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Title: The Influence of Parasite Ecology on the Genetic Structure of Parasite Populations

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Michael S. Blouin

Parasites are unique study organisms for evolutionary ecologists. Yet, molecular ecology studies on parasites have lagged far behind those on free-living organisms. The goal of the review in Chapter 2 was to illustrate areas of research that may be of particular interest in relation to the parasitic life style and to highlight areas that require additional study. While parasite molecular ecology is still in its infancy, it is obvious that molecular techniques have numerous applications for understanding the basic biology of parasites (e.g., elucidating life cycles). Reciprocally, parasite ecology is useful for predicting genetic patterns within and among parasite populations. Chapters 3 and 4 examine hypotheses about the influence of parasite ecological characteristics on the genetic structure of parasite populations. Chapter 3 is a broad scale analysis in which life cycle patterns were used to predict the potential for gene flow among geographic populations. Three freshwater trematode species that cycle exclusively in aquatic hosts are much more subdivided among streams than another trematode species from the same locations but whose life cycle includes highly mobile terrestrial hosts. These results show how variation in life cycles can shape parasite evolution by predisposing them to vastly different genetic structures. Chapter 4 focuses on a local scale dynamics in the salmonid trematode *Plagioporus shawi*. The transmission dynamics and mating system (selfing vs. outcrossing) of a parasite will determine the levels and patterns of genetic diversity within populations. The results of this study challenge the previous paradigm that segregation of parasites into infrapopulations (all the parasites in a single host) cause low genetic diversity. We found high levels of genetic diversity, and that *a posteriori* inference of population structure overwhelmingly supports the component population (all the parasites among a host population) as the deme. Furthermore, genetic data indicate *P. shawi* is largely outcrossing. Aquatic transmission and the use of multiple intermediate hosts likely promote high genetic diversity and well-mixed infrapopulations.

The Influence of Parasite Ecology on the Genetic Structure of Parasite Populations

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APPROVED:

Redacted for privacy

Major Professor, representing Molecular and Cellular Biology

Redacted for privacy

Director of the Molecular and Cellular Biology Program

Redacted for privacy

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Charles David Criscione, Author

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CONTRIBUTION OF AUTHORS

Dr. Michael S. Blouin and Dr. Robert Poulin provided intellectual contributions to the review paper presented Chapters 2. Dr. Blouin was directly involved in the design and data analysis of Chapters 3 and 4.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX TABLES

The Influence of Parasite Ecology on the Genetic Structure of Parasite Populations

Chapter 1

General introduction

> "The general patterns envisaged for parasite species include small, relatively homozygous populations with little gene flow between populations, which results in many specialized races, rapid evolution and speciation without geographic isolation, and an abundance of sibling species. Short generation times, with large fluctuations in population sizes, and narrow environmental tolerances contribute importantly to the evolutionary potential of parasitic species...
>
> These generalizations, reached by using the inductive processes, need critical evaluation based on more information on natural populations in order to test the generality of the concepts proposed here...
>
> In sexually reproducing parasites population structure requires much attention. What is the effective size, the distance moved by the dispersal phase of parasites, the frequency of gene flow from one population to another, the behavior of individuals which influence mating patterns within and between populations? The genetics of parasite species and races should receive much more attention."
>
> Price (1977)

The ecological characteristics of parasites can be very useful for predicting patterns of genetic structure within and among populations of parasites. In fact, Price (1977, 1980) relied exclusively on several ecological characteristics of parasites to derive his conclusions on the genetic structure of parasite populations. For example, he suggested that parasites exist in non-equilibrium (extinction-recolonization dynamics) conditions because individual hosts represent ephemeral habitats. Thus, he predicted that parasite populations would have low genetic diversity. It should be noted that while Price generalized to all parasites, a large proportion of his ecological examples were based on phytophagous insects. Although there were several allozyme studies on parasites in the 1980's and early 1990's (reviewed by Nadler 1990 and Bush *et al.* 2001), few of these studies shed light on the ecological factors important for determining the genetic structure of parasite populations (see Nadler 1995). However, these studies did indicate that parasites have levels of genetic diversity that were comparable to free-living invertebrates (Nadler 1990). There were a handful of studies in the 1990's that showed interesting genetic

patterns for parasites (e.g., Nadler *et al.* 1990; Mulvey *et al.* 1991; Blouin *et al.* 1995; see Chapters 2, 3, 4 for discussions of these studies). However, because there were so few studies on such taxonomically diverse parasites, few generalizations about the effects of parasite ecology on genetic structure could be made. In a review of parasite population genetics, Nadler (1995) revisited the aspect of using parasite ecology as a means for understanding microevolutionary processes in parasites. Even though over 15 years had passed since Price's original predictions, Nadler's (1995) arguments were also largely verbal because of the lack of empirical data. However, Nadler (1995) recognized that there was a range of ecological diversity among parasites. Thus, a broad spectrum of genetic structures should be expected. Despite the recent advances in the field of molecular ecology since 1990 (e.g., sequencing capabilities, microsatellites), Price's (1977, 1980) and Nadler's (1995) predictions largely remain untested. Thus, the field of parasite molecular ecology lags far behind the current state of molecular ecology in free-living organisms.

As an aside, there is a bit of irony in that there are few population genetic studies on parasites. That is because Sewall Wright, one of the leading pioneers in population genetics theory, actually began his graduate career as a student in parasitology. In fact, his first publication was an anatomical description of the digenean trematode, *Microphallus opacus* (Wright 1912). In William Provine's (1986) biography of Sewall Wright, Provine reports that Wright "greatly enjoyed" his parasite research. I guess it is safe to say that it was all for the better that Wright chose a path in statistical genetics, but who knows what he could have done for the field of parasitology. At least now, Wright's advances in population genetics theory in conjunction with current molecular methodologies can be applied to questions about the evolutionary biology of parasites.

The theme of my dissertation is summarized in the title "The influence of parasite ecology on the genetic structure of parasite populations". Since Nadler's (1995) review, there have been several new studies on the genetics of parasite populations. Chapter 2 is the most recent review in parasite molecular ecology. In this chapter, my coauthors and I propose a broad range of questions that can be used to elucidate ecological and microevolutionary processes in parasites, and highlight areas of research that require additional study. Parasites display a huge diversity of life cycles and life styles. Thus, parasites provide great opportunity for comparative studies to test parasite-specific questions or questions about evolution in general.

Chapters 3 and 4 address population genetics questions driven by the ecological characteristics of parasites. In Chapter 3, I integrate an ecological hypothesis (autogenic-

allogenic hypothesis) about the dispersal capabilities of parasites to examine factors that affect gene flow among parasite populations. Autogenic parasites complete their life cycles within a freshwater system, whereas allogenic parasites pass through terrestrial hosts in their life cycles. Thus, allogenic parasites have a means of dispersal to circumvent barriers to aquatic systems. The prediction at the intraspecific level is that gene flow should be greater in allogenic species.

Chapter 4 revisits Price's (1977, 1980) prediction that the ephemeral nature of individual host habitats (parasite infrapopulations) should cause fragmented parasite gene pools. In this chapter, I present different aspects of parasite transmission that can lead to vastly different predictions than proposed by Price. For example, adults of most macroparasite species release offspring (eggs or larvae) into the external environment. 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. Thus, the transient subdivision of parasite breeders into infrapopulations should not result in fragmented gene pools. We examine these predictions within populations of the salmonid trematode *Plagioporus shawi*. Furthermore, little is know about the mating systems of parasites in natural systems. In response to this situation, we use genetic data to infer the levels of outcrossing in populations of *P. shawi*.

Chapter 2

Molecular ecology of parasites: elucidating ecological and microevolutionary processes

Charles D. Criscione
Robert Poulin
Michael S. Blouin

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Abstract

We review studies that have used molecular markers to address ecological and microevolutionary processes in parasites. Our goal is to highlight areas of research that may be of particular interest in relation to the parasitic life style, and to draw attention to areas that require additional study. Topics include species identification, phylogeography, host specificity and speciation, population genetic structure, modes of reproduction and transmission patterns, and searching for loci under selection.

Introduction

Parasitism is one of the most common life styles among eukaryotes (Poulin & Morand 2004). Yet, molecular ecology studies on parasites have lagged far behind those on free-living organisms. Molecular markers offer great tools for studying processes such as transmission, the evolution of host specificity and patterns of speciation. Molecular ecology studies also have very practical applications such as in studying the evolution and control of drug resistance. Another reason to study parasites is that they display a huge diversity of life cycles and life styles (modes of reproduction, dispersal abilities, effective sizes, and so on). These provide great opportunity for comparative studies to test parasite-specific questions or questions about evolution in general. Nevertheless, parasitologists have been slow to adopt the methods of molecular ecology, and for some reason, the field of parasitology has yet to attract many evolutionary biologists.

In this review we highlight studies that used molecular markers to address questions about parasite ecology or evolution. We do not provide an exhaustive review, but instead use selected examples to illustrate areas of research that may be of particular interest in relation to the parasitic life style and highlight areas that require additional study. To facilitate discussion, we limit our review to organisms typically covered in parasitology texts (protozoan and metazoan parasites of animals) (Poulin 1998; Bush *et al.* 2001). Phylogenetic studies on recognized species or studies on cospeciation between parasites and hosts certainly provide insight into parasite evolution; however, phylogenetics is beyond the scope of this review.

Species identification: the fine line between population genetics and phylogenetics

Delimiting species of parasites is often difficult owing to their limited morphological characters. Additionally, the primary samples may consist of unidentifiable egg or larval stages from a particular host. Thus, genetic identification of species is by far the most utilized application of molecular techniques in parasitology (McManus & Bowles 1996). Here we briefly draw attention to three key uses of molecular markers in species identification.

First, the identification of known species from morphologically indistinguishable life stages (e.g., eggs, larvae) is important for disease transmission studies and clinical diagnostics. Fecal samples can be screened for eggs to assess the parasite diversity in the host (e.g., Schnieder *et al.* 1999; Wimmer *et al.* 2004). Such screening may be a necessity in hosts where invasive sampling of adult parasites is not an option (e.g., humans, endangered species). Correct identifications are critical for designing control programs or applying drug treatments. The study by Singh *et al.* (2004) illustrates the importance of correct identifications. Malarial infections in

humans from Malaysian Borneo were previously identified via microscopy as *Plasmodium malariae*, which is morphologically very similar to *P. knowlesi* (a parasite of macaque monkeys). Small subunit rDNA sequences obtained from infected patients, however, were identical to *P. knowlesi*. This identification now enables research to focus on potential transmission dynamics between humans and macaque monkeys (Singh *et al.* 2004).

A second use of species identification is to elucidate parasite life cycles (e.g., Cribb *et al.* 1998; Jousson *et al.* 1999; Bartoli *et al.* 2000). For example, which species serve as the INTERMEDIATE HOSTS (see Table 2.1 for Glossary) for larval stages of a parasite? Traditional methods for working out the life cycle, such as experimental infections, may be hampered if potential hosts cannot survive in the laboratory. PCR detection techniques (e.g., use of species specific primers) or sequencing provide a rapid means to screen potential hosts as illustrated by the study of Jousson *et al.* (1999). Adult specimens for 16 known species of trematodes and unidentifiable larval specimens were collected from fish DEFINITIVE HOSTS and mollusk intermediate hosts, respectively. Sequences of the internal transcribed spacer rDNA matched many of the larval specimens to their corresponding adult form, thus, identifying the natural intermediate hosts.

Table 2.1. Glossary of parasite terms.

Abundance: the number of individuals of a particular parasite in/on an individual host regardless of whether or not the host is infected.
Component population: all individuals of a parasite species in a specified life history phase (e.g., mature adults) at a particular place and time.
Definitive host: the host in or on which a parasite sexually reproduces.
Infrapopulation: all individuals of a parasite species in or on an individual host at a particular time.
Intensity: the number of individuals of a particular parasite in/on a single infected host.
Intermediate host: the host in or on which a parasite undergoes some developmental change, but does not reach sexual maturity.
Macroparasite: multicellular parasites such as nematodes, platyhelminthes, acanthocephalons, pentastomes, and arthropods.
Prevalence: is the number of hosts infected with one or more individuals of a parasite species divided by the number of hosts examined for that parasite species.
Paratenic host: host in which development does not occur, but which may serve to bridge an ecological gap in a parasite's life cycle.
Reservoir host: host in which a parasite can survive and reproduce, but is not considered the normal host.

