were conducted. One analysis was conducted including all host species and the second included just the keyhole limpet hosts. These analyses were conducted in ARLEQUIN version 3.5.1.2 (http://cmpg.unibe.ch/software/arlequin35/) and significance at each level was tested by 10,000 permutations of alleles, individual parasites or host individuals for each respective level of the nested design. Our primary interest was in testing for among host species genetic differentiation. However, the hierarchical analysis was necessary because any local scale patterns of non-random transmission among host individuals that is not factored out could lead to the false inference of among host species differentiation. Finally, a post hoc analysis where we did hierarchical pairwise analyses between each host species (pairwise F_{CT} estimates and their respective *P* values) was conducted.

Table 1
Microsatellite diversity of *Proctoeces* cf. *lintoni* in limpets, *Fissurella* spp. and in the fish *Sicyases sanguineus*. Data are summarised for the 175 parasites genotyped (once repeated clones were removed, see Section 3) at nine loci and by host population.

Host species	N_H	N_P	H_s	N_{all}	R_{all}
Fissurella crassa	8 (2–8)	35	0.901	18.67	16.82
Fissurella maxima	7 (4–8)	41	0.913	18.67	16.53
Fissurella latimarginata	7 (1–8)	36	0.910	18.67	16.61
Fissurella cumingi	7 (3–8)	37	0.917	20.30	18.22
Sicyases sanguineus	7 (1–10)	26	0.909	16.70	16.78

N_H, number of hosts examined, followed by range of parasites genotyped from each host individual in parentheses; N_P, number of genotyped parasites; H_s, mean gene diversity per locus; N_{all}, mean number of alleles per locus; R_{all}, mean allelic richness per locus.

3. Results

From a total of 36 dissected hosts we genotyped 179 individuals of *P.* cf. *lintoni*. The evaluation of data quality found no null alleles or evidence of stuttering or scoring errors in any of the nine microsatellite loci tested.

There were only three repeated MLGs in the data set and all were identified as true clones (P_{sex} values < 0.05); one of these was repeated three times in two specimens from *F. cumingi*, a second was found in two individuals from *F. maxima* and the third in two individuals from *F. latimarginata*. Thus, the percentage of truly unique MLGs (i.e., different clonal lines) to genotyped individuals was 97.7% (175/179). Population genetic analyses with and without repeated clones can yield insights into trematode transmission before and after passage of the FIH (Prugnolle et al., 2005). Furthermore, analysis without clones is needed to make inferences about the mating system of the previous adult generation (Prugnolle et al., 2005). We did not notice any difference in analyses with or without the repeated clones, thus only analyses with repeated clones removed (i.e., $n = 175$) are reported. In part, no difference was observed as only four individuals (randomly chosen) needed to be removed from the data set.

Mean H_s, N_{all} and, R_{all} across the nine loci did not differ among the parasites from the different host species (Table 1, ANOVA; P values 0.75–0.95). Overall, there was no evidence for deviations from Hardy–Weinberg expectations (Table 2). In fact, only one of 45 locus-by-host species tests (Prolin 06 in *F. maxima*) showed significantly excessive heterozygosity ($P = 0.03$, Table 2). The multilocus estimate of F_{IS} in *S. sanguineus* (−0.039) was marginally significant ($P = 0.041$). However, these values are not significant if a Bonferroni correction is applied for multiple tests (Rice, 1989). Moreover, the multilocus F_{IS} over the entire data set was virtually zero and not significant (Table 2). Global tests for genotypic disequilibrium were not significant between all pairs of loci.

The clustering analyses did not detect any underlying structure. For example, the mean ln $P(D)$ was highest at $K = 1$ and continually declined to $K = 4$ in the Bayesian analysis of STRUCTURE (data not shown). The PCoA also showed no evidence of clustering with a random scattering of overlapping individuals from each host species (Fig. 2).

