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MINIMAL SELFING, FEW CLONES, AND NO AMONG-HOST GENETIC STRUCTURE IN A HERMAPHRODITIC PARASITE WITH ASEXUAL LARVAL PROPAGATION

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Abstract.—Little is known about actual mating systems in natural populations of parasites or about what constitutes the limits of a parasite deme. These parameters are interesting because they affect levels of genetic diversity, opportunities for local adaptation, and other evolutionary processes. We expect that transmission dynamics and the distribution of parasites among hosts should have a large effect on mating systems and demic structure, but currently we have mostly speculation and very few data. For example, infrapopulations (all the parasites in a single host) should behave as demes if parasite offspring are transmitted as a clump from host to host over several generations. However, if offspring are well mixed, then the parasite component population (all the parasites among a host population) would function as the deme. Similarly, low mean intensities or a high proportion of worms in single infections should increase the selfing rate. For species having an asexual amplification stage, transmission between intermediate and definitive (final) hosts will control the variance in clonal reproductive success, which in turn could have a large influence on effective sizes and rates of inbreeding. We examined demic structure, selfing rates, and the variance in clonal reproductive success in natural populations of *Plagioporus shawi*, a hermaphroditic trematode that parasitizes salmon. Overall levels of genetic diversity were very high. An a posteriori inference of population structure overwhelmingly supports the component population as the deme, rather than individual infrapopulations. Only a single pair of 597 adult individuals was identified as clones. Thus, the variance in clonal reproductive success was almost zero. Despite being hermaphroditic, *P. shawi* appears to be almost entirely outcrossing. Genetic estimates of selfing (<5%) were in accordance with the proportion of parasites from single infections. Thus, it appears that individual flukes outcross whenever possible and only resort to selfing when alone. Finally, our data support the hypothesis that aquatic transmission and the use of several intermediate hosts promotes high genetic diversity and well-mixed infrapopulations.

Key words.—Clones, deme, genetic structure, *Oncorhynchus*, *Plagioporus shawi*, selfing rate, Trematoda.

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An important goal in evolutionary biology is to understand how factors such as the boundaries of demes and the mating systems in natural populations shape patterns and levels of genetic diversity within populations (e.g., Charlesworth 2003). For parasites, however, we know very little about actual mating systems in wild populations or what constitutes the limits of a deme (Criscione et al. 2005). For example, does selfing or outcrossing predominate within demes of hermaphroditic parasites? To assess the mating system, the boundaries of the deme must be determined. The question of what constitutes a parasite deme has been raised repeatedly because the limits of a parasite deme may not be related to only geographic isolation but also isolation among hosts within a geographic location (Lydeard et al. 1989; Nadler 1995; Bush et al. 2001; Jarne and Theron 2001; Sire et al. 2001). Here we define a deme as a cohesive genetic unit that has a recurrence of generations such that random genetic drift can occur over successive generations. For most macroparasite species (e.g., acanthocephalans, platyhelminths, nematodes), sexually mature adults are separated among infrapopulations of definitive hosts. Infrapopulations are all the parasites of a species in or on an individual host, whereas a component population refers to all parasites in a host population at a given place and time (Bush et al. 1997). Hence, do individual infrapopulations or do component populations constitute the deme?

In reality there is probably a continuum among parasite species along which various transmission processes lead to either infrapopulations or component populations behaving as demes (Fig. 1). For example, adults of most macroparasite species release offspring (eggs or larvae) into the external environment and, in general, do not multiply within or on their definitive host (Hudson et al. 2002). If transmission leads to a large mixing of parasite offspring before recruitment into definitive hosts, then individual infrapopulations will not have a succession of generations (Fig. 1A). As a result, the component population will be the evolving unit. On the other end of the continuum, if offspring are transmitted as a clump from host to host over several generations, then the component population behaves more like a traditional subdivided population with infrapopulations as demes (Figs. 1B,C).

The transmission processes presented in Figure 1 lead to different predictions about the patterns and levels of genetic diversity in a component population of parasites. These predictions can be tested with molecular data. When the component population functions as the deme (Fig. 1A), the component population should have high diversity and infrapopulations should be undifferentiated (as measured by F_{ST} or related statistics). When infrapopulations are demes (i.e., little mixing of parasite offspring), two models of population subdivision lead to different predictions. The classic island model (Wright 1969) predicts that increasing isolation among infrapopulations increases component population diversity because infrapopulations will tend toward fixation for different genetic variants (Fig. 1B; reviewed by Pannel and Charlesworth 2000). This prediction has been reiterated in recent papers modeling parasite populations (e.g., Prugnolle