A related use of molecular markers is the search for cryptic species (morphologically similar, but genetically distinct) (e.g., Hung *et al.* 1999; Jousson *et al.* 2000; Leignel *et al.* 2002). Here the goal is not to use markers to distinguish among known species, but to prospect for new species (Blouin 2002). The finding of cryptic parasite species has become very common as more phylogeographic and genetic structure studies are carried out on parasites (Anderson *et al.* 1998). For instance, in studies on trematodes in salmon (Criscione & Blouin 2004) and on avian malaria parasites (Bensch *et al.* 2004), the observation of complete disequilibrium between nuclear and mitochondrial markers proved that cryptic species can co-occur in the same individual hosts.

DNA-based identification and discovery of parasite species also has implications for our understanding of global biodiversity (Brooks & Hoberg 2000; Poulin & Morand 2004). The limited morphological characters of many parasitic helminths and protozoans have probably resulted in a gross underestimation of the true number of species in biodiversity surveys (e.g., Bensch *et al.* 2004; Westenberger *et al.* 2004). For instance, mtDNA analyses suggest that the number of species of avian malaria approaches 10,000 rather than the 175 that were previously identified via morphology (Bensch *et al.* 2004).

An emerging area using molecular methods for species identification is molecular epidemiology and epizootiology (the study of disease transmission in humans and wildlife). For example, one can ask if a species of human parasite infects a RESERVOIR HOST (host in which a parasite can survive and mature, but is not considered the normal host; Bush *et al.* 2001). Reservoir hosts are significant because they may maintain parasites in the absence of the primary host. Anderson (2001) concluded that cross-infections were rare between *Ascaris* nematodes of humans and pigs in sympatric areas. Thus, control programs for human *Ascaris* need not concentrate on infection control in pigs. Other molecular epidemiology applications include detecting mixed species/strain infections or subpatent infections, surveying wildlife for human parasites, and estimating PREVALENCE (percent of hosts infected) (e.g., Perkins *et al.* 1998; Dubey *et al.* 2004; Njiru *et al.* 2004).

Phylogeography

Reconstructing the historical biogeography of populations (e.g., vicariance events) and identifying major genetic subdivisions within species are major objectives of phylogeographic analyses (Avise 2000). Furthermore, comparative phylogeography can identify historically and evolutionary independent geographic regions (Bermingham and Moritz 1998). There have been relatively few traditional phylogeographic studies on parasites (Table 2.2). Given that parasites

are closely tied to their hosts, it might be expected that parasites and their hosts would share similar phylogeographic patterns. Indeed, congruence between host and parasite phylogeography has been found, although it is not always perfect (Table 2.2). The degree of congruence will depend on which host in a parasite's life cycle is compared and on the nature of the transmission dynamics between the host and parasite (Neiberding *et al.* 2004). For instance, malaria parasites of Caribbean lizards showed complex patterns of genetic fragmentation that were inconsistent with those of their lizard hosts (Perkins 2001). Population extinctions and dispersal by the vector host may contribute to this discordance (Perkins 2001).

Phylogenetic studies that test for cospeciation between parasites and hosts have expanded our understanding of parasite evolution (Page 2003). Likewise, much can be gained by testing whether a single species of parasite and its host have similar phylogeographic structure. As an example, Neirberding *et al.* (2004) illustrates how cophylogeographic patterns between a parasite and its host can be used to calibrate a molecular clock for parasites. Additional comparative phylogeographic studies between hosts and parasites are needed to help illuminate the microevolutionary processes that result in interesting macroevolutionary patterns such as cospeciation

Phylogeographic data can also be used to understand the history of colonization by an exotic parasite. These data could indicate potential source populations or the timing of invasion, or could be used to test whether multiple introductions have occurred. Knowing the origin of the parasite and levels of genetic diversity in its native range may be useful for implementing control measures or for evaluating the potential for drug resistance in introduced locations. For example, molecular data suggest that the human malarial parasite, *Plasmodium falciparum*, has a most recent common ancestor that dates back ~100,000 years, and then underwent a recent population expansion in Africa followed by separate colonizations into South America and Asia. (Hartl *et al.* 2002; Mu *et al.* 2002; Joy *et al.* 2003). There appear to have been multiple independent introductions into South America (Anderson *et al.* 2000; Joy *et al.* 2003). The human filarial nematode *Onchocerca volvulus* also recently colonized South America. Molecular data suggest that New World populations originated from an African savannah strain during the slave trade (Zimmerman *et al.* 1994). Likewise, mtDNA data support a recent introduction of the trematode *Schistosoma mansoni* into America from Africa (Despres *et al.* 1993).

Table 2.2. Summary of phylogeographic studies of non-human parasites (see text for human parasite examples).

Parasite species	Host species	Reference	General conclusions and phylogeographic breaks
Gyrodactylus salaris (monogene)	*Thymallus thymallus* (European grayling)	Meinila *et al.* (2004)	The distribution of mtDNA haplotypes was consistent with postglacial recolonization patterns of the host
Heligmosomoides polygyrus (nematode)	*Apodemus sylvaticus* (field mouse)	Nieberding *et al.* (2004)	Congruence with major host breaks (Western Europe, Italian, and Sicilian), but parasite also had further breaks within some regions. Host and parasite recolonized northwestern Europe from Iberian refuge.
Leptorhynchoides spp. -three cryptic species found (acanthocephalan)	*Lepomis* spp. (sunfish)	Steinauer (2004)	Significant phylogenetic associations with previously reported breaks (East-West of the Apalachicola River and Gulf-Atlantic drainages) for several taxa and the parasite's hosts (see Avise 2000). Data suggest allopatric speciation.
Paranoplocephala arctica (cestode)	*Dicrostonyx* spp. (collard lemmings)	Wickstrom *et al.* (2003)	Nearctic and Palaearctic clades, which correspond with major division of the hosts, but parasite lacks complete congruence with host relationships.
Perkinsus marinus (protozoan)	*Crassostrea virginica* (American oyster)	Reece *et al.* (2001)	Atlantic-Gulf Coast break; a pattern also seen in its host (see Avise 2000).
Plasmodium azurophilum (malaria)	*Anolis* spp. (lizards)	Perkins (2001)	Patterns not consistent with lizard hosts. Vector mediated dispersal may contribute to discordance.

Host-specificity and speciation

Parasites are interesting systems in which to study patterns and mechanisms of speciation. Adaptation to different tissues or body locations in a single host species could select for reproductive isolating mechanisms between groups of parasites. Such may be the case for two species of *Plasmodium* that infect lizards (Perkins 2001). One infects red blood cells while its sister species infects white blood cells in the same hosts (Perkins 2001). Adaptation to different sympatric hosts could also select for intrinsic barriers to reproduction. Alternately, non-overlapping ecologies between sympatric host species (distinct transmission cycles) could result in simple physical separation between groups of parasites that ultimately leads to speciation (McCoy 2003). We are not aware of any molecular studies that explicitly test mechanisms of speciation in parasites. However, Trouve *et al.* (1996, 1998) observed potential assortative mating and hybrid breakdown as a result of crossing *Echinostoma caproni* collected from Egypt and Madagascar. Mechanisms of reproductive isolation that result from host specificity can potentially be tested in the laboratory by running the life cycle of a parasite through different intermediate or definitive host species for several generations. As far as elucidating patterns of speciation, some genetic studies have found parasite populations that are subdivided between host species in sympatry. Examples include ticks on communally nesting seabirds (McCoy *et al.* 2001) and lice on doves (Johnson *et al.* 2002). However, these studies only show that host-race formation in sympatry is a potential diversifying force in parasites. More work is needed to determine the importance of extrinsic vs. intrinsic barriers, and to rule out other explanations such as geographic speciation followed by secondary contact of host species.

Population genetic structure

The ability of parasites to adapt to local environments or hosts, evolve drug resistance, or speciate will be affected by gene flow among populations and genetic drift within. Thus, having estimates of migration rates and effective population sizes (N_e), and understanding what factors control those parameters in parasites, are key for understanding microevolutionary processes in these taxa.

Gene flow

Host vagility should be a major determinant of parasite gene flow because many parasites lack free-living stages (e.g., malaria) or have low dispersal capability in their free-living stages (Price 1980; Nadler 1995). For example, three freshwater trematode species that cycle

exclusively in aquatic hosts are much more subdivided than another trematode species from the same locations but whose life cycle includes highly mobile terrestrial hosts (Criscione and Blouin 2004). Two other studies that compared the genetic structures of parasites having hosts with different dispersal capabilities also supported host movement as a key determinant of parasite gene flow (Blouin *et al.* 1995; McCoy *et al.* 2003). A corollary hypothesis is that gene flow in a parasite with a complex life-cycle will be controlled by the most mobile host (Jarne & Theron 2001; Prugnolle *et al.* 2005b). Other possible determinants of gene flow such as the dispersal ability of free-living larval stages or the degree of host specificity (e.g., many reservoir hosts may increase dispersal chances; Nadler 1995) have not been tested.

Much can be learned from comparing genetic structure in parasites and in their hosts. For example, host-parasite systems can be used to study the interaction between gene flow and the ability of natural selection to promote local adaptation. Theoretical models show that coevolution between host and parasite is affected by the relative rates of gene flow in the parasite and in the host (Lively 1999; Gandon 2002). These models predict that local adaptation by parasites to their host is facilitated by higher parasite migration (because parasite migration can import novel alleles that may counteract a host's evolutionary response to the parasite). Support for this prediction was found in two snail-trematode systems in which there was greater parasite gene flow and locally adapted parasites (Dybdahl & Lively 1996; Davies *et al.* 1999). Studying movement in parasites and hosts is also important for understanding the evolution of drug resistance. For example, the wide spread occurrence of drug-resistant *P. falciparum* was shown to result from the recent spread of a few selected alleles, rather than from the independent evolution of new resistance alleles in multiple locations (Nair *et al.* 2003; Roper *et al.* 2003). Finally, genetic structure in parasites could potentially be used to inform us about genetic structure in their hosts. Parasites have been used as biological tags for stock identification of migratory marine organisms (reviewed by MacKenzie 2002). Here the presence or absence of parasite species is used to identify the geographic origin of hosts that carry those parasites. If a parasite is more finely subdivided than its host, then one could potentially use the genotypes of a single species of parasite to assign hosts to their population of origin with higher probabilities than by using the host's own genotypes. Whether a parasite is more or less subdivided than its dispersing host should depend on factors such as the INTENSITY and prevalence of infection in migrating hosts, and on the effective sizes of host and parasite populations.

Genetic diversity within populations and effective population size

Price (1980) predicted that parasite populations would be largely homozygous and have low genetic diversity. His prediction stems from the idea that hosts are ephemeral habitats and represent patchy environments for the parasite. Therefore, parasite populations should be subject to large population fluctuations and chance colonization events that promote inbreeding and fractionated gene pools. However, most animal MACROPARASITES (helminths and arthropods) have levels of allozyme and mtDNA diversity that are similar to or higher than what has been reported in free-living animals (Blouin *et al.* 1992; Criscione & Blouin 2004; Bush *et al.* 2001). Why then do we see deviations from Price's predictions?

Price's perception may have stemmed from the idea that all parasites of a given species within or on an individual host (i.e., an INFRAPOPULATION; Bush *et al.* 1997) undergo genetic drift like a traditional deme. Many of Price's examples were phytophagous insects that can have many recurrent generations on a single host plant. In contrast, most animal macroparasites release offspring into the external environment. Offspring are mixed and then recruited back into new definitive hosts. So the question of whether the COMPONENT population (all the parasites of a given species in an entire host population; Bush *et al.* 1997) or the infrapopulation is best considered the relevant unit of evolution has been raised repeatedly (Lydeard *et al.* 1989; Nadler 1995; Sire *et al.* 2001). In reality there is probably a continuum. If offspring are well mixed, then the transmission process only separates adult breeders into infrapopulations each generation but does not result in recurrent generations within individual infrapopulations. On the other end of the continuum, if offspring re-infect their natal host (e.g., lice, pinworms) or 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. Such species would be more likely to fit Price's predictions. For instance, lice on pocket gophers recruit back to their natal host, and infrapopulations of these lice have low heterozygosity and are highly subdivided (Nadler *et al.* 1990).

N_e has a large influence on overall levels of genetic diversity in populations, and on the fate of alleles under selection. Therefore, N_e affects local adaptive potential, including the ability of a parasite population to evolve drug resistance. Yet N_e is an almost completely ignored parameter in parasitology, and we know almost nothing about what factors control the N_e of parasite populations. Prugnolle *et al.* (2005a) modeled how several features of the complex life cycle of trematodes affect the distribution of genetic variation within and among infrapopulations. In particular, they showed how an increase in selfing rate and in the variance in the reproductive

success between clones reduces N_e of the component population, while clumped transmission of offspring from host to host increases N_e (by creating non-transient population subdivision). Criscione & Blouin (2005) present a conceptual model for making demographic estimates of N_e in parasites. This model highlights several factors such as aggregated distributions, crowding effects, and host immunity that can influence the N_e of parasite component populations via effects on sex ratios and variation in reproductive success. An interesting result is that several features of parasite life cycles probably act in concert to reduce N_e below that expected in a single free-living population of equivalent census size. Conversely, dormant stages (e.g., long-lived nematode eggs) or PARATENIC hosts (hosts in which a parasite does not develop, but can be maintained for long periods until reaching a definitive host) may buffer the effects of genetic drift (Nadler 1995; Nunney 2002).