In the hierarchical analyses with or without the parasites from the fish host included (Table 3), there was significant genetic differentiation at the level of individual hosts within host species (F_{SC}). However, a post hoc analysis for each host species separately revealed that only in the keyhole limpet *F. crassa* was there statistically significant among-individual host structure ($P < 0.01$). Thus, the significance of F_{SC} was entirely driven by the parasites sampled from *F. crassa* hosts. At the level of host species (F_{CT}), there was no significant genetic differentiation among all host species or just the limpet species (Table 3). However, there was a large change in the P value when including the fish host ($P = 0.083$) and when excluding the fish host ($P = 0.365$). As a post hoc analysis, the hierarchical pairwise analyses between each host species were performed (Table 4). In these pairwise tests, only two of the comparisons had significant genetic differentiation ($P < 0.05$) and both of these involved the fish host (Table 4).

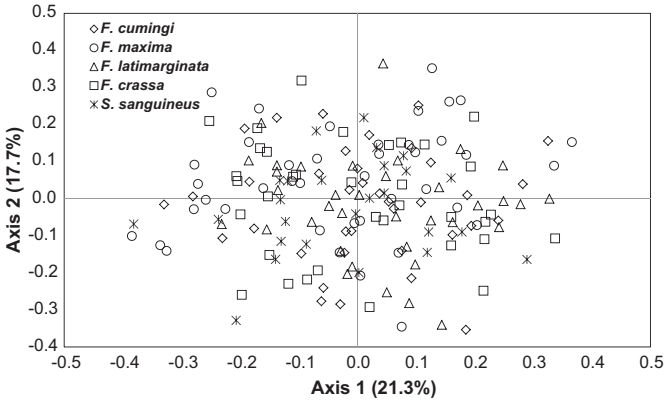

Fig. 2. Principal coordinates analysis based upon individual parasite multilocus genotypes without previous information on the origin of the host. There is no evidence of any underlying genetic structure. The parasites were obtained from different species of keyhole limpet of genus *Fissurella* (*F. cumingi*, *F. maxima*, *F. latimarginata* and *F. crassa*) and from the clingfish *Sicyases sanguineus*.

4. Discussion

We did not find any support for the idea that a precocious life cycle predisposes parasites to inbreeding. Inclusion of all parasites from all hosts yielded a multilocus F_{IS} of 0.005 with very little variance across loci (Table 2), which strongly suggests that *P.* cf. *lintoni* is a predominately outcrossing hermaphrodite with no inbreeding and no substructure among host species. These results strongly contrast to the only other precocious digenean, *Coitocaecum parvum*, for which there are multilocus genotypic data (Lagrue et al., 2009). This fluke has a three host life cycle (snail to amphipods to fish). In the SIH amphipods, *C. parvum* has facultative maturation and sexual reproduction. However, its life history contrasts with *P.* cf. *lintoni* in that upon penetration of the SIH, *C. parvum* cercariae form encysted metacercariae. Thus, even if more than one trematode infects an amphipod, individuals that precociously mature can only self-mate as they remain enclosed in the cyst. Indeed, *C. parvum* shows high levels of inbreeding with F_{IS} among 12 microsatellite loci ranging from 0.73 to 0.99 (Lagrue et al., 2009; we note the latter range excludes potentially duplicated loci in their data set; see Detwiler and Criscione, 2011). As noted by Lefebvre and Poulin (2005) and as indicated by our data, the lack of a cyst wall for *P.* cf. *lintoni* in its keyhole limpet SIH likely enables outcrossing in the SIH (although progeny-array data are needed to confirm this latter hypothesis) as well as in the fish DH.

Several studies have shown how host species may impact upon parasite differentiation on a local scale, possibly via intrinsic host-race formation mechanisms (e.g., McCoy et al., 2003; de Meeus et al., 2010; Gorton et al., 2012). Even though a facultatively precocious life cycle sets the stage for isolating mechanisms (extrinsic or intrinsic; McCoy et al., 2003) among the different keyhole limpet host species and between the keyhole limpet and fish hosts, we found no strong evidence that supports genetic differentiation among host species for the parasite *P.* cf. *lintoni*. It is interesting to note that Lagrue and Poulin (2009a) did not find any evidence that precocious reproductive development in *C. parvum* was heritable (i.e., the strategy adopted by offspring was independent of that used by their parents). A lack of heritability for precocious reproduction in *P.* cf. *lintoni* would likely slow both intrinsic and

Table 2
The inbreeding coefficient (F_{IS}) is given for each locus and multilocus per host population in the limpets, *Fissurella* spp. and in the fish *Sicyases sanguineus* and overall F_{IS}.