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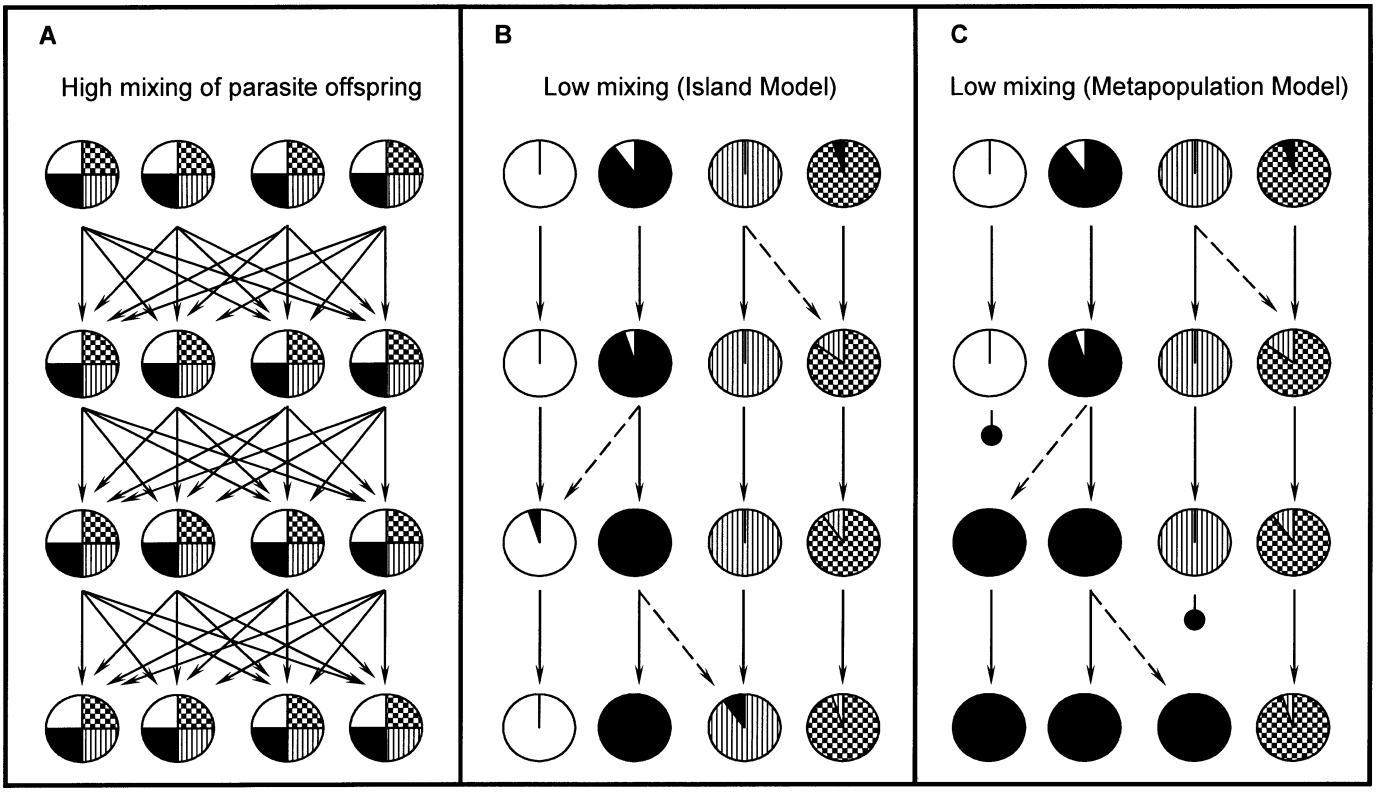

FIG. 1. Schematic showing the effects of parasite transmission on the genetic diversity of parasite populations. Circles represent infrapopulations in definitive hosts. Patterns within circles are different genetic variants of parasites. Four generations (rows from top to bottom) of adult parasites are illustrated. Solid arrows indicate major paths of recruitment for parasite offspring into definitive hosts. Dashed arrows show limited recruitment. Recruitment paths that terminate in small circles illustrate the extinction of an infrapopulation. Parasite offspring may pass through intermediate hosts before reaching definitive hosts. (A) A high amount of mixing among parasite offspring before recruitment into definitive hosts prevents differentiation among infrapopulations and maintains genetic diversity in the parasite component population (e.g., four infrapopulations of a given generation). Thus, the component population functions as the deme when offspring are well mixed. (B) Low mixing under an island model predicts high genetic differentiation among infrapopulations. However, with clumped transmission from one host to another over generations, genetic drift causes the fixation of different parasite genetic variants among infrapopulations. Thus, the component population will have high genetic diversity with low mixing under an island model (e.g., Prugnolle et al. 2005a,b). (C) Low mixing with extinction-recolonization dynamics predicts low levels of genetic diversity in the component population because of an increase in the variation in reproductive success among infrapopulations. In both (B) and (C), infrapopulations behave as demes because there is a stable recurrence of generations. Note that (A) is illustrated as an island model. However, high levels of migration (i.e., a high amount of mixing among parasite offspring) under a metapopulation model lead to the same predictions about genetic diversity as high migration under an island model (Pannel and Charlesworth 1999). Furthermore, the component population also functions as the deme under a metapopulation model with high mixing of parasite offspring. These schematics represent the extremes of different transmission processes. In reality, there is likely a continuum among parasite species along which infrapopulations or component populations define the boundaries of a deme.

et al. 2005a,b). These models assume that the infrapopulation remains stable over time. A metapopulation model, however, is an island model with extinction-recolonization dynamics (Pannel and Charlesworth 1999) and thus does not assume infrapopulations are permanent over time. The metapopulation model seems more realistic given that parasite transmission is dependent on such factors as host behavior, immunity, or physiological condition. For example, infrapopulations may go extinct if the host dies or deposits parasite offspring into unsuitable habitat. The metapopulation model predicts low genetic diversity for the component population because extinction-recolonization increases the variance in reproductive success among individuals across the component population (Fig. 1C). However, differentiation (F_{ST}) among hosts can increase or decrease depending on the source and number of colonizers and the extinction rates of infrapopulations (reviewed by Pannel and Charlesworth 2000). Although several studies have examined genetic structure (F_{ST}-based analyses) among infrapopulations (reviewed in Criscione et al. 2005), there has never been an explicit test to define the boundaries of a parasite deme.

Elucidation of mating systems and the effects of inbreeding on genetic diversity in natural populations of parasites is another sorely neglected topic (Criscione et al. 2005). Low intensities (the number of parasites in an infected host) may increase the rate of selfing in hermaphroditic species or may increase the chance of biparental inbreeding if parasite siblings are transmitted together. However, there exist almost no data on how parasite transmission affects inbreeding (Criscione et al. 2005). As a simple prediction, hermaphroditic parasites should outcross whenever possible and only self when alone if there are fitness costs to being inbred. That

inbreeding depression is an issue for selfing helminths is shown by laboratory experiments on a tapeworm that demonstrate higher infection success for outcrossed individuals (Christen et al. 2002; Christen and Milinski 2003). In addition, digenean trematodes have a unique aspect of reproduction in that there is an asexual amplification stage in the snail intermediate host. Models show that a high variance in clonal reproductive success can decrease the effective size of a parasite component population and increase differentiation among definitive hosts (Prugnolle et al. 2005a,b). Furthermore, mating between clones is equivalent to selfing in a hermaphroditic species. Thus, the questions of how abundant clones are and how often clones are transmitted together are important for understanding trematode mating systems and local levels of genetic diversity.