As a starting null hypothesis, one might expect a simple relationship between N_e and the census number of adult parasites in definitive hosts. A crude estimate of the standing number of breeders at any one time would be the number of hosts times the mean ABUNDANCE (number of parasites per host examined; Bush *et al.* 1997). Interestingly, mtDNA diversity in outcrossing nematode species that have direct life cycles appears to be a simple function of mean intensity, which should be correlated with the number of parasites in the component population (Fig. 2.1). However, many parasites have much more complex life cycles. Thus, more comparative studies among species or populations would be useful (e.g., Blouin *et al.* 1999). For example, comparisons of short-term genetic estimates of N_e among parasite populations that differ in key traits (e.g., levels of aggregation or abundance) would help identify the ecological determinants of N_e. Unfortunately, we are not aware of any short-term N_e estimates for parasites that utilize techniques such as the temporal method (Schwartz *et al.* 1998). There are a handful of long-term estimates based on DNA diversity (Blouin *et al.* 1992; see Hartl *et al.* 2002 for malaria references). However, these are crude estimates of long-term species-wide or metapopulation-wide N_e, and require an estimate of the mutation rate. Such long-term estimates can be interesting, but they provide little insight into current ecological determinants of N_e.

The effects of mating systems on within-population genetic diversity (reviewed by Charlesworth 2003) have also received little attention in parasites. Among plants and snails, outcrossing species tend to have higher levels of diversity than selfing species (Jarne 1995, Charlesworth 2003). Nematode parasites appear to have a similar pattern, but this conclusion is based on only a handful of studies (Blouin 1998). More comparative studies are needed to generalize these conclusions among parasites. It is worth noting that almost any variation in

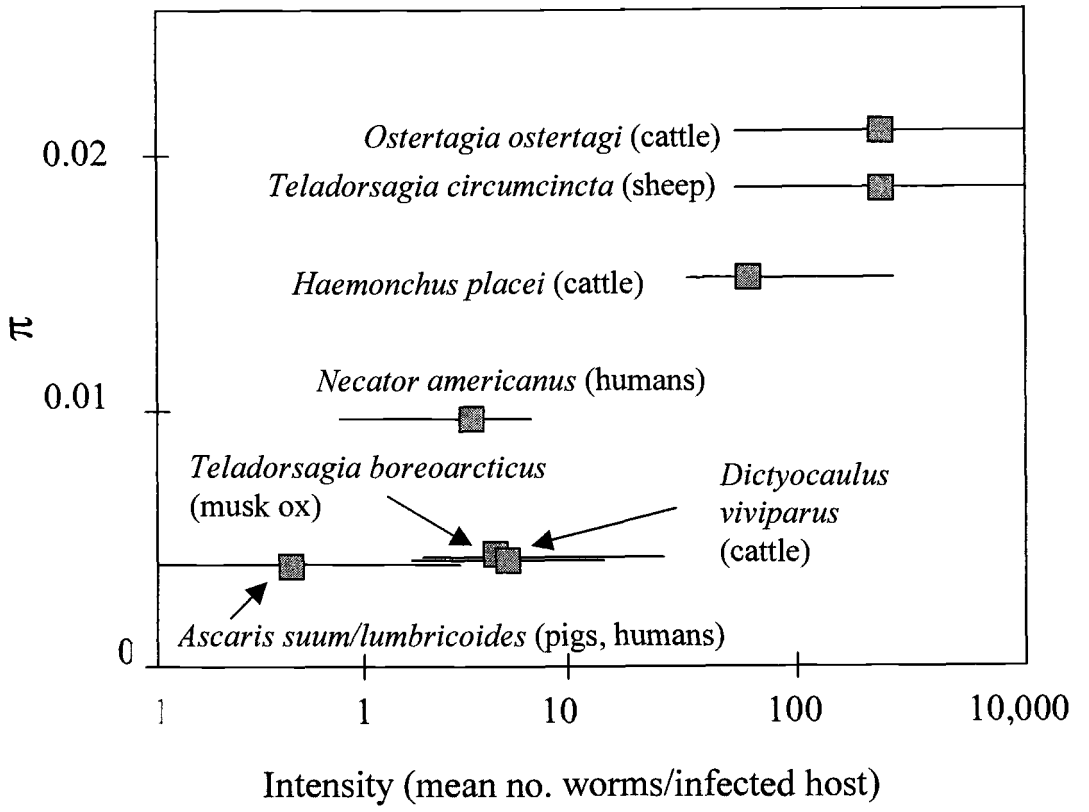

Fig. 2.1. Average population mtDNA diversity (π; average number of substitutions per site) versus mean infection intensities for several species of nematodes. Host species are in parentheses. Horizontal bars give the range of typical intensities for that species. mtDNA diversities based on whole-molecule RFLP or on *Cox1* sequences are reported as given. mtDNA diversities based on *Nad4* gene sequence were adjusted down by 20% to account for the higher substitution rate of *Nad4* (Blouin 2002). RFLP: *Ascaris suum/lumbricoides*; *Cox1*: *Dictyocaulus viviparous, Necator americanus*; *Nad4*: *Haemonchus placei*; *Ostertagia ostertagi*; *Teladorsagia boreoarcticus*; *Teladorsagia circumcincta.*

reproductive mode (e.g., asexual, monoecious, haplodiploidy, dioecy; Bush *et al.* 2001) can be found among animal parasites. Therefore, numerous opportunities exist to test how life history variation influences genetic diversity.

Modes of reproduction, mating systems, and transmission patterns

The interplay between transmission dynamics, mode of reproduction (sexual vs. asexual), and mating system (selfing, biparental inbreeding or outcrossing) in parasites is important for several reasons (see also Prugnolle *et al.* 2005a). Firstly, transmission and mating patterns influence inbreeding rates (see Prugnolle *et al.* 2004a; Christen *et al.* 2002; Christen & Milinski 2003 for how inbreeding may affect parasite infection success). For example, clumped recruitment of siblings into a definitive host ("sib-transmission") can increase the chance of biparental inbreeding (Anderson *et al.* 1995; Nadler 1995). This effect will be exacerbated if the effective number of breeders per infrapopulation is small (Anderson *et al.* 1995). Populations of hermaphroditic parasites that have low mean intensities may have a higher rate of selfing than populations with high intensities. Likewise, transmission that leads to clones in the same definitive host can increase the effective selfing rate (e.g., as in *P. falciparum*, Anderson *et al.* 2000; or in trematodes where asexual multiplication in the snail leads to the synchronous release of many genetically identical cercariae that could infect the same definitive host). Indeed, it has been hypothesized that trematodes keep second intermediate hosts in their life cycle, rather than going directly from snail to definitive host, as an inbreeding avoidance mechanism (Rauch *et al.* 2005). Here the second intermediate host "collects" different cercarial genotypes over time, and these different genotypes then mate when a definitive host ingests the second intermediate host (Rauch *et al.* 2005). Secondly, mode of transmission and mating system also control the opportunity for kin selection. Recent models predict that relatedness among parasites within a host is a key determinant of optimal strategies of parasite growth, manipulation of host behavior, or virulence (Frank 1996; Brown 1999; Parker *et al.* 2003). These predictions remain untested.

Inferring modes of reproduction and mating systems

Molecular markers can be very useful when the mode of reproduction is still in doubt. For example, Viney *et al.* (1993) and Viney (1994) demonstrated where sexual reproduction occurs in the life cycle of the nematode *Strongyloides ratti*, and showed that the parthenogenetic phase was mitotic rather than meiotic. Populations of protozoan parasites are frequently classified as being clonal (limited genetic exchange that is not sufficient to erode linkage

disequilibrium), panmictic (frequent sexual recombination), or epidemic (basic panmictic structure, but some genotypes expand clonally due to favorable conditions) (see MacLeod *et al.* 2001). Populations of the same protozoan species often show different degrees of clonality (reviewed by Tibayrenc & Ayala 2002). For example, populations of African trypanosomes in wildlife and livestock are mostly epidemic in genetic structure. However, clonal structure has arisen several times in human hosts (MacLeod *et al.* 2001). Similarly, *Toxoplasma gondii* has a mix of clonal and sexual propagation that may be associated with domestic and wild environments (Ajzenberg *et al.* 2004).

There have been relatively few studies that used molecular markers to infer mating patterns. Mixed mating systems with high rates of outcrossing were demonstrated using progeny-array analysis from experimental infections in a trematode, *Echinostoma caproni* (Trouve *et al.* 1999) and from *in vitro* matings in a cestode, *Schistocephalus solidus* (Luscher & Milinski 2003). Deviations from Hardy-Weinberg equilibrium have been used to estimate outcrossing rates in natural populations of flatworms. The trematode *Lecithochirium rufoviride* and cestode *Proteocephalus exiguus* had inferred outcrossing rates of 70% and 84%, respectively (Snabel *et al.* 1996; Vilas & Paniagua 2004), whereas the cestode *Echinococcus granulosus* had a very low inferred rate of outcrossing (1.1%) (Lymbery *et al.* 1997).

Of course multiple factors can cause deviations from Hardy-Weinberg or linkage equilibrium within parasite infrapopulations, so in many cases inferring the mating system from just Hardy-Weinberg considerations can be problematic. Large and variable (often negative) deviations from Hardy-Weinberg equilibrium may appear if clones transmit together and are not identified as such (Prugnolle *et al.* 2005a). Hosts that sample from spatially or temporally separated pools of infective larvae could show a Wahlund effect in their infrapopulations (Vilas *et al.* 2003). Clumped transmission of sibs can produce variable, and often negative values of F_{IS} if samples consist of a few sibships (Balloux 2004). Clumped transmission of sibs can also produce positive F_{IS} owing to inbreeding in subsequent generations if offspring continue to transmit together.

Inferring the ecology of transmission

Data on the non-random distribution of genotypes among infrapopulations or social host groups can be very useful for understanding the ecology of transmission from host to host. For example, humans in Guatemala were infected with ascarid nematodes having identical mtDNA haplotypes more often than expected by chance alone (Anderson *et al.* 1995). Thus, transmission

may be clustered within human households, rather than occurring randomly throughout each village. The distribution of clones within and among hosts can be informative about transmission in some parasites (e.g., trematodes). For example, Theron *et al.* (2004) studied the transmission of the trematode *Schistosoma mansoni* between its snail intermediate host and rat definitive host in Guadeloupe. Individual snails carried an average of 1.1 unique genotypes, while individual rats carried an average of 34. Thus, rats are infected from about 30 snails, rather than in one or a few infection events. Similarly, only a few clones of the trematode *Diplostomum pseudospathaceum* are normally found in each snail, while a fish second intermediate host can harbor dozens of unique genotypes (Rauch *et al.* 2005).

To date most studies have relied on F_{ST} (or related analogs) to describe patterns of genetic differentiation among hosts (Nadler *et al.* 1990; Anderson *et al.* 1995; Nadler *et al.* 1995; Fisher & Viney 1998; Davies *et al.* 1999; Hawdon *et al.* 2001; Sire *et al.* 2001; Patterson *et al.* 2000; Brouwer *et al.* 2001; Curtis *et al.* 2002; Prugnolle *et al.* 2002; Vilas *et al.* 2003; Braisher *et al.* 2004; Theron *et al.* 2004). The usual explanation for a significant F_{ST} is genetic drift, but for many parasite species infrapopulations may not behave as traditional demes. Instead, variation in allele frequencies among infrapopulations informs us more about recruitment patterns and aspects of the mating system (Sire *et al.* 2001; Prugnolle *et al.* 2005a).