Locus	Overall $n = 175$ F_{IS}	*Fissurella crassa* $n = 35$ F_{IS}	*Fissurella maxima* $n = 41$ F_{IS}	*Fissurella latimarginata* $n = 36$ F_{IS}	*Fissurella cumingi* $n = 37$ F_{IS}	*Sicyases sanguineus* $n = 26$ F_{IS}
Prolin 02	0.007	0.0540	−0.004	0.0340	−0.026	−0.044
Prolin 04	0.027	0.0210	0.079	−0.0200	0.024	0.018
Prolin 05	−0.013	0.0890	−0.023	−0.0360	−0.078	−0.042
Prolin 06	−0.031	0.0005	−0.093[a]	0.0150	−0.027	−0.056
Prolin 07	0.012	−0.0080	0.010	0.0180	0.058	−0.064
Prolin 09	0.012	−0.0160	0.084	−0.0120	0.028	−0.078
Prolin 10	0.037	−0.0070	0.040	−0.0050	0.094	0.056
Prolin 14	−0.025	−0.0790	0.101	−0.0280	−0.062	−0.092
Prolin 15	0.017	0.0660	−0.005	0.0240	0.044	−0.062
Multilocus F_{IS}	0.005	0.0140	0.021	−0.0004	0.008	−0.039
Multilocus P value	0.680	0.4500	0.180	0.9900	0.620	0.041[a]

[a] $0.01 <$ 2-tailed $P \leqslant 0.05$.

Table 3
Hierarchical *F*-statistics analysis for all hosts (fish and limpets) together and for *Fissurella* spp. only.

	All hosts		*Fissurella* spp.	
	F-Statistic	*P* Value	*F*-Statistic	*P* Value
Parasites within hosts (F_{IS})	−0.002	0.587	0.003	0.362
Host individual within host species (F_{SC})	0.006	0.013[a]	0.009	0.003[a]
Host species to total (F_{CT})	0.002	0.083	0.0004	0.365

[a] Significant at $P < 0.05$.

Table 4
Pairwise F_{CT} analyses in limpets, *Fissurella* spp., and in the fish *Sicyases sanguineus*. Below the diagonal are F_{CT} values, above are *P* values.

Host species	*F. cumingi*	*F. maxima*	*F. latimarginata*	*F. crassa*	*S. sanguineus*
Fissurella cumingi	–	0.111	0.734	0.922	0.024[a]
Fissurella maxima	0.002	–	0.070	0.331	0.017[a]
Fissurella latimarginata	−0.001	0.003	–	0.351	0.059
Fissurella crassa	−0.003	0.001	0.001	–	0.131
Sicyases sanguineus	0.005[a]	0.006[a]	0.004	0.004	–

[a] Significant at $P < 0.05$.

extrinsic isolating mechanisms among host species relative to the situation if the trait was heritable. Thus, it would be of interest to test the heritability of precocious reproduction in *P.* cf. *lintoni*.

We did observe a few significant results: among individual hosts of the keyhole limpet *F. crassa* and two pairwise comparisons involving the fish host (Table 4). Because *F. crassa* inhabits the uppermost intertidal zone, flukes infecting this keyhole limpet species will only be exposed periodically to conditions when transmission is possible (i.e., high tides). Such periodic opportunities for transmission may create more local recruitment patterns among individual hosts and therefore lead to the differentiation we observed. However, our sample sizes were small on an individual host basis and thus more extensive sampling is needed to test the latter hypothesis. Significance driven by the fish host samples could be explained by the fact that the fish were sampled within a 2 km range of the limpets. If *P.* cf. *lintoni* has a wide distribution along the Chilean coast, then the parasites acquired by the fish may not be from the same locality as those of the keyhole limpets sampled. Any geographic influence on the genetic structure (e.g., isolation by distance) could then drive the differentiation observed (Table 4). More data are needed to test for possible geographic structure. Nonetheless, there was overall Hardy–Weinberg equilibrium even upon inclusion of the parasites from the fish samples (Table 1). Moreover, there was no significant structure among the host species in hierarchal analyses (Table 3). Thus, taken collectively, we find no support for parasite differentiation among host species.