Here we address the following three questions with a microsatellite DNA study on the hermaphroditic parasite *Plagioporus shawi*, a digenean trematode that matures in the intestines of salmonid fishes (*Oncorhynchus* spp.). What unit along the continuum from individual infrapopulations to the entire component population functions as the deme? What is the selfing rate, and is it predicted by the proportion of worms in single infections? What is the variance in reproductive success of clones? To test whether infrapopulations function as the deme, we used relatedness estimates and a Bayesian method of clustering individuals into populations (Corander et al. 2003, 2004) that does not rely on a priori expectations of population delineation (as does the estimation of F_{ST} among hosts). We conclude that the entire component population of *P. shawi* functions as a deme. Genetic estimates of selfing are in the range predicted from the proportion of worms in single infections and indicate that *P. shawi* is largely outcrossing. Very few clones were found, indicating low variance in clonal success and/or high mixing of clones before transmission to the definitive host. Finally, comparisons with previous studies suggest that aquatic transmission and the use of multiple intermediate hosts promote a high degree of mixing of parasite offspring and high opportunity for outcrossing.

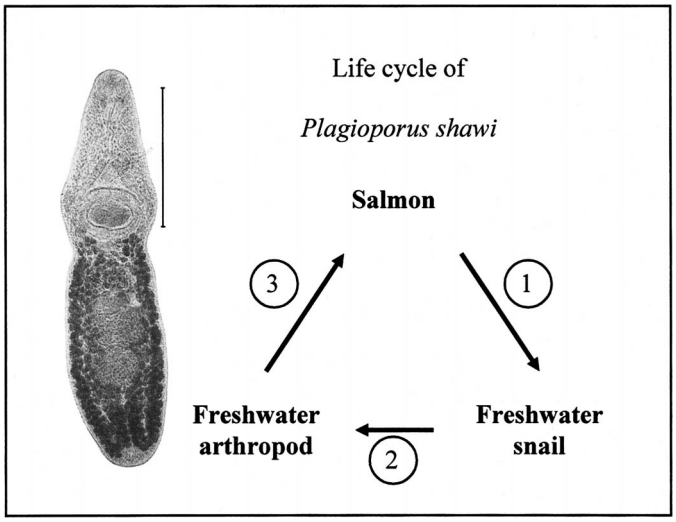

FIG. 2. Life cycle *of Plagioporus shawi* (Schell 1975). The adult flukes (picture shown; scale bar = 1mm) infect the intestines of salmonid fishes. Eggs pass into the freshwater stream via host feces (1). A miracidium hatches and penetrates a freshwater snail. In the snail, the miracidium becomes a mother sporocyst, which generates several daughter sporocysts. The daughter sporocycsts in turn produce many cercariae, which leave the snail (2) and penetrate an aquatic arthropod. The developmental stages within the snail are the asexual process polyembryony. The fish definitive host becomes infected (3) upon ingesting an infected second intermediate host. Notice that steps 1, 2, and 3 provide chances for mixing of parasite offspring before recruitment back into a definitive host. However, only steps 2 and 3 allow mixing of clonal flukes produced in the snail before recruitment into the definitive host.

MATERIALS AND METHODS

Species Background

Plagioporus shawi completes its life cycle within a freshwater stream (Fig. 2). Sexually mature adults infect the intestines of salmonids and pass eggs into the freshwater via host feces. A miracidium hatches and penetrates a freshwater snail, where a period of asexual reproduction occurs prior to cercarial development. Cercariae leave the snail and penetrate aquatic arthropods (e.g., amphipods, caddis larvae), where they encyst as metacercariae. The life cycle is completed when a fish ingests an infected arthropod (Schell 1975). It is important to note that digenean trematodes progress through asexual developmental stages in their first intermediate host (see Whitfield and Evans 1983). This process is often called ''polyembryony'' (Bush et al. 2001). The description by Schell (1975) of mother and daughter sporocysts in the snail indicates that *P. shawi* also goes through clonal reproduction within its snail host. The length of development in the different life cycle stages suggests that *P. shawi* has one to two generations per year (Schell 1975). The geographic range of *P. shawi* extends west of the Cascade Mountains from northern California to northern Washington, with reports in eastern Washington and western Idaho (Hoffman 1999). A previous study with mitochondrial DNA suggested that *P. shawi* has limited gene flow among freshwater drainages (Criscione and Blouin 2004).

Sampling

Geographic and temporal stability of our results were examined by sampling two creeks from separate river systems from March to April in 2002 and 2004. Cascade Creek (44°19.20′N, 123°50.89′W) feeds into the Alsea River, and Mill Creek (44°44.95′N, 123°47.60′W) into the Siletz River. Both the Siletz (SIL) and Alsea (ALS) Rivers are located in the coastal mountain range of central Oregon and drain into the Pacific Ocean. To insure that the parasites originated from the respective stream, we sampled outmigrating smolts (juvenile salmonids leaving the drainage for the ocean) rather than returning adults. Collections were made in conjunction with Oregon salmonid-monitoring projects under the permits OR2002-019 and OR2004-1431. Twelve steelhead trout (*Oncorhynchus mykiss*) and 30 cutthroat trout (*Oncorhynchus clarki*) were collected in both time periods from SIL, whereas 30 cutthroat were sampled from ALS in both time periods. Standard parasitological techniques (Pritchard and Kruse 1982) were used to recover individuals of *P. shawi* from

hosts. Individual flukes (trematodes) were identified with wet mounts prior to storage in 70% ethanol.

Genotyping

Protocols for DNA extraction, polymerase chain reaction, and genotyping are given in Criscione and Blouin (2005). Flukes from ALS and SIL were genotyped at nine microsatellite loci (m26, m41, m48, d04, d09, d13, d36, d43, d47; GenBank accession numbers AY894897–AY894905), which are described in Criscione and Blouin (2005). Failed amplifications and significant deviations from Hardy-Weinberg suggested the presence of null alleles at d13 and d47 in SIL. Thus, d13 and d47 were omitted from SIL analyses. Controls with fish DNA were always negative (Criscione and Blouin 2005). All collected flukes were genotyped, except 14 individuals from ALS-2002, which were used as a pooled sample in primer development (Criscione and Blouin 2005). These 14, which came from a single host, were included in the estimates of prevalence (percent hosts infected) and intensity (number of parasites per infected host).