There are, however, several methodological concerns when interpreting estimates of F_{ST} among infrapopulations. First, small sample sizes of parasites per host can lead to imprecise or biased estimates of F_{ST} (Waples 1998). Unfortunately, small sample sizes may be unavoidable because some parasites naturally have low mean intensities or highly aggregated distributions (i.e., few hosts are heavily infected, but most hosts have few parasites; Shaw *et al.* 1998). In the latter case, researchers could sample only from large infrapopulations (e.g., Vilas *et al.* 2003), but then sampling from a non-random sample of hosts may be an issue. Second, many studies rely on samples of offspring (e.g., excreted eggs) because collecting adult parasites from each infrapopulation is impractical (e.g., Fisher & Viney 1998; Patterson *et al.* 2000; Brouwer *et al.* 2001; Curtis *et al.* 2002). The problem here is that one usually does not know the effective number of breeders that contributed those offspring. Sampling a large number of sibs from a limited number of breeders can inflate the apparent F_{ST} among infrapopulations because of chance allele frequency differences among families (Allendorf & Phelps 1981). One way around this problem that has never been applied to parasites is to partition offspring into sibships using marker data, and to then replace sibships with reconstructed parental genotypes for the purposes of estimating allele frequencies (Blouin 2003 and references therein). Note also that this

approach could be used to estimate the number of breeding adults infecting a host when direct counts are impossible (e.g., how many adult schistosomes occur in an infected human?). Such data can be essential for parameter estimation in epidemiological studies and for estimating the effective size of parasite populations (Criscione and Blouin 2005).

Even if we ignore the above methodological concerns, interpreting the causes of among-infrapopulation F_{ST} in the absence of other information remains problematic. If parasite isolation among geographic locations or host social groups is not accounted for, F_{ST} among infrapopulations will be inflated (e.g., Nadler *et al.* 1995; Curtis *et al.* 2002). Multiple copies of the same clone in infrapopulations can also inflate the among-infrapopulation F_{ST} (Theron *et al.* 2004; Prugnolle *et al.* 2005a). Assuming one has adjusted for clones and simple geographic isolation, a non-random distribution of parasite genotypes among infrapopulations could still be caused by several processes such as sib-transmission or even host factors that select against certain parasite genotypes (Anderson *et al.* 1995). Presumably it is processes such as these that one is interested in studying, but simply demonstrating significant F_{ST} among infrapopulations does not distinguish among them.

Much more could be gained by using polymorphic markers and modern analytical techniques such as methods for pedigree reconstruction (Blouin 2003; Jones and Ardren 2003) and methods for *a posteriori* assignment of individuals into natural groupings (Pritchard *et al.* 2000; Dawson & Belkhir 2001). Pedigree methods can directly assess the degree to which sibs are transmitted together and will be essential for testing hypotheses about kin selection in parasites. One could test whether deviations from Hardy-Weinberg equilibrium within infrapopulations result from the presence of related individuals, of inbred but unrelated individuals, or of groups of unrelated individuals from distinct gene pools (Ritland 1996; Amos *et al.* 2001; Castric *et al.* 2002). As some studies have shown that parasites are capable of mate choice (Trouve *et al.* 1999; Luscher & Milinski 2003), it will also be interesting to test if sib-transmission results in the evolution of inbreeding avoidance behaviors (Prugnolle *et al.* 2004b).

Searching for loci under selection

Many methods are available for identifying the signature of selection in genomes (see Conway & Polley 2002 and Anderson 2004 for recent reviews). These methods can provide important contributions in two areas of parasitology. One is the identification of potential drug resistance genes (Anderson 2004). For example, intense selection on a resistance gene in *P. falciparum* was inferred from patterns of linkage disequilibrium and heterozygosity around the

target locus (Wootton *et al.* 2002; Nair *et al.* 2003). A second application is the identification of genes involved in host-parasite interactions. For instance, several loci encoding antigens in *P. falciparum* have been found to be under balancing selection, thus, indicating potential interactions with host immunity (Conway & Polley 2002). Searching for genes involved in host-parasite interactions is an exciting and growing field that should benefit greatly from the availability of entire sequenced genomes of several hosts and parasites (e.g., El-Sayed *et al.* 2004; Ghedin *et al.* 2004).

Concluding remarks

The disciplines of molecular ecology and parasitology have much to offer each other. Molecular methods can elucidate ecological and microevolutionary processes in parasites, and parasites are unique study organisms for evolutionary ecologists. For example, the interactions between hosts and parasites raise interesting questions about the evolution of parasitic life styles and host defenses. The fitness effects of parasites on hosts are of obvious concern to medical science and commercial industries (e.g., aquaculture, livestock). Parasites are also extremely diverse (Poulin & Morand 2004). By diversity, we include not only the myriad of taxa that have independently evolved a parasitic life style, but also the diversity in life cycles, modes of reproduction, host species, and ecosystems utilized by parasites. For example, hermaphroditic, parthenogenetic, and asexual modes of reproduction are frequent and have evolved independently among several parasite taxa. This diversity should captivate biologists because parasites present numerous opportunities for the study of ecological and evolutionary theory. Nevertheless, there has been relatively little work on parasites using the methods of molecular ecology. Indeed, even traditional phylogeography studies have been done on only a handful of parasite species. Parasite molecular ecology is still in its infancy, but it promises to be a rewarding field for those who embrace it.

Chapter 3

Life cycles shape parasite evolution: comparative population genetics of salmon trematodes

Charles D. Criscione
Michael S. Blouin

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Abstract

Little is known about what controls effective sizes and migration rates among parasite populations. Such data are important given the medical, veterinary, and economic (e.g., fisheries) impacts of many parasites. The autogenic-allogenic hypothesis, which describes ecological patterns of parasite distribution, provided the foundation on which we studied the effects of life cycles on the distribution of genetic variation within and among parasite populations. The hypothesis states that parasites cycling only in freshwater hosts (autogenic life cycle) will be more limited in their dispersal ability among aquatic habitats than parasites cycling through freshwater and terrestrial hosts (allogenic life cycle). By extending this hypothesis to the level of intraspecific genetic variation, we examined the effects of host dispersal on parasite gene flow. Our a priori prediction was that for a given geographic range, autogenic parasites would have lower gene flow among subpopulations. We compared intraspecific mitochondrial DNA variation for three described species of trematodes that infect salmonid fishes. As predicted, autogenic species had much more highly structured populations and much lower gene flow among subpopulations than an allogenic species sampled from the same locations. In addition, a cryptic species was identified for one of the autogenic trematodes. These results show how variation in life cycles can shape parasite evolution by predisposing them to vastly different genetic structures. Thus, we propose that knowledge of parasite life cycles will help predict important evolutionary processes such as speciation, coevolution, and the spread of drug resistance.

Introduction

In light of emerging diseases, the spread of drug resistance, and the potential effects of habitat alterations and climatic changes on parasite transmission (Crompton 1999; Daszak *et al.* 2000; Dobson & Foufopoulos 2001; Harvell *et al.* 2002; Roper *et al.* 2003), it will be critical to develop predictors of genetic drift and gene flow among populations of parasites. Nevertheless, remarkably little is known about effective sizes and migration rates for parasite populations, or about what factors (parasite or host life history characteristics) control those parameters (Poulin 1998; Blouin *et al.* 1999). Host dispersal has been proposed as a major determinant of parasite gene flow (Blouin *et al.* 1995). Only two studies have examined this issue (Blouin *et al.* 1995; McCoy *et al.* 2003), and both found support for the hypothesis. Nevertheless, one study involved the movement of livestock by humans (Blouin *et al.* 1995), and the other could not completely separate the effects of host dispersal from host nesting behaviors (McCoy *et al.* 2003). Thus, it remains unclear how important host movement is as a determinant of parasite gene flow in natural systems. Data from wild populations are needed to provide a baseline on which to predict parasite responses to altered systems (Harvell *et al.* 2002) (e.g., the spread of drug resistance in treated parasite populations). In our study, we compare the genetic structures of three described species of digenean trematodes (*Deropegus aspina*, *Nanophyetus salmincola*, and *Plagioporus shawi*) that infect wild populations of salmonid fishes (*Oncorhynchus* spp.) in the Pacific Northwest of the U.S.A. These species differ in key features of their life cycles that allow us to examine whether host movement predicts parasite gene flow in a natural system.

The autogenic-allogenic hypothesis (Esch *et al.* 1988) states that parasites cycling through freshwater and terrestrial hosts (allogenic life cycle) will have a greater ability to disperse among aquatic habitats than parasites cycling only through freshwater hosts (autogenic life cycle). Originally, this hypothesis was used to describe ecological patterns of parasite species distributions and community structure. Here, we extend the autogenic-allogenic hypothesis to the level of intraspecific genetic variation. Our a priori prediction was that the autogenic species would have lower gene flow among subpopulations and thus, greater population subdivision over a common geographic range than the allogenic species.

The life cycles of our study species illustrate the different gene flow potentials between the autogenic and allogenic modes of transmission. Sexually mature adults of the autogenic *P. shawi* (Suborder Xiphidiata: Family Opecoelidae) 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). Adults of *D. aspina* (Suborder Hemiurata: Family Derogenidae) infect the stomachs of salmonids, and although the life cycle has not been completely determined, it is almost certain that *D. aspina* is an autogenic species (McCauley & Pratt 1961). In contrast, sexually mature adults of the allogenic *N. salmincola* (Suborder Xiphidiata: Family Troglotrematidae) infect the intestines of fish-eating, terrestrial birds and mammals (Bennington & Pratt 1960; Hoffman 1999). Eggs are passed into the freshwater where snails become infected with miracidia and eventually, release cercariae. Cercariae penetrate salmonids and encyst as metacercariae in the internal organs and musculature. All three trematodes utilize the same salmonid hosts and have almost identical geographic distributions that are limited to the Pacific Northwest (Hoffman 1999). Therefore, their population histories are likely to have been exposed to similar population structuring events such as non-natal migrations of anadramous salmonids or past geological events. The key difference that predicts low gene flow for *D. aspina* and *P. shawi* is that their life cycles are confined to a freshwater system (autogenic transmission), whereas *N. salmincola* can be transported into or out of a freshwater habitat via its terrestrial hosts (allogenic transmission).

Materials and Methods

Parasite Collections

We sampled all four trematode species (a cryptic species of *D. aspina* was found, see below) from juvenile salmonids from four rivers in the Pacific Northwest of the U.S.A. (Fig. 3.1A). Standard parasitological techniques (Pritchard & Kruse 1982) were used to recover the digeneans from the following hosts: *Oncorhynchus mykiss* (steelhead trout), *Oncorhynchus clarki* (cutthroat trout), *Oncorhynchus kisutch* (coho salmon), *Oncorhynchus tshawytscha* (Chinook salmon). To insure that the parasites originated from the respective stream, we sampled out-migrating smolts (juveniles leaving the drainage for the ocean) rather than returning adults. All four species of parasites occur in all host species sampled. There was no evidence of genetic structuring among the host species. Sampling was conducted from March to July of 2002 in conjunction with Oregon and Washington salmonid-monitoring projects under the permits OR2002-019 and 02-033, respectively. Digeneans were identified with wet mounts prior to storage in 70% ethanol.

Fig. 3.1. Sampling locations and ND1 mtDNA genealogies. (**A**) Rivers sampled in Washington (WA) and Oregon (OR): Bingham Creek (white), North Fork Nehalem River (horizontal waves), West Fork Smith River (polka dots), Winchuck River (vertical lines). (**B-E**) Statistical parsimony networks. Each connection is a single mutational step with black circles representing inferred haplotypes. Observed haplotypes are shown as shaded circles. Shading scheme indicates the geographic locations (as in **A**) from which haplotypes were sampled. Haplotypes shared among locations are shown as proportional pie diagrams. Sizes of circles are proportional to the number of individuals (as indicated in the circles) with that haplotype; blank pie slices or circles indicate a single individual. Reticulations in the networks represent all most parsimonious connections and result from homoplasies in the sequence data. For the autogenic species (**B-D**), notice the strong geographic structure caused by low gene flow (i.e., related haplotypes are more likely found in the same subpopulation). High gene flow, instead, results in a lack of structure as shown by the network (**E**) of the allogenic *N. salmincola*.

A
Pacific Ocean
WA
OR
N
250 km
B
Deropegus aspina A
C
Deropegus aspina B
D
Plagioporus shawi
E
Nanophyetus salmincola

The digeneans sampled in this study have an asexual amplification stage in the snail. Consequently, sampling multiple parasites per host, or multiple hosts from the same microhabitat, might artificially increase genetic subdivision among populations. To ensure that we sampled parasites as randomly as possible from the river, we (1) sampled out-migrating smolts, and (2) we used only one parasite per host individual (e.g., the 91 samples of *N. salmincola* each came from a separate host). The one exception was for *D. aspina* B from the Nehalem River (horizontal waves population, Fig. 3.1A) where two worms were taken each from six hosts. The fact that four of these pairs had different sequences gives us further confidence that non-random sampling of clones was not a problem in this study. Twenty-two or 23 individuals of each trematode species was sequenced per subpopulation.