It has been hypothesised that strictly aquatic parasites with intermediate hosts will behave as panmictic populations among hosts because ample opportunity exists for random mixing of unrelated individuals during transmission to the DH (Criscione and Blouin, 2006). Even with the precocious life cycle, these data for *P.* cf. *lintoni* support the aquatic-mixing hypothesis. There is extremely high allelic and genetic diversity (Table 1), suggesting a large effective population size for this parasite. Similar results were found among adults of the aquatic, non-precocious trematode *Plagioporus shawi* (Criscione and Blouin, 2006). In addition, few repeated clonal genotypes were observed; 97.7% of genotyped worms were unique MLGs. This is similar to reports for other digeneans in aquatic environments (Criscione et al., 2011; Gorton et al., 2012). The lack of multiple individuals of the same clone also suggests there is substantial mixing of clones before infection of the marine SIH. Similar results were found in other marine trematodes among their invertebrate SIH (*Maritrema novaezealandensis* in crabs and amphipods, Keeney et al. (2007); and *Gymnophallus* sp. in cockles, Leung et al. (2009)).

Lefebvre and Poulin (2005) highlighted four factors that may drive the evolution of facultative precocious reproduction: (i) internal host resources are suitable for egg production, (ii) instability of the environment reduces transmission to the next host, (iii) unavailability of the vertebrate host, and (iv) the total time spent in the intermediate host. Our study was not designed to test these hypotheses and we do not have data to comment on factor iv. Nonetheless, multiple factors may be at play in this system. For example, fecundity can be as high in the limpets as it is in the fish hosts (Oliva and Alvarez, 2011). Therefore, there appear to be host resources in the SIH for suitable egg production (factor i). Also, it is possible that because the intertidal environment is dynamic (i.e., exposed to periods of air), there is instability in transmission to the fish host (factor ii) or periods of unavailability of the fish hosts (factor iii). In addition, based on the patterns observed in this study, we present a fifth hypothesis for the evolution of a facultative precocious reproduction; i.e., the interplay between transmission (offspring mixing potential) and the mating system drives precocious reproduction. For example, Rauch et al. (2005) suggested that parasite life cycles may have evolved the use of second and subsequent intermediate hosts in order create a greater mixing pool of potential mates in the DH (see also Brown et al., 2001). In *P.* cf. *lintoni*, it appears that substantial mixing occurs even prior to infection in the SIH (possibly due to the marine environment dispersing the free-living cercarial stage). Although our data are not appropriate for addressing life cycle evolution itself, it is interesting to speculate that facultative precocious reproduction may have evolved (or may be evolving) because a third host is no longer needed to help promote mixing.

The interplay between mating and the transmission process also raises an additional mechanism that may trigger when maturation occurs in the SIH. Because individuals of *P.* cf. *lintoni* are not encysted in the SIH it would be interesting to test whether the presence of potential mates cues maturation in the SIH. This mechanism, however, is not expected for *C. parvum* because worms are encysted in the SIH. Rather, both time spent in the SIH and the chemical cues of predators, which are periodically absent, have been shown to alter maturation in *C. parvum* (Lagrue and Poulin 2009b).

In conclusions, in this study we tested whether a facultatively precocious life cycle could influence local scale (tens of meters) genetic structure. We found no such effect of life cycle variation on

local scale genetic patterns among individual parasites (i.e., no inbreeding) or among host species. In part, this may be due to the unencysted stage in the SIH and the high mixing potential of an aquatic environment. Moreover, the very high genetic diversity suggests a very large population size of the marine trematode *P.* cf. *lintoni*. It will be interesting to determine whether regional connectivity along the Chilean coast contributes to the large local effective population sizes. However, the latter needs to be tested as both limpets and clingfish have limited home ranges, which could promote parasite genetic structure among spatially separated local populations.

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