Prevalence and Intensity Analyses

To evaluate if the distribution of parasites among hosts was stable over time and among geographic populations, we compared prevalence and mean intensity of infection among samples. Comparisons of mean intensities are important for understanding any differences among samples in selfing or biparental inbreeding. Logistic regression was used to test for differences in prevalence, where the model included year and location and the interaction between year and location. Similarly, a two-way ANOVA, where the model included year and location and the interaction between year and location, was used to test for differences in intensity. Log-transformed intensities were used because the data were not normally distributed. Residual plots were examined visually for violations of normality or homogeneity of variance. Systat version 9.01 (SPSS, Inc., Chicago, IL) was used for both statistical analyses.

Identification of Clones

Identical multilocus genotypes were identified by searching for repeated data entries in Microsoft (Redmond, WA) Access. The program MLGSIM (Stenberg et al. 2003) was used to test if identical multilocus genotypes were likely to have occurred by chance given sexual reproduction. Rejection of sexual reproduction indicates that the identical multilocus genotypes are likely the result of clonal reproduction.

Molecular Analyses for Assessing the Boundaries of the Deme

We assessed whether individual infrapopulations or the component population of *P. shawi* define the limits of the deme by using three methods. First, BAPS version 3.1, which uses Bayesian inference to delineate populations (Corander et al. 2003, 2004), was used to cluster individual parasites. BAPS version 3.1 finds clusters that are in Hardy-Weinberg equilibrium with the assumption that loci are in linkage equilibrium. Details of BAPS version 3.1 can be found at http://www.rni.helsinki.fi/~jic/bapspage.html. An expectation for the maximum number of clusters (K) is needed as a prior. If infrapopulations are acting as demes, then a logical choice for a maximum K is the number of infected hosts from which parasites were genotyped. Therefore, maximum K was set to 24, 24, 35, and 32 for ALS-2002, ALS-2004, SIL-2002, and SIL-2004, respectively. As mentioned in the BAPS manual, if K is set too large, the algorithm make get stuck on a local mode. The program, however, allows multiple inputs of K and will return the optimal partition after conducting a separate analysis for each K. The optimization algorithm is stochastic. Therefore, different results can be obtained for the same value of K. Thus, we used 10 replicates for each K-value ranging from two to the maximum K mentioned above. BAPS also identifies which individual parasites belong to each cluster in the optimal partition. Thus, after obtaining the optimal partition, we tested the null hypothesis of no association between the BAPS-identified cluster to which each parasite was assigned and the actual infrapopulation from which it was collected. Clumped transmission would cause a significant association, while substantial mixing each generation would lead to no association. To analyze the contingency tables of infrapopulations by BAPS-identified parasite clusters, we used the program RxC (program distributed by author, M. P. Miller, and freely available from http://www.marksgeneticsoftware.net/). RxC employs the metropolis algorithm to obtain an unbiased estimate of the exact P-value (i.e., Fisher's exact test) for any sized contingency table. The following Markov chain parameters were used to test significance: 5000 dememorizations, 5000 batches, 5000 permutations per batch. In the SIL samples, two host species, *O. mykiss* and *O. clarki*, were sampled. Therefore, we also ran exact tests to see if there was an association between BAPS-identified parasite clusters and host species.

F-statistics (or related analogs) and tests of population differentiation are commonly used to describe genetic structure among infrapopulations (e.g., Prugnolle et al. 2005c). To provide a basis of comparison to previous studies, our second method for evaluating the deme employed the use of the unbiased estimator F_{ST} (Weir and Cockerham 1984; calculated by FSTAT ver. 2.9.3, Goudet 1995). The G-based test (Goudet et al. 1996) with 15,000 permutations of individual parasites among hosts was used to test for differentiation among infrapopulations. Complete multilocus genotypes were permuted, as opposed to individual alleles, to mimic the recruitment of individual parasites. The 95% confidence intervals of F_{ST} were calculated by bootstrapping over loci with FSTAT.

If transmission of parasites is clumped, then it should be possible to detect related parasites within infrapopulations, especially when the number of breeders per infrapopulation (approximated with mean intensities) is small. We examined the average relatedness (R) of parasites within hosts using Relatedness 5.08 (program distributed by author, K. F. Goodnight, and freely available from http://www.gsoftnet.us/GSoft.html), which uses the algorithm of Queller and Goodnight (1989). To assess the sampling error of infrapopulations, jackknife resampling over hosts was used to calculate the 95% confidence intervals. We also wanted to test if the average relatedness within hosts was different from that ex-

pected with random parasite recruitment. For this test, we permuted individuals among hosts 10,000 times using the program SPAGEDI version 1.2 (Hardy and Vekemans 2002).

Mating System and Genetic Diversity Analyses

Based on the deme analyses, we concluded that the component population of *P. shawi* functions as the evolving unit (see Results and Discussion). Thus, in each location by time period sample, analyses of mating system and genetic diversity included all genotyped parasites from all hosts. Genotypic disequilibrium for pairs of loci (36 and 21 pairwise comparisons for ALS and SIL, respectively) was tested using GENEPOP web version 3.4 (http://wbiomed.curtin.edu.au/genepop; Raymond and Rousset 1995). Significance levels were determined using the Markov chain method (5000 dememorizations, 5000 batches, 5000 iterations per batch). The Weir and Cockerham (1984) estimator of F_{IS} for each locus and of the multilocus F_{IS} was calculated in SPAGEDI. Deviations from Hardy-Weinberg equilibrium were tested with two-tailed tests of 10,000 randomizations of alleles among individuals. Significance was set at $P \leq 0.05$. A sequential Bonferroni correction was applied for the multiple comparisons in the genotypic disequilibrium tests within each population sample (e.g., significance determined at $P \leq 0.05/21 = 0.0024$ in SIL-2002). Likewise, a sequential Bonferroni correction was used within each population sample to test for deviations from Hardy-Weinberg equilibrium across multiple loci (e.g., $P \leq 0.05/7 = 0.0071$ in SIL-2002). Gene diversity (H_s; Nei 1987) and allelic richness (rarefied to the ALS-2002 sample size of 94) were calculated with FSTAT.