Extraction, DNA Amplification, and Sequencing

To examine genetic structure, we used a 636-639 bp region of the NADH-dehydrogenase subunit 1 (ND1) mitochondrial gene for all species. For DNA extractions, individual worms were placed in 100 µl of 5% chelex containing 0.2 mg/ml of proteinase K, incubated for 2 h at 56°C, and boiled at 100°C for 8 min. PCR amplifications were performed with 50 µl reactions containing 5 µl of extraction supernatant, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.4 µM each primer, 1 unit Taq DNA polymerase (Promega). Forward and reverse ND1 primers for *N. salmincola* were MB352 (5'-CGT AAG GGK CCT AAY AAG-3') and MB399 (5'-CTT ACA AAA TAG TCA TAG CG-3'), respectively. For *P. shawi*, the primers were MB352 and the reverse MB405 (5'-AAC ACA CTT TCA AAT ATT AAC C-3'). A forward primer, MB411 (5'-CAT ATG ATG TTR TCT TCT AG-3'), anchored in the NADH-dehydrogenase subunit 2 gene (see Le *et al.* 2002 for mitochondrial gene order in parasitic flatworms) was used with the reverse ND1 primer MB415 (5'-CAA AAC AAT AAC TAA GGC CC-3') to amplify the ND1 region in the *Deropegus* spp. The general PCR scheme was 95°C for 3 min, once; 94°C for 45 sec, 54°C for 30 sec, 72°C for 45 sec, 35 times; 72°C for 7 min, once. The following exceptions were *N. salmincola* with 40 cycles, *P. shawi* with a 55°C annealing temperature, and *Deropegus* spp. with a 52°C annealing temperature and 1 min extension. PCR products were purified with the Ultra Clean™ PCR clean-up™ Kit (MO BIO Laboratories, Inc.) and then sent to Nevada Genomics Center (Nevada, U.S.A.) for sequencing. Reverse primers were used as the sequencing primers. The same overlapping ND1 region was analyzed for all species and consisted of 639 bp for *N.*

salmincola and 636 bp for the other three species. There were no indels in any intraspecific alignment.

Upon finding two divergent ND1 lineages for *D. aspina* (see Results and Discussion), we sequenced the nuclear internal transcribed spacer 1 gene (ITS1) of the ribosomal DNA to test for the presence of cryptic species (morphologically similar, but genetically distinct). ITS1 for the two *Deropegus* lineages was amplified using the primers s18 and 5.8s1 from Jousson *et al.* (2000). PCR was as above, but with 1 μl of extraction supernatant, 1.5 mM $MgCl_2$, a 60.1°C annealing temperature, and a 1 min extension time. Purification and sequencing were as above. Two complete ITS1 sequences were initially obtained for both ND1 lineages by sequencing with both primers. Subsequent sequencing with 5.8s1 was used to identify the ITS1 type of the remaining individuals. Sequence data sets are deposited in GenBank under accession numbers AY269445-AY269510 (*D. aspina* A), AY269511-AY269599 (*D. aspina* B), AY269600-AY269690 (*N. salmincola*), and AY269691-AY269782 (*P. shawi*) for the ND1 sequences and AY269443, AY269444 for the ITS1 of the *Deropegus* species.

Data Analysis

We used analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) to test for genetic subdivision among the four parasite subpopulations (Fig. 3.1A). The resulting Φ-statistics are related to *F*-statistics, but estimate the average correlations of mutation frequencies at different levels of population subdivision (Excoffier 2001). ARLEQUIN v2.001 (Schneider *et al.* 2000) was used for the AMOVA analyses and to calculate the species-wide average number of nucleotide differences per site between two sequences, π. Statistical parsimony networks were computed with the program TCS v1.13 (Clement *et al.* 2000). We also used coalescent methods (Beerli & Felsenstein 2001) to obtain estimates of migration. An *N*-island migration model was used in the program MIGRATE v1.6.9 (Beerli & Felsenstein 2001) to estimate the average subpopulation θ ($\theta = 2N_e\mu$, where N_e is the effective size and μ is the mutation rate per nucleotide site) and $2N_em$ (2 times the average number of immigrants into each subpopulation, where m is the proportion of immigrants into a subpopulation per generation). The $2N_e$ (instead of $4N_e$) results from the fact that mitochondrial DNA (mtDNA) is haploid and all trematode species examined are hermaphroditic. Based on the results of MODELTEST v3.06 (Posada & Crandall 1998), base frequencies, transition-transversion ratios, and gamma shape parameters were estimated under the HKY model for all species, and then used as input into MIGRATE. Default

settings in Migrate were used to obtain initial estimates of θ and $2N_em$. A second run was conducted using the initial parameter estimates and increasing the short and long sampling increments to 100 as recommended in the program manual (http://evolution.genetics.washington.edu/lamarc.html). Results of the second run are those reported. Estimated parameters did not deviate appreciably upon use of different random number seeds or exclusion of inputted estimates obtained from MODELTEST.

Results and discussion

Mitochondrial DNA sequences for *D. aspina* revealed two divergent haplogroups that had fixed differences at 51 out of 636 sites (8% divergence), hereafter referred to as mtDNA types A and B. The mtDNA type A haplogroup was not found in the Winchuck River (vertical lines population, Fig. 3.1A). However, both haplogroups were sympatric in the other three rivers (Fig. 3.1A) and could be found infecting the same individual hosts. To test whether these two mtDNA haplogroups represent cryptic species, we sequenced the nuclear ITS1 from each of 73 individuals for which we already had mtDNA sequence. Within each sympatric location, equal numbers of individuals of each mtDNA type were sequenced (a total of 34 of each type) plus five mtDNA type B individuals were sequenced from the Winchuck River. We found exactly two ITS1 sequences, which differ at 15 of 822 sites (1.8% divergence). There was perfect concordance between mtDNA type and ITS1 type, a result that suggests no (or very little) introgression between the two lineages of *D. aspina*. Therefore, we considered the two mtDNA haplogroups to represent genetically distinct species, *D. aspina* A and *D. aspina* B, and analyzed their genetic structures separately.

High Φ_{ST} values (0.17-0.55) that were statistically different from null distributions of no genetic structure (estimated via random permutations of haplotypes among subpopulations) (Excoffier *et al.* 1992) were found for all three autogenic species (Table 3.1). A result of subdivision is that alleles are more closely related (i.e., coalesce to a common ancestor) within than among subpopulations (Hudson 1990). Thus, for *D. aspina* A, *D. aspina* B, and *P. shawi*, related haplotypes are much more likely to be found within the same subpopulation. This geographic structuring is strikingly illustrated in the genealogical relationships of the ND1 haplotypes (Figs. 3.1B-D). In contrast, the allogenic *N. salmincola* had a very low, non-significant Φ_{ST} (0.01) (Table 3.1). Related haplotypes, therefore, are just as likely to occur among as within subpopulations. The lack of geographic structure is evident in the ND1 network of *N. salmincola* (Fig. 3.1E).

The *N*-island migration model in MIGRATE, which assumes symmetric migration among all subpopulations, has the advantage of providing a single estimate of migration that can be used to assess the overall migration abilities of the autogenic and allogenic species. However, the values in Table 3.1 should not be considered absolute numbers, but rather relative measures (Whitlock & McCauley 1999). Migration values for *D. aspina* A, *D. aspina* B, and *P. shawi* were 90 to 400 times lower than estimated for *N. salmincola* (Table 3.1). These coalescent estimates of migration were congruent with Φ_{ST} values in showing that the autogenic species had much less gene flow than the allogenic *N. salmincola* (Table 3.1).

Table 3.1. Genetic population subdivision (Φ_{ST}), estimated parameters ($2N_em$ and average subpopulation θ) and species wide nucleotide diversity (π). *N* is the total sample size over four subpopulations. For each species, 22 or 23 individuals were sequenced per location (*D. aspina* A was only found in 3 subpopulations). Asterisk signifies Φ_{ST} values that are statistically significant ($P < 0.0001$) from 10,000 random permutations of haplotypes among populations; ns, $P > 0.05$.

Species	Total *N*	Φ_{ST}	$2N_em$	Average subpopulation θ	Species wide π
Autogenic					
Deropegus aspina A	66	0.172^{*}	0.76	0.0038	0.005
Deropegus aspina B	89	0.553^{*}	0.17	0.0038	0.011
Plagioporus shawi	92	0.393^{*}	0.24	0.0044	0.008
Allogenic					
Nanophyetus salmincola	91	0.013^{ns}	70.44	0.0244	0.008

It is noteworthy that the four species all have a similar value of species-wide π (Table 3.1) and of maximum pairwise difference between haplotypes (14, 13, 13, and 10 bp for *N. salmincola*, *P. shawi*, *D. aspina* B, and *D. aspina* A, respectively). In other words, the ND1 trees (Figs. 3.1B-E) have about the same total depth, indicating that they all have similar species-wide effective sizes (Wakeley 2000), even though N_e within subpopulations is substantially smaller in the autogenic species (assuming μ is constant for the average subpopulation θ values in Table 3.1). This pattern is again consistent with low gene flow for the autogenic species because reduced migration among subpopulations can substantially increase species-wide N_e beyond the sum of the subpopulation effective sizes (Nei & Takahata 1993; Wakeley 2000, 2001).

A corollary of the autogenic-allogenic hypothesis is that, owing to lower gene flow in autogenic species, we expect a higher rate of allopatric speciation over a given geographic range

in autogenic than in allogenic species. Thus, one should be more likely to find a pair (or complex) of sister species in samples of autogenic than in samples of allogenic species collected over the same geographic range. While our study does not permit a formal test of this expectation, it is interesting that we found that one of our autogenic species (*D. aspina*) is actually a pair of cryptic species.

By using species with similar ranges, and sampling from the same hosts and from the same populations, we controlled for almost every important variable except life cycle differences among the species. The tradeoff for this design was that we had access to only one allogenic species. We recognize that with only a single allogenic species our data do not provide a conclusive test of the hypothesis that allogenic species have higher gene flow among aquatic habitats than autogenic species. Nevertheless, the difference in genetic structures between the three autogenic and one allogenic species was extreme and in the predicted direction. Thus, our results strongly agree with the autogenic-allogenic hypothesis. Our study is now the third to examine the effects of host movement on gene flow in parasites. When taken together, this body of work suggests an emerging consensus that host movement is a major determinant of parasite gene flow.

Our study demonstrates that variation in life cycles can predispose parasites to different genetic structures, thereby affecting the evolutionary potential of those parasites. Thus, this study has several important implications. First, information on life cycles can help predict local adaptive potential, as parasite gene flow is an important component in the coevolutionary process (Gandon *et al.* 1996; Lively 1999). Second, little is known about factors that cause speciation in parasites (Poulin & Morand 2000). The observation that life cycles strongly affect parasite population genetic structure provides a potential explanation for why some parasitic taxa are more species rich than others (Poulin & Morand 2000). Lastly, gene flow has been shown to play a key role in the dispersal and persistence of drug resistant alleles among parasite populations (Caprio & Tabashnik 1992; Roper *et al.* 2003). Therefore, prediction of parasite gene flow from life cycle patterns will help in evaluating the potential for the evolution and spread of drug resistance in parasites (Anderson & May 1991).

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Chapter 4

Molecular analyses refute old paradigms of parasite mating systems and transmission dynamics

Charles D. Criscione

Michael S. Blouin

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Abstract

Understanding what factors shape genetic diversity within populations is an important goal in evolutionary biology. For parasites, however, key questions such as what defines the limits of a deme or what mating systems predominate within natural populations remain unanswered. For example, a standing paradigm for parasites is that subdivision of parasites into infrapopulations (all the parasites in a single host) causes low genetic diversity. Such may be true if parasite offspring are transmitted as a clump from host to host over several generations (i.e., the infrapopulation is the deme). However, if offspring are well mixed, then the parasite component population (all the parasites among a host population) functions as the deme. We examined these questions in natural populations of a hermaphroditic trematode, *Plagioporus shawi*, of salmonid fishes. We found high levels of genetic diversity, and *a posteriori* inference of population structure overwhelmingly supports the component population as the deme. Furthermore, genetic data indicate *P. shawi* is largely outcrossing. Genetic estimates of selfing (< 5%) were in accordance with the proportion of parasites from single infections. These results challenge the previous paradigm. We predict that aquatic transmission and use of several intermediate hosts will promote high genetic diversity and well-mixed infrapopulations.