The proportion of flukes present in single infections provides a rough prediction of the selfing rate because infrapopulations define discrete mating boundaries for *P. shawi*. Gravid flukes from single infections were observed in this study. The selfing rate was estimated two ways from the genetic data. The first is based on the relationship $F_{IS} = S/(2 - S)$, where S is the proportion of selfed offspring assuming inbreeding equilibrium (Hedrick 2005). Multilocus estimates of F_{IS} were used in the above equation. The second method uses the distribution of estimated individual inbreeding coefficients (IIC). Recent studies have used IIC to provide a fine-scale analysis of mating systems (Sweigart et al. 1999; Ruzzante et al. 2001; Vogl et al. 2002; Muir et al. 2004). However, these studies rely on an arbitrary cutoff for the IIC to define the proportion of selfed or inbred individuals. Here, we use a maximum likelihood (ML) method to estimate what proportion of individuals of the observed IIC distribution is the result of a selfing event. This method is based on the method of Queller et al. (2000), who estimated the proportion of unrelated individuals from an observed distribution of relatedness coefficients. We simulated 2000 individuals under monoecy with random mating (without selfing) and with complete selfing using BottleSim version 2.6 (Kuo and Janzen 2003). Individuals were drawn from a population with the observed microsatellite allele frequencies. Two measures of IIC, Loiselle et al. (1995) and Ritland (1996), were calculated for the simulated and observed individuals using SPAGEDI. IIC were binned into intervals of width 0.02 for the likelihood calculations.

TABLE 1. Contingency table of BAPS-identified parasite clusters by host species from SIL-2004 ($P = 0.54$, SE = 0.0005).

	Parasite cluster							
Host species	1	2	3	4	5	6	7	$\sum$[1]
Oncorhynchus clarki	2	7	0	2	111	2	0	124
Oncorhynchus mykiss	1	4	1	1	49	1	1	58
$\sum$[2]	3	11	1	3	160	3	1	182

[1] Row sums are the total number of parasites genotyped from 21 infected *O. clarki* and 11 *O. mykiss*.
[2] Column sums are the cluster sizes from BAPS version 3.1.

RESULTS

Prevalence and Intensity

Prevalence was high at 83%, 80%, 83%, and 76% for ALS-2002, ALS-2004, SIL-2002, and SIL-2004, respectively. The interaction between year and location and the main effects of year and location were not significant (P values > 0.05). Mean intensities were low (4.3 ± 3.9 SD, 5.2 ± 3.6, 5.6 ± 4.2, 5.7 ± 6.2 for ALS-2002, ALS-2004, SIL-2002, and SIL-2004, respectively) and were also not significantly different between years or locations ($P > 0.05$). Thus, prevalence and mean intensities were similar between sites and between years.

Identified Clones

A single pair of individuals with identical multilocus genotypes was identified from one host in ALS-2004. After 1×10^6 simulations in MLGSIM, the P_{sex}-value (the likelihood of finding at least as many identical multilocus genotypes as observed in a panmictic population) of the observed pair fell outside of the distribution of simulated P_{sex}-values ($P < 0.002$). Thus, the two individuals were considered to be clones. Both individuals were included in the prevalence and intensity analyses. However, one of the clones was removed for all subsequent genetic analyses because clonal reproduction in trematodes does not reflect reproductive events in the previous parental generation.

Assessing the Boundaries of the Deme

The numbers of optimal partitions from BAPS were nine, six, eight, and seven for ALS-2002, ALS-2004, SIL-2002, and SIL-2004, respectively. However, the majority of individuals in each sample belonged to one cluster (83%, 93%, 93%, and 88% for ALS-2002, ALS-2004, SIL-2002, and SIL-2004, respectively). With one exception in SIL-2004 (Table 1), the remaining clusters from all population samples had only one to four individuals. The column sums of Table 1 show an example of the cluster sizes from SIL-2004. All exact tests among infrapopulations or among host species (SIL samples) were not significant ($P > 0.05$). Thus, there was no association between BAPS-identified clusters and individual infrapopulations or host species (SIL samples). Sample sizes for the exact test between fish species in SIL-2004 were 115 flukes from 25 infected cutthroat trout and 82 from 10 infected steelhead trout. The contingency table and sample sizes for the exact test among host species in SIL-2004 are shown in Table 1.

TABLE 2. Structure among (F_{ST}) and relatedness (R) within infrapopulations.

Population	Host N[1]	Parasite N[2]	F_{ST} (95% CI)[3]	Average within-host R (95% CI)[4]
ALS-2002	24	94	0.002 (−0.008, 0.011)	−0.0115 (−0.037, 0.014)
ALS-2004	24	123	0 (−0.008, 0.006)	−0.0061 (−0.022, 0.01)
SIL-2002	35	197	0 (−0.009, 0.006)	−0.0026 (−0.015, 0.01)
SIL-2004	32	182	0.01 (0.005, 0.014)	0.0142 (−0.004, 0.032)

[1] The number of infected hosts from which parasites were genotyped.
[2] The total number of genotyped parasites.
[3] Confidence intervals for F_{ST} were obtained by bootstrapping over loci.
[4] Confidence intervals for R were obtained by jackknifing over hosts.

Randomly mating populations will still have individuals on the tails of a hypothetical statistical distribution. Given that BAPS forms clusters with the assumptions of Hardy-Weinberg equilibrium and that loci are in linkage equilibrium, we suspect that the satellite groups possibly represent these tails. Several lines of evidence indicate that the additional clusters are not biologically meaningful. First, examination of the genotypes of the individuals excluded from the main cluster did not reveal any systematic pattern for their exclusion. For example, these individuals were not largely homozygous across loci. Nor did individuals in the same satellite groups appear to share rare alleles. Second, except for two individuals in SIL-2002 (each in their own cluster), all satellite clusters were genetically more similar to the main cluster than to any other cluster. This latter result is based on the Kullback-Leibler divergence matrix, which can be used as a measure of relative genetic distance between clusters, provided by BAPS version 3.1. Thus, it appears that the satellite clusters stem from the main cluster. This result is consistent with the idea that these individuals are in the tails of the random mating distribution. Finally, datasets based on simulations of randomly mated populations produced qualitatively similar results (see Supplementary Table 1 available online at http://dx.doi.org/10.1554/05-421.1.s1). The observed number of clusters and proportion of individuals in the main cluster observed in our four population samples always fell within the range observed in our simulated datasets (Supplementary Table 1).

There was also no sign of genetic structuring among hosts or that related parasites were transmitted together (Table 2). All permutation tests of population differentiation among hosts were not significant ($P > 0.05$). All confidence intervals, but the F_{ST} for SIL-2004, overlapped with zero (Table 2). Likewise, the confidence intervals for the average within-host relatedness contained zero (Table 2). The permutations performed in SPAGEDI showed that within-host relatedness was not significantly different than expected from random recruitment. Therefore, both a priori and a posteriori analyses strongly support the conclusion that the entire component population functions as a single, well-mixed deme.