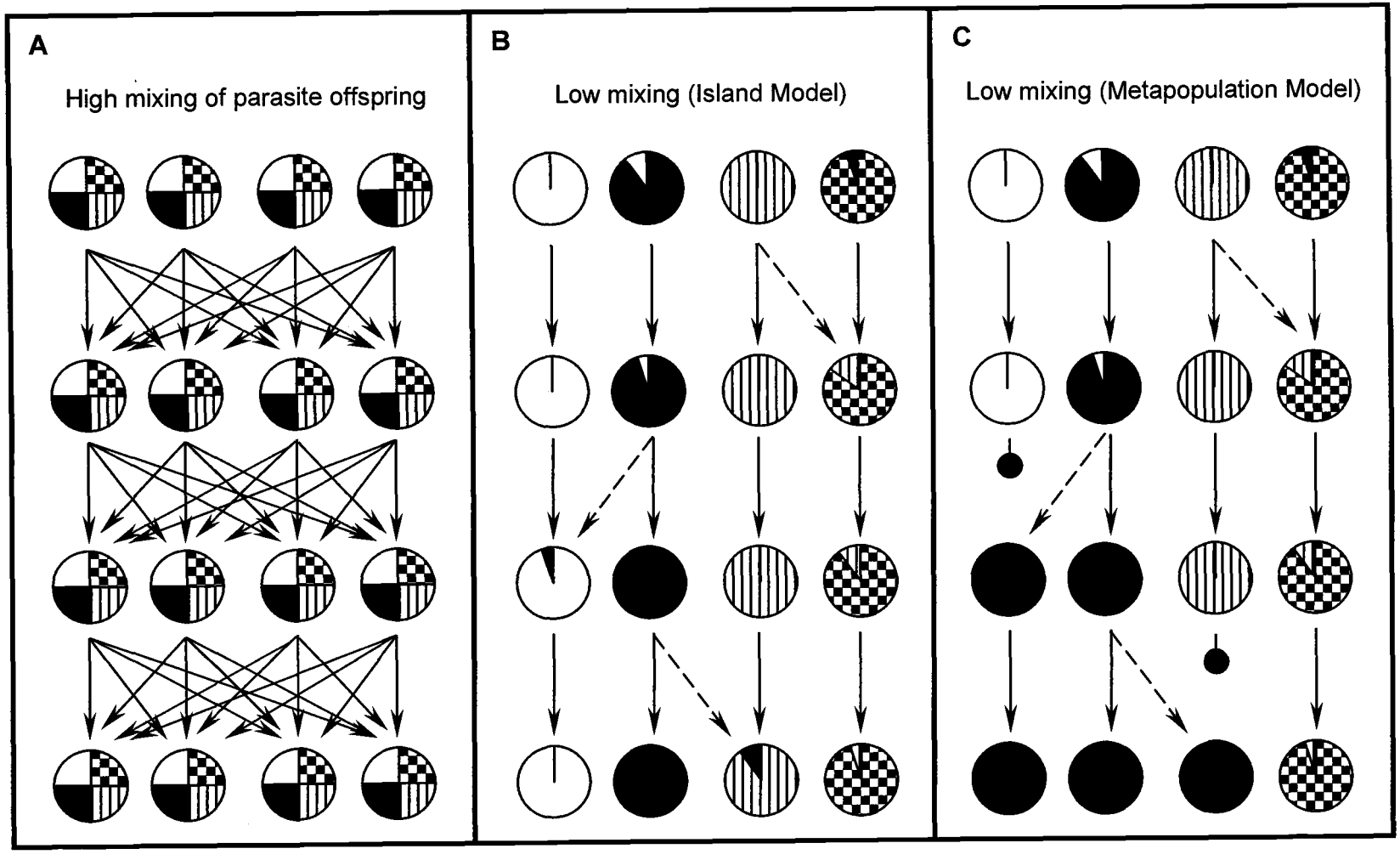
Introduction

Recognizing the boundaries of demes and the mating system in natural populations is key for understanding levels and patterns of genetic diversity (Charlesworth 2003). For parasites, 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? In addition, the question of what constitutes a parasite deme has been raised repeatedly (Lydeard *et al.* 1989; Nadler 1995; Bush *et al.* 2001; Jarne and Theron 2001; Sire *et al.* 2001) because the limits of a parasite deme may not just be related to geographic isolation, but also isolation among hosts within a geographic location. 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 (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. 4.1). 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. 4.1A). 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. 4.1B,C).

The paradigm that infrapopulations cause fractionated gene pools (Price 1977, 1980) remains unverified. The transmission processes presented in Figure 4.1 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. 4.1A), 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

Fig. 4.1. 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. 2005). **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. Price's (1977, 1980) views (the standing paradigm) of parasite transmission dynamics and predictions of low genetic diversity for parasite populations are akin to those in the metapopulation model. In both **B** and **C**, infrapopulations behave as demes because there is a stable recurrence of generations. 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.


A
High mixing of parasite offspring
B
Low mixing (Island Model)
C
Low mixing (Metapopulation Model)

subdivision lead to different predictions. The classic island model (Wright 1969) predicts that increasing isolation among infrapopulations increases component population diversity because infrapopulations will become fixed for different genetic variants (Fig. 4.1B) (reviewed by Pannel & Charlesworth 2000). This prediction has been reiterated in recent papers modeling parasite populations (Prugnolle *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 & 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. 4.1C). However, differentiation (F_{ST}) among hosts can increase or decrease depending on the source and number of colonizers, and extinction rates of infrapopulations (reviewed by Pannel & Charlesworth 2000). It should be noted that Price (1977, 1980) viewed hosts as ephemeral habitats and his predictions are similar to those of the metapopulation model. 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). In addition, digenean trematodes have a unique aspect of reproduction in that there is asexual amplification stage in the snail intermediate host. Models show that clones can increase differentiation among definitive hosts (Prugnolle *et al.* 2005a,b). Thus, the questions of how abundant clones are and how often clones are transmitted together are important for understanding trematode transmission dynamics (Theron *et al.* 2004).

Here we address the above 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.). To test whether infrapopulations result in fragmented gene

pools, we use 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 component population of *P. shawi* functions as a deme rather than individual infrapopulations. In addition, genetic and demographic estimates of selfing indicate that *P. shawi* is largely outcrossing. Comparisons with regards to the transmission dynamics of flatworms and clonal structure of trematodes are made to previously studied systems.

Material and Methods

Studied Species

Plagioporus shawi completes its life cycle within a freshwater stream. 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). 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, USA (Hoffman 1999). A previous study with mitochondrial DNA suggests that *P. shawi* has limited gene flow among freshwater drainages (Criscione & Blouin 2004).

Sampling

Geographic and temporal stability of our results were examined by sampling two creeks from separate river systems from March to April of 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 (USA), and drain into the Pacific Ocean. To insure that the parasites originated from the respective stream, we sampled out-migrating 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 & 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, PCR, and genotyping are given in Criscione & 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 & 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 from ALS-2002, which were used as a pooled sample in primer development (Criscione & Blouin 2005). These 14, which came from a single host, were included in demographic analyses.

Demographic Analyses

To evaluate the demographic stability of transmission over time and among geographic populations, we compared prevalence (percent hosts infected) and mean intensity of infection. A nested logistic regression where year was nested within location was used to test for differences in prevalence. Similarly, a nested ANOVA was used to test for differences in log-transformed intensity. Residual plots were examined visually for violations of normality or homogeneity of variance. JMPIN v4.04 (SAS Institute Inc.) was used for both statistical analyses. As noted above, comparisons of mean intensities are important for understanding differences in selfing or biparental inbreeding among samples. Many macroparasites have aggregated abundance distributions (the number of parasites per host examined) such that few hosts are infected with many parasites whereas most hosts have few parasites (Shaw *et al.* 1998). The index of dispersion test as described in Krebs (1999) was used to see if *P. shawi* displays a pattern of aggregation among hosts. This is a simple test to see if the variance to mean ratio of abundance is greater than one. Significance was determined from a two-tailed chi-squared test because the index of dispersion (I) could be less than one, thus indicating a uniform distribution (Krebs 1999). A Bonferroni correction was applied for multiple tests (i.e., $P < 0.006$ would indicate significant

aggregation). Note that tests of intensity do not include uninfected hosts whereas tests of abundance do (Bush *et al.* 1997).

Identification of clones

Identical multilocus genotypes were identified by searching for repeated data entries in Microsoft® 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 v3.1, which uses Bayesian inference to delineate populations (Corander *et al.* 2003, 2004), was used to cluster individual parasites. BAPS v3.1 finds clusters that are in Hardy-Weinberg equilibrium with the assumption that loci are in linkage equilibrium. Details of BAPS v3.1 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 v3.1 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 ten replicates for each K value ranging from two to the maximum K mentioned above. After obtaining the optimal partition from BAPS v3.1, we tested the null hypothesis of no association between BAPS-identified parasite clusters and individual infrapopulations. Thus, we could assess if there was clumped transmission with regards to the identified parasite clusters. We used the G-based exact test (Goudet *et al.* 1996) as implemented in GENEPOP web v3.4 (http://wbiomed.curtin.edu.au/genepop; Raymond & Rousset 1995). This was done by coding each parasite cluster as a unique genotype from a single locus and then running the population genotypic differentiation option. All three Markov chain parameters were set to 5,000. In the SIL samples, two host species, *O. mykiss* and *O. clarki*, were sampled. Therefore, we also ran an

exact test 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 within and 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 unbiased estimators of F_{IS} (average within infrapopulations) and F_{ST} (among infrapopulations) (Weir & Cockerham 1984; calculated by FSTAT v2.9.3, Goudet 1995). Significance of F_{IS} was tested by permuting alleles among individuals within infrapopulations (15,000 permutations). 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, in order to mimic the recruitment of individual parasites. The 95% confidence intervals of F_{IS} and F_{ST} were calculated by bootstrapping over loci with FSTAT v2.9.3.

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 (by K. F. Goodnight), which uses the algorithm of Queller & 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 expected with random parasite recruitment. For this test, we permuted individuals among hosts 10,000 times using the program SPAGEDI v1.2 (Hardy & 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 v3.4. Markov chain parameters were set to 5,000. The Weir & Cockerham (1984) estimator of F_{IS} for each locus and the multilocus F_{IS} was calculated in SPAGEDI v1.2. Deviations from Hardy-Weinberg equilibrium were tested with two-tailed tests of 10,000 randomizations of alleles among individuals. Significance was determined at $P \leq 0.05$. A Bonferroni correction was applied for multiple tests. Gene diversity

(H_s; Nei 1987) and allelic richness (rarefied to the ALS-2002 sample size of 94) were calculated with FSTAT v2.9.3.

Selfing rate was estimated with one demographic and two genetic methods. The first method takes advantage of the fact that infrapopulations define discrete mating boundaries for *P. shawi*. Thus, the proportion of flukes present in single infections can provide a rough estimate of the selfing rate. Gravid flukes from single infections were observed in this study. This method is only a crude approximation because selfing may occur within hosts infected with more than one trematode or because flukes from single infections may have outcrossed, but the mate was subsequently lost from the host. The second method is based on the relationship $F_{IS} = S/(2\text{-}S)$ where S is the proportion of selfed offspring assuming inbreeding equilibrium (Hedrick 2004). Multilocus estimates of F_{IS} were used in the above equation. The third method uses the distribution of 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 monoecy with complete selfing using BOTTLESIM v2.6 (Kuo & 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 v1.2. IIC were binned into intervals of width 0.02 for the likelihood calculations.

Results

Demographic Results

Prevalence was high (range 76-83%; Fig. 4.2) and was not significantly different between years within locations or between locations (P values > 0.05). Mean intensities were low (range 4.3-5.6; Fig. 4.2) and were not significantly different between years within locations or between locations (P values > 0.05). The index of dispersion was significantly greater than one in all samples (P values < 0.001, Fig. 4.2), thus indicating that the observed abundance distributions of *P. shawi* were aggregated.