Genetic Diversity and Selfing Rates

After Bonferroni corrections, there was neither support for genotypic linkage between pairs of loci nor deviations from Hardy-Weinberg among all loci in each population sample. (Table 3 shows the by-locus measures of H_s, allelic richness, and F_{IS}.) Furthermore, the multilocus estimates of F_{IS} were not significantly different from those expected given random allocation of alleles among individuals (Table 4). Mean H_s was high and above 0.8 in all population samples (Table 4). Likewise, the mean allelic richness was high and above 21 in all population samples (Table 4). The mean allelic richness in the SIL samples was slightly higher than the ALS samples because d13 and d47, which had fewer alleles than most of the other loci (Table 3), were excluded from the SIL analyses.

Although no population was significantly out of Hardy-Weinberg equilibrium, the nominal values of multilocus F_{IS} were slightly positive (Table 4). Thus, there may be low levels of selfing or biparental inbreeding in these populations. The proportion of worms in single infections suggests a potential range of selfing from about 1% to 10% (Table 4). In accordance with this range, the inbreeding equilibrium measure of selfing, S, ranges from 2.4% to 4.5% (Table 4). The

TABLE 3. Gene diversity (H_s), number of alleles (A), and F_{IS} by locus.

	ALS-2002 (94)[1]			ALS-2004 (123)			SIL-2002 (197)			SIL-2004 (182)		
Locus	H_s	A	F_{IS}	H_s	A[2]	F_{IS}	H_s	A	F_{IS}	H_s	A	F_{IS}
m41	0.476	21	−0.027	0.399	22 (19.0)	−0.038	0.508	22 (16.9)	−0.010	0.443	22 (16.9)	0.020
m26	0.840	22	0.037	0.825	22 (21.6)	0.005	0.759	21 (19.8)	0.064	0.798	21 (19.8)	0.077
m48	0.960	35	−0.008	0.958	38 (35.2)	−0.010	0.958	34 (31.1)	−0.023	0.957	33 (30.7)	0.007
d36	0.902	28	−0.026	0.880	29 (26.5)	0.020	0.911	34 (29.6)	0.003	0.894	31 (27.3)	−0.032
d04	0.813	10	0.045	0.832	13 (12.5)	−0.016	0.851	15 (13.3)	0.040	0.843	15 (13.0)	−0.016
d09	0.955	30	0.042	0.957	30 (29.1)	0.032	0.953	32 (28.0)	0.036	0.954	33 (28.7)	0.038
d43	0.913	25	0.021	0.904	21 (20.5)	0.011	0.901	28 (24.0)	−0.020	0.909	30 (25.1)	−0.003
d13	0.856	14	0.055	0.846	15 (14.6)	0.049	—	—	—	—	—	—
d47	0.828	17	0.050	0.820	16 (15.6)	0.048	—	—	—	—	—	—

[1] The total number of parasites genotyped.
[2] In ALS-2004, SIL-2002, and SIL-2004, the number of alleles (A) is followed in parentheses by the allelic richness rarefied to the ALS-2002 sample size of 94.

TABLE 4. Mean gene diversity (H_s), mean allelic richness, multilocus F_{IS}, selfing rates, and percentage of flukes in single infections (SI).

Population[1]	Mean H_s	Mean allelic richness[2]	Multilocus F_{IS}	S[3]	ML-Ritland[4]	ML-Loiselle[5]	SI
ALS-2002	0.838	22.4	0.023	4.5%	3.3%	0%	10.6%
ALS-2004	0.825	21.6	0.014	2.8%	1.1%	1.3%	0.8%
SIL-2002	0.834	23.2	0.013	2.6%	1.4%	1.8%	3.0%
SIL-2004	0.828	23.1	0.012	2.4%	3.2%	1%	2.2%

[1] All calculations for ALS were based on nine loci, and seven loci were used for SIL.
[2] Mean allelic richness is the average allelic richness among loci (values rarefied to a sample size of 94; see Table 3). Because ALS-2002 had a sample size of 94, the mean allelic richness is equal to the mean number of alleles per locus.
[3] S is the proportion of selfed offspring and was calculated with the formula $F_{IS} = S/(2 - S)$, where F_{IS} is the multilocus estimate.
[4] ML-Ritland is the maximum likelihood estimate of the proportion of selfed individuals using the individual inbreeding coefficient of Ritland (1996) as calculated in SPAGEDI version 1.2 (Hardy and Vekemans 2002).
[5] ML-Loiselle is the maximum likelihood estimate of the proportion of selfed individuals using the individual inbreeding coefficient of Loiselle et al. (1995) as calculated in SPAGEDI version 1.2 (Hardy and Vekemans 2002).

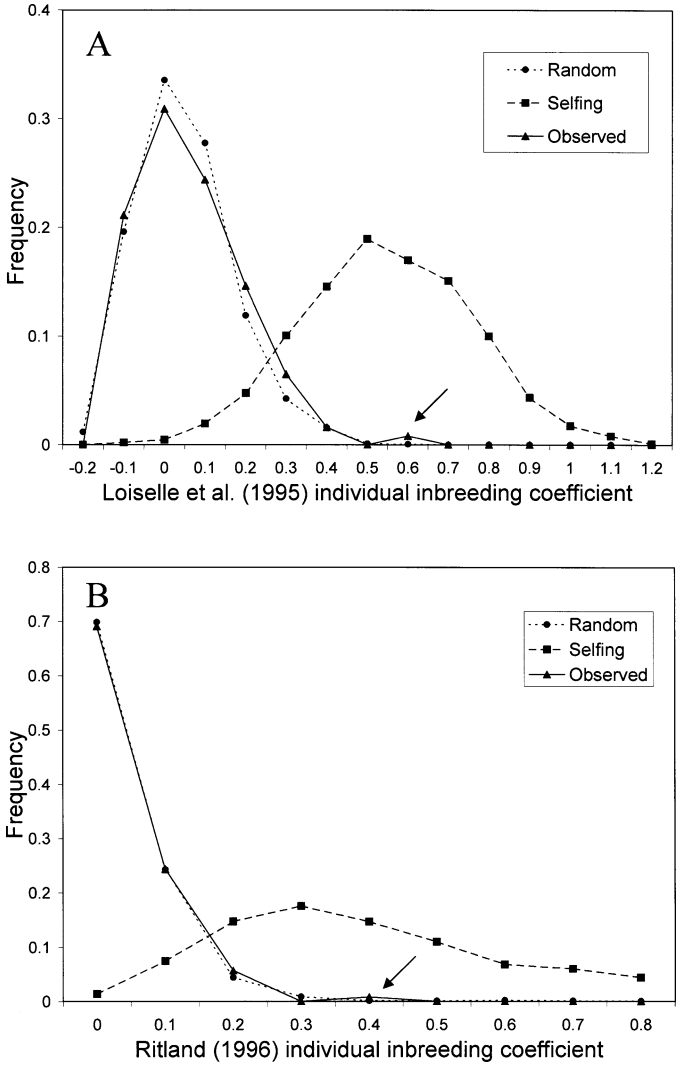

FIG. 3. Frequency distributions of individual inbreeding coefficients (IIC) for ALS-2004. (A) Loiselle et al. (1995) IIC. (B) Ritland (1996) IIC. IIC were simulated under random mating and full selfing. The observed distributions of IIC closely approximate the IIC distributions simulated under random mating. The arrows indicate individuals that are likely the result of selfing.

ML estimates of the fraction of selfed individuals using either the Ritland (1996) or Loiselle et al. (1995) IIC were lower than the estimates from S, ranging from 0% to 3.3% (Table 4). The distributions of observed IIC closely approximated the IIC distributions that were simulated under random mating (Fig. 3). There was the occasional individual(s), however, whose score extended beyond the random mating distribution (Fig. 3), thereby suggesting a very low proportion of individuals that are the result of selfing.

DISCUSSION

What Is the Deme?

Our results overwhelmingly support the component population, rather than individual infrapopulations, as the functional deme in *P. shawi* (Fig. 1A). The BAPS inference of population structure grouped over 80% of the individuals into a single cluster within each population sample. The lack of association between BAPS-identified clusters and individual hosts or host species (e.g., Table 1) indicates that infrapopulations are not causing fractionated parasite gene pools and that there is no host specificity between cutthroat and steelhead trout. A lack of differentiation among infrapopulations (G-based test) and an average within-host relatedness of zero (Table 2) further support the hypothesis that a large degree of mixing occurs among parasite offspring before they recruit into definitive hosts.

Mating System and Clonal Reproduction

Little is known about mating systems in wild populations of parasites. Indeed, we are aware of only three studies that examined whether a hermaphroditic macroparasite is largely outcrossing or selfing. Deviations from Hardy-Weinberg were used to infer a 70% outcrossing rate for the trematode *Lecithochirium rufoviride* (one allozyme locus; Vilas and Paniagua 2004), 84% for the cestode *Proteocephalus exiguus* (three allozyme loci; Snabel et al. 1996), and 1.1% for the cestode *Echinococcus granulosus* (four allozyme loci; Lymbery et al. 1997). In *P. shawi* estimates of outcrossing rate derived from F_{IS} ranged from 95.5% to 97.6% (Table 4). If we assume that all inbred individuals result from selfing events and not biparental inbreeding (a reasonable assumption given the average within-host relatedness was zero), then

we estimate selfing rates from 2.4% to 4.5% (Table 4). Direct ML estimates of the fraction of individuals that resulted from a selfing event (e.g., Fig. 3) were very similar, ranging from 0% to 3.3% (Table 4). All genetic selfing estimates are surprisingly close to those predicted by the fraction of individuals that are found in single-worm infections (Table 4). This result supports the simple prediction that parasites should outcross whenever possible and resort to selfing only when alone.

We identified only a single pair of individuals as clones of a total of 597 genotyped parasites. There are several interesting implications from this result. First, clonal propagation results in no more than one representative at most per definitive host. Thus, in *P. shawi* multiple copies of clones are rarely recruited into definitive hosts or else they are being thoroughly mixed among hosts. This result stands in sharp contrast to results from studies on liver flukes of deer (*Fascioloides magna*; Mulvey et al. 1991) and shistosomes of rats (*Schistosoma mansoni*; Theron et al. 2004), in which identical multilocus genotypes were common. Second, clonal reproduction in the first intermediate host (snails) has the potential to greatly increase the variance in reproductive success among individual genotypes (Prugnolle et al. 2005a,b). In *P. shawi*, nevertheless, clonal reproductive success is very low and the variance in reproductive success of clones is basically zero. Third, clonal reproduction can lead to inbreeding if identical clones transmit together. However, the selfing rates estimated above are unlikely to be influenced by the mating between clones. Although these phenomena may be important in other parasites, clonal amplification appears to have little effect on the genetic composition of populations of *P. shawi*.

Genetic Diversity

The question of what controls genetic diversity in parasite populations has received little empirical attention (e.g., Blouin 1998). Among plants and snails, selfing species tend to have lower levels of diversity than outcrossing species (Jarne 1995; Charlesworth 2003). Nevertheless, here we found that a hermaphroditic trematode has very high levels of genetic diversity, with mean $H_s > 0.8$ (Table 4) and allelic richness ranging from 10 to 35 (Table 3). In this case the species appears to outcross preferentially and to occur in well-mixed populations that are not subdivided. *Schistosoma mansoni* is the only other trematode for which comparable microsatellite data are available. Even though *S. mansoni* is dioecious, it is substantially less diverse than *P. shawi*. Populations of *S. mansoni* in rats on Guadeloupe have mean H_s of around 0.5 and a range of two to 19 alleles among seven loci (Prugnolle et al. 2005c). If we estimate allelic richness in *P. shawi* after rarifying our sample size to the same as that in Prugnolle et al. (2005c; about 40 individuals), allelic richness is still higher in *P. shawi*, ranging from eight to 28 alleles.