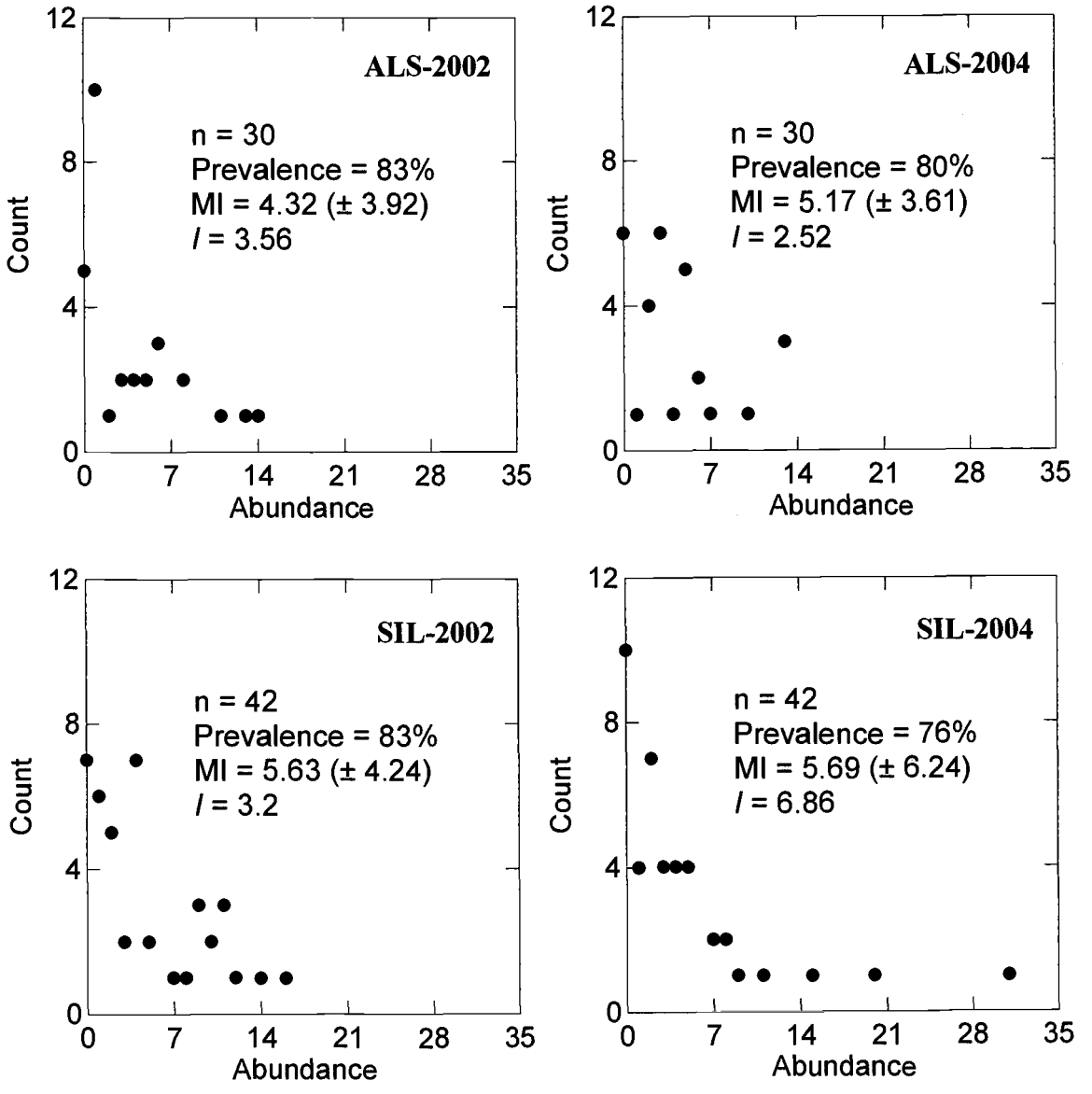


Fig. 4.2. Abundance distributions and demographic summary statistics of *P. shawi* in ALS and SIL from 2002 and 2004. Each figure shows the count of hosts (y-axis) that contain different numbers of parasites including uninfected hosts (x-axis). *n* is the number of hosts examined, prevalence is the percent hosts infected, MI (± standard deviation) is mean intensity, and *I* is the index of dispersion (variance to mean ration of abundance). Notice that *P. shawi* has aggregated abundance distributions and that all samples have low mean intensities.

Identified clones

A single pair of individuals with identical multilocus genotypes was identified from one host in ALS-2004. After one million 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 pair was considered to be clones. Both individuals were included in the demographic 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.

Results for Assessing the Boundaries of the Deme

The numbers of optimal partitions from BAPS v3.1 were 9, 6, 8, and 7 for ALS-2002, ALS-2004, SIL-2002, and SIL-2004 respectively. The majority of individuals, however, 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 (see Table 4.1), the remaining clusters from all population samples had only one to four individuals. The column sums of Table 4.1 show an example of the cluster sizes from SIL-2004. All exact tests among infrapopulations or among host species (SIL samples) were not significant ($P > 0.05$). The contingency table for the exact test among host species in SIL-2004 is shown in Table 4.1.

Table 4.1. Contingency table of BAPS-identified parasite clusters by host species from SIL-2004

	Parasite cluster							
Host species	1	2	3	4	5	6	7	Σ
Oncorhynchus clarki	2	7	0	2	111	2	0	124
Oncorhynchus mykiss	1	4	1	1	49	1	1	58
Σ	3	11	1	3	160	3	1	182

Note: *G*-like test: $P = 0.8$, SE = 0.0003. Column sums are the cluster sizes from BAPS v3.1. Row sums are the total number of parasites genotyped from 21 infected *O. clarki* and 11 *O. mykiss*.

There was no sign of genetic structuring within or among hosts based on the analyses with *F*-statistics (Table 4.2). All permutation tests (within and among hosts) were not significant ($P > 0.05$). All confidence intervals, but the F_{ST} for SIL-2004, overlapped with zero (Table 4.2).

Likewise, the confidence intervals for the average within-host relatedness contained zero (Table 4.2). The permutations performed in SPAGEDI v1.2 showed that within-host relatedness was not significantly different than expected from random recruitment.

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. (See Tables A1 and A2 in the appendix for allele frequencies, and 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 4.3). Mean H_s was high and above 0.8 in all population samples (Table 4.3). Likewise, the mean allelic richness was high and above 21 in all population samples (Table 4.3). 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 A2), were excluded from the SIL analyses.

Although all population samples were in Hardy-Weinberg equilibrium, the multilocus estimates of F_{IS} were slightly positive (Table 4.3). Thus, there may be low levels of selfing or biparental inbreeding in these populations. The demographic estimates of selfing suggest a potential range of about one to ten percent (Table 4.3). In accordance with this range, the inbreeding equilibrium measure of selfing, S, ranges from 2.4 to 4.5% (Table 4.3). In general, the ML estimates of selfed individuals using either the Ritland (1996) or Loiselle *et al.* (1995) IIC were lower than the estimates from S (Table 4.3). The distributions of observed IIC closely approximated the IIC distributions that were simulated under random mating (Fig. 4.3). There was the occasional individual(s), however, that extended beyond the random mating distribution (Fig. 4.3), thereby suggesting a very low proportion of individuals that are the result of selfing.

Table 4.2. Summary statistics for structure within and among infrapopulations

Population	Host N [a]	Parasite N [b]	F_{IS} (95% C.I.)	F_{ST} (95% C.I.)	Average within-host R (95% C.I.)
ALS-2002	24	94	0.021 (-0.002; 0.041)	0.002 (-0.008; 0.011)	-0.0115 (-0.037; 0.014)
ALS-2004	24	123	0.014 (-0.001; 0.028)	0 (-0.008; 0.006)	-0.0061 (-0.022; 0.01)
SIL-2002	35	197	0.013 (-0.008; 0.038)	0 (-0.009; 0.006)	-0.0026 (-0.015; 0.01)
SIL-2004	32	182	0.003 (-0.023; 0.031)	0.01 (0.005; 0.014)	0.0142 (-0.004; 0.032)

Note: Confidence intervals for F-statistics were obtained by bootstrapping over loci, whereas confidence intervals for relatedness (R) were obtained by jackknifing over hosts.

[a] The number of infected hosts from which parasites were genotyped.

[b] The total number of genotyped parasites

Table 4.3. Mean gene diversity (H_s), mean allelic richness, multilocus F_{IS}, and selfing rates

Population	Mean H_s	Mean allelic richness	Multilocus F_{IS}	S [a]	ML-Ritland[b]	ML-Loiselle[c]	% of flukes in single infections
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%

Note: All calculations for ALS were based on nine loci, whereas seven loci were used for SIL. Mean allelic richness is the average allelic richness among loci (values rarefied to a sample size of 94; see table A2). Because ALS-2002 had a sample size of 94, the mean allelic richness is equal to the mean number of alleles per locus.

[a] S is the proportion of selfed offspring, and was calculated with the formula $F_{IS} = S/(2\text{-}S)$, where F_{IS} is the multilocus estimate.

[b] 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 v1.2 (Hardy & Vekemans 2002).

[c] 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 v1.2 (Hardy & Vekemans 2002).

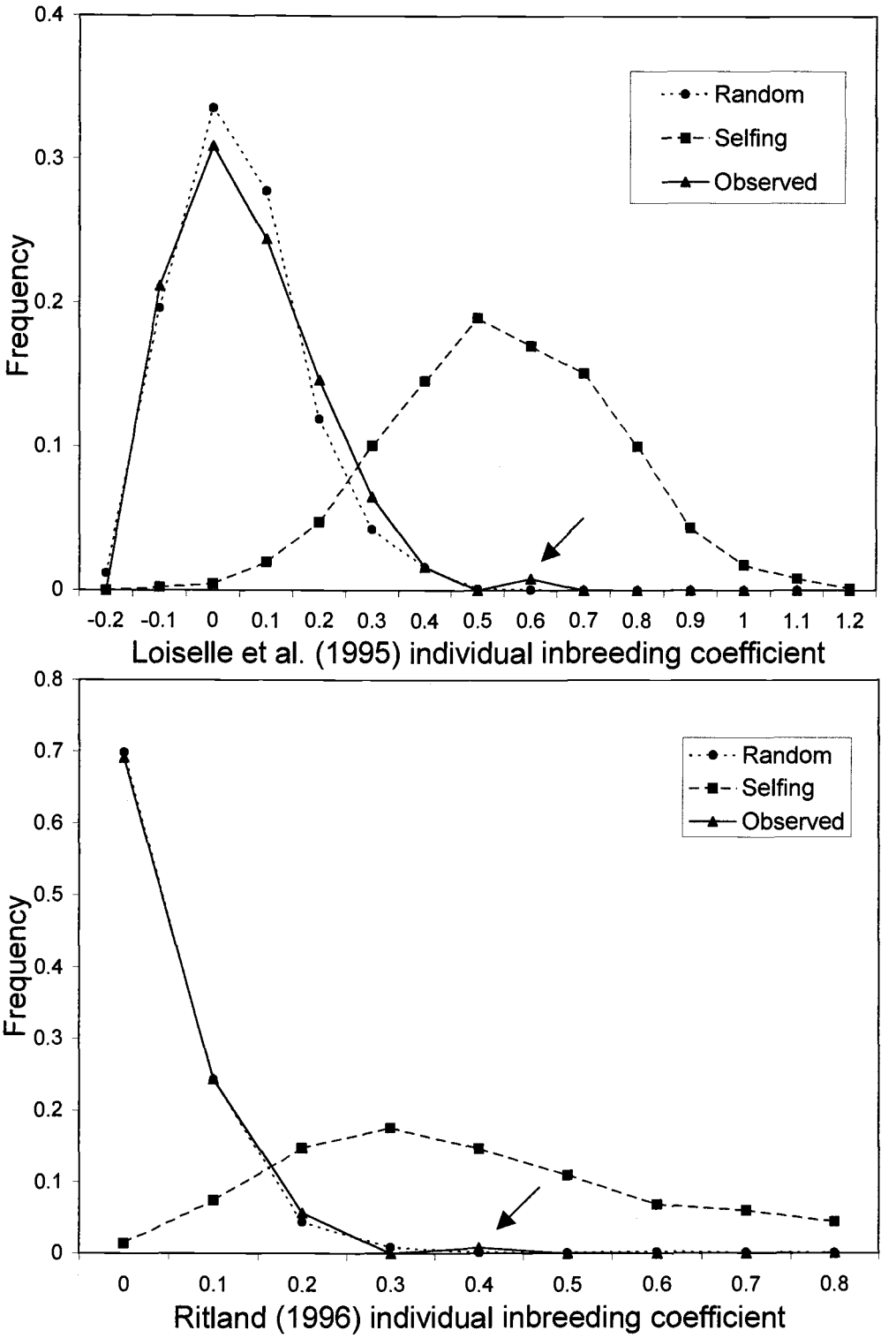


Fig. 4.3. Frequency distributions of individual inbreeding coefficients (IIC) for ALS-2004. **A**: Loiselle et al. (1995) IIC. **B**: Ritland (1996) IIC. IIC were simulated under random mating and full selfing. The observed distributions of IIC closely approximate the IIC distributions simulated under random mating. The arrows indicate individuals that are likely the result of selfing.

Discussion

What is the deme?