Plagioporus Versus Other Flatworms: Effects of Aquatic Versus Terrestrial Environment

Plagioporus shawi shows higher genetic diversity than *S. mansoni* and less co-occurrence of clones than in either *S. mansoni* or *F. magna*. Both *F. magna* and *S. mansoni* have semiterrestrial transmission and two-host life cycles (Mulvey et al. 1991; Sire et al. 2001; Prugnolle et al. 2002; Theron et al. 2004). After leaving an aquatic snail, clonal cercariae of *F. magna* encyst on vegetation as metacercariae. Thus, it is likely that a deer host will ingest a clump of metacercariae consisting of a many copies of the same clone. Cercariae of *S. mansoni* are released into shallow pools and directly penetrate the rat host. Therefore, clumped transmission of clones may be likely if a rat remains temporarily stationary in a wet area. Furthermore, eggs of trematodes having terrestrial definitive hosts may often be deposited into habitat unsuited for transmission, thereby potentially causing greater reproductive skew among infrapopulations or possibly even infrapopulation extinction (Fig. 1C). Such clumped transmission, extinction dynamics, or clonal variation in reproductive success may cause a greater variance in reproductive success among individual genotypes and further explain lower genetic diversity in species such as *S. mansoni* (Theron et al. 2004; Prugnolle et al. 2005c). However, in a purely aquatic species such as *P. shawi*, eggs will always be deposited into water, and the aquatic environment is conducive to the dispersal of low mobility larval stages such as cercariae. *Plagioporus shawi* also has a three-host life cycle that includes several potential arthropod second intermediate hosts (Schell 1975). After leaving the snail, cercariae of *P. shawi* first penetrate an arthropod that is then eaten by the fish definitive host. Arthropod intermediate hosts may harbor multiple metacercariae (Schell 1975). It may be that the second intermediate hosts of *P. shawi* accumulate several distinct cercarial genotypes before being eaten, as is the case with the trematode *Diplostomum pseudospathaceum* (Rauch et al. 2005). Furthermore, definitive hosts may consume many intermediate hosts. Thus, in aquatic trematodes having second intermediate hosts there may be more opportunity for genotypes to be widely dispersed and mixed before they enter a definitive host. As indicated by the model of Prugnolle et al. (2005a), mixing before and after asexual reproduction can decrease F_{ST} among hosts. Sampling intermediate hosts for *P. shawi* could help determine if mixing is occurring at one or all potential dispersal stages in the life cycle (Fig. 2). However, due to the lack of clones, it appears that a large degree of mixing does occur after infection of the snail hosts.

In the comparison of outcrossing rates among different flatworms, the two species having high outcrossing rates, *L. rufoviride* and *P. exiguous*, have aquatic transmission and use multiple intermediate hosts, while the highly inbred *E. granulosus* has a completely terrestrial life cycle with direct transmission between intermediate and definitive hosts. *Echinococcus granulosus* amplifies asexually in the sheep intermediate host and is transmitted from the sheep to the canid definitive host by direct ingestion. This mode of transmission obviously creates ample opportunity for mating between individuals of the same clone, a form of selfing.

Therefore, we have identified intriguing differences in patterns of transmission and genetic diversity among parasite species that might be explained by the use of second intermediate hosts and by whether the life cycle is primarily terrestrial or aquatic. More comparative studies are needed, but we predict that, in general, aquatic species and those having

multiple intermediate hosts will have the highest local genetic diversity and genetically most well-mixed infrapopulations.

Plagioporus Versus Animals with Alternating Sexual and Asexual Reproductive Life Cycles

Many organisms (e.g., aphids, rotifers, cnidarians) have alternating sexual and asexual reproductive life cycles that are similar to digenean life cycles (see Prugnolle et al. 2005b). Many of these species differ from digeneans in that breeding adults may be able to reproduce sexually and asexually, such that asexual lines can continue across multiple generations. Digeneans have an obligate sexual phase that will break clonal lines. Despite this difference, a few comments on interesting parallels are in order. First, the patterns and levels of genetic diversity that we find for *P. shawi* are often reported in aphid plant parasites (e.g., Guillemaud et al. 2003; Halkett et al. 2005) and have been observed in the nonparasitic rotifer *Brachionus plicatilis* (Gómez and Carvalho 2000). This highlights that animal parasitism does not preclude random mating or high genetic diversity. Second, transmission patterns seem to influence mating patterns in other organisms that alternate between sexual and asexual reproduction. The use of different plant species for the sexual and parthenogenetic phases can impact aphid mating systems (Hales et al. 1997). This is similar to our hypothesis about multiple intermediate hosts increasing mixing, but here the aphid dispersal (not host dispersal) is responsible for creating the mixing opportunities. For example, significant heterozygote deficits in populations of the aphid *Macrosiphoniella tanacetaria* were attributed, in part, to inbreeding (Massonnet et al. 2002). Few winged morphs and the use of a single host plant may have limited dispersal, thereby increasing the potential for inbreeding. In contrast, *Myzus persicae* and *Rhopalosiphum padi* have primary (sexual stage) and secondary (parthenogenetic phase) host species. Populations of the cyclical parthenogenetic variants of these species were found to be mostly in Hardy-Weinberg equilibrium and to have no or few clones (Guillemaud et al. 2003; Halkett et al. 2005). Here, the migrations to the different host species may promote admixture (Hales et al. 1997).

Summary

Our molecular data clearly show that the transient compartmentalization of *P. shawi* into infrapopulations each generation does not result in fragmented gene pools. *Plagioporus shawi* component populations appear to exist as a single, mostly randomly mating unit, and selfing only occurs as a last resort in isolated individuals. Furthermore, our prevalence, intensity, and genetic data were consistent over space and time, and so our results do not represent an atypical sample. It is interesting to note the contrast between our data on *P. shawi* and one of the general predictions made by Price (1977, 1980) concerning the genetic structure of parasite populations. He predicted that because parasites inhabit ephemeral and patchy environments (i.e., infrapopulations in hosts), parasite populations should be subject to large population fluctuations and chance colonization events that promote inbreeding and fractionated gene pools. These predictions are similar to what current-day metapopulation models predict for genetic structure within and among demes when there is low mixing (Fig. 1C; Pannel and Charlesworth 2000). *Plagioporus shawi*, however, obviously has a large degree of mixing of parasite offspring before recruitment into definitive hosts and, thus, does not fit Price's prediction. Whether the within-population genetic structure observed for *P. shawi* is more typical of parasites from aquatic environments than from terrestrial environments is an intriguing hypothesis that deserves further testing.

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