Our results overwhelmingly support the component population, rather than individual infrapopulations, as the functional deme in *P. shawi* (Fig. 4.1A). The BAPS inference of population structure grouped over 80% to90% 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 4.1), 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 4.2) 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 & 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 4.3). 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 4.3). Direct ML estimates of the fraction of individuals that resulted from a selfing event (e.g., Fig. 4.3) were very similar, ranging from 0 to 3.3% (Table 4.3). All genetic selfing estimates are surprisingly close to those predicted by the fraction of individuals that are found in single-worm infections (Table 4.3). 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 out of a total of 596 genotyped parasites (Table 4.2). Thus, in *P. shawi* multiple copies of clones are rarely recruited into definitive hosts or 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. 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), and can lead to inbreeding if identical clones transmit together. 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 4.3) and allelic richness ranging from 10-35 (Table A2). In this case the species appears to preferentially outcross and to occur in well-mixed, non-subdivided populations. *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 vs. other flatworms: effects of aquatic vs. 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 semi-terrestrial 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 causing infrapopulation extinction (Fig. 4.1C). Such clumped transmission and extinction dynamics may cause a greater variance in reproductive success among individuals and further explain lower genetic diversity in species such as *S.*

mansoni (Theron *et al.* 2004; Prugnolle *et al.* 2005*c*). On the other hand, 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. Second intermediate hosts may accumulate many distinct cercarial genotypes before being eaten (e.g., Rauch *et al.* 2005), and 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.

It is also interesting that 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. *E. 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 genetic diversity and genetically most well-mixed infrapopulations.

Price's paradigm and transmission dynamics

Price (1977, 1980) 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 (Pannel & Charlesworth 2000). At first glance, trematodes such as *P. shawi* would seem to possess the ecological characteristics that fit Price's predictions. It has the aggregated abundance distribution among hosts (Fig. 4.2) that is typical for macroparasites (Shaw *et al.* 1998). It has low mean intensities (Fig. 4.2) and is hermaphroditic,

both of which should increase the chance for inbreeding or founder effects. Furthermore, the clonal reproductive stage in the snail host could create a large reproductive skew among individuals (Prugnolle *et al.* 2005a,b), not to mention the opportunity for extreme inbreeding. Nevertheless, 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 random-mating unit. Furthermore, our genetic and demographic data were consistent over space and time, and so do not result from an atypical sample. Whether this genetic structure is more typical of parasites from aquatic environments than from terrestrial environments remains to be tested. Regardless, these data provide a sound challenge to previous generalizations about the genetic structure and mating systems of parasites.

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Chapter 5

General conclusion

I addressed the influence of parasite ecological characteristics on the genetic structure of parasite populations. Chapter 2 provides a current review of parasite molecular ecology, while Chapters 3 and 4 examine ecological-based predictions of among and within population genetic structure of parasites, respectively. As highlighted in Chapter 2, several molecular ecology studies on parasites have appeared since Nadler's (1995) review. However, there are still significant gaps in our knowledge about the genetics of parasite populations. In addition, many of the existing studies are concentrated on human related parasites such as *Schistosoma mansoni* and *Plasmodium falciparum*. Studies on human parasites often enjoy abundant resources to address evolutionary questions, and provide an obvious benefit in dealing with parasite control (e.g., knowledge on drug resistance evolution). However, it may be hard to extrapolate the results of anthropogenic systems to more natural settings of parasite transmission. Thus, there is a need to study wild populations to develop a full understanding of factors that influence the genetic structure of parasite populations.

In Chapter 3, I predicted that life cycle variation (explained by the autogenic-allogenic hypothesis) would affect the genetic structure among natural populations of salmonid trematodes. The results of this study show that the three autogenic species have highly subdivided subpopulations, whereas the one allogenic species has high gene flow among subpopulations. Furthermore, there is now mounting evidence (in conjunction with previous studies on nematodes of domestic animals (Blouin *et al.* 1995) and tics of seabirds (McCoy et al. 2003), that parasite gene flow is a function of host vagility. More importantly, this study highlights how life cycles may be useful in predicting patterns of local adaptation or the potential for the spread of drug resistance.

The effects of local transmission dynamics among individual hosts on the genetic diversity within parasite populations were examined in Chapter 4. I tested Price's (1977, 1980) paradigm that subdivision of parasites among hosts will result in fractionated parasite gene

pools, and thus low genetic diversity in the component population. By outlining different modes of transmission (a large amount of mixing of parasite offspring before recruitment into definitive hosts versus clumped transmission), however, different predictions than those of Price can be reached. In particular, high mixing among parasite offspring leads to the component population as behaving as the functional deme rather than individual infrapopulations. *A posteriori* inference of population structure overwhelmingly supported the component population as the deme for the trematode *Plagioporus shawi*. Comparisons with data from other trematode studies suggest that the aquatic transmission of *P. shawi* promotes well-mixed infrapopulations. Furthermore, genotype frequencies in component populations of *P. shawi* were not significantly different than expected under Hardy-Weinberg equilibrium. Genetic estimates of selfing were less than five percent and within the range of the proportion of flukes present in single infections. Similar selfing estimates between the genetic and demographic methods suggests that *P. shawi* only self fertilizes when alone. These data refute several generalizations made by Price (1977, 1980) concerning the genetic structure and mating systems of parasites.

Both the broad scale (among geographic populations) and local scale (within geographic populations) studies presented here illustrate the impact of parasite ecological characteristics on the genetic structure of parasite populations. More studies, however, are still needed to develop a thorough understanding of factors that influence microevlutionary processes in parasites. Given that parasites are ecologically and taxonomically diverse, broad generalizations such as those presented by Price (1977, 1980) are unlikely to hold. Instead, a diversity of population genetic patterns that reflect different life history characteristics of parasites is likely to emerge. A diversity of patterns, however, should be viewed as a positive argument for studying parasite molecular ecology because there will be numerous opportunities for studying ecological and evolutionary theory in a variety of different historical and selective backgrounds.

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Appendix

Appendix A. Supplementary materials, Chapter 4

Table A1. Allele frequencies by locus

Locus and alleles	ALS-2002	ALS-2004	SIL-2002	SIL-2004
Locus m41				
98	0.0372	0.0325	0.0025	0
102	0.0053	0	0	0
103	0	0.0041	0	0
106	0.0106	0.0122	0	0
107	0.016	0.0122	0.0025	0.0027
111	0.7181	0.7724	0.6904	0.7363
115	0.0851	0.0569	0.1193	0.1209
118	0	0	0.0025	0
119	0.0106	0	0.0228	0.0137
123	0.0053	0.0041	0.0102	0.0082
127	0.0053	0	0	0
147	0.0053	0	0.0025	0.0055
151	0	0.0041	0.0025	0
155	0	0.0041	0.0025	0.0027
159	0	0	0	0.0027
163	0	0	0	0.0027
167	0	0.0041	0.0025	0.0027
171	0.0053	0	0	0
175	0	0.0081	0	0.0027
179	0.016	0.0041	0.0025	0
183	0.0053	0	0.0228	0.0137
187	0	0.0041	0.0178	0.0192
191	0.0213	0.0163	0.0152	0.0137
195	0.0106	0.0203	0.0203	0.011
199	0.0053	0.0041	0.0127	0.0055
203	0	0.0081	0.0127	0.0027
207	0.016	0.0041	0.0152	0.0082
211	0	0.0122	0.0076	0.0082
215	0.0053	0.0041	0.0025	0.0082
219	0	0.0041	0.0102	0.0055
223	0	0	0	0.0027
227	0.0053	0.0041	0	0
231	0.0053	0	0	0
235	0.0053	0	0	0
Locus m26				
69	0.0745	0.0569	0.0558	0.1236
73	0.0426	0.0244	0.0152	0.011
77	0.3723	0.3902	0.4721	0.4148
81	0.0372	0.0447	0.0152	0.0137
85	0.0372	0.0772	0.0685	0.0412
89	0.0638	0.0569	0.0482	0.0659
93	0.0319	0.0122	0.0254	0.0137

97	0.0053	0.0203	0.0051	0.0165
101	0.0106	0.0244	0.0102	0.0055
105	0.0053	0.0122	0.0355	0.0275
109	0.0372	0.0325	0.0228	0.0577
113	0.0266	0.0407	0.033	0.0385
117	0.0479	0.0244	0.0431	0.033
121	0.0372	0.0366	0.0203	0.0412
125	0.0266	0.0163	0.0406	0.0247
129	0.0372	0.0569	0.0381	0.0275
133	0.0372	0.0285	0.0076	0.0165
137	0.0372	0.0081	0.0152	0.011
141	0.0106	0.0122	0.0102	0
145	0.0053	0	0.0152	0
149	0.0106	0.0122	0	0.0055
153	0	0.0081	0	0.0082
157	0	0.0041	0	0
161	0.0053	0	0.0025	0
165	0	0	0	0.0027
Locus m48				
84	0.016	0.0081	0.0025	0
88	0.0053	0.0081	0.0025	0
92	0.016	0.0203	0.0787	0.0632
96	0.0213	0.0203	0.0102	0.0055
100	0.0053	0.0041	0.0076	0.011
104	0.0053	0.0122	0.0102	0.011
108	0.0053	0.0041	0.0305	0.022
112	0.016	0.0041	0.0305	0.0192
116	0.0426	0.0122	0.0279	0.0412
120	0.0532	0.0163	0.0305	0.0275
124	0.0266	0.0325	0.033	0.033
128	0.0266	0.0488	0.0508	0.044
132	0.0638	0.0366	0.0533	0.0495
136	0.0691	0.0854	0.0381	0.0495
140	0.0479	0.061	0.0508	0.0604
144	0.0638	0.0447	0.0457	0.0577
148	0.0798	0.0691	0.0736	0.044
152	0.0532	0.0528	0.0711	0.0522
156	0.0585	0.0407	0.0406	0.0687
160	0.0426	0.0813	0.0533	0.0797
164	0.0053	0.0325	0.0482	0.044
168	0.0266	0.0447	0.0305	0.0247
172	0.0319	0.0447	0.0228	0.0275
176	0.0213	0.0366	0.0254	0.0467
180	0.0426	0.0325	0.033	0.0302
184	0.0426	0.0203	0.0178	0.0137

188	0.0213	0.0325	0.0152	0.0055
192	0.016	0.0285	0.0279	0.0082
195	0	0	0	0.0082
196	0.0106	0.0244	0.0102	0.0247
200	0.0213	0.0041	0	0.0082
204	0	0.0041	0.0076	0.0055
208	0.0106	0	0.0076	0
212	0.0053	0.0041	0.0076	0
216	0.016	0.0041	0	0.0082
220	0	0.0041	0.0025	0.0027
224	0.0053	0.0081	0	0
228	0	0	0.0025	0.0027
232	0.0053	0	0	0
236	0	0.0041	0	0
240	0	0.0041	0	0
244	0	0.0041	0	0
Locus d13				
284	0.0106	0.0081	-	-
285	0	0.0081	-	-
286	0.016	0.0163	-	-
287	0.0106	0.0163	-	-
289	0.0798	0.0732	-	-
290	0.0106	0.0081	-	-
291	0.1596	0.2195	-	-
292	0.2766	0.252	-	-
293	0.133	0.1626	-	-
294	0.1011	0.0732	-	-
295	0.0957	0.0691	-	-
296	0.0319	0.0203	-	-
297	0.0372	0.0447	-	-
299	0.0266	0.0244	-	-
301	0.0106	0	-	-
305	0	0.0041	-	-
Locus d36				
135	0	0	0.0025	0
137	0	0	0.0228	0.011
139	0.0213	0.0325	0.0178	0.0247
140	0	0.0041	0	0
141	0.0319	0.0285	0.0254	0.0165
143	0.0691	0.065	0.0635	0.1016
144	0.016	0.0325	0.0355	0.0385
145	0.117	0.187	0.1701	0.1841
146	0.0053	0.0203	0.0102	0.0082
147	0.2447	0.2602	0.198	0.2088
148	0.0106	0.0041	0.0127	0.011

149	0.1011	0.0772	0.0863	0.1016
150	0.0053	0.0041	0.033	0.011
151	0.0532	0.0203	0.0355	0.0247
152	0	0	0.0076	0.0055
153	0.0213	0.0244	0.0381	0.0577
155	0.0479	0.0407	0.0279	0.0137
157	0.0479	0.0488	0.0203	0.0165
159	0.0319	0.0285	0.0228	0.0192
161	0.0372	0.0041	0.0228	0.022
162	0	0	0	0.0027
163	0.0266	0.0081	0.0178	0.011
165	0.0213	0.0285	0.0102	0.0165
167	0.0213	0.0203	0.0178	0.0192
169	0	0.0122	0.0076	0.0137
171	0.0106	0.0041	0.0178	0.011
173	0	0.0041	0.0102	0
175	0	0.0041	0.0076	0.0082
177	0	0.0041	0.0025	0.0027
179	0.0053	0	0.0025	0
181	0.0106	0	0.0127	0
183	0	0.0041	0.0025	0
185	0.0053	0	0	0.0027
189	0.0053	0.0041	0	0
191	0	0	0.0025	0.0027
194	0.0053	0	0	0
203	0	0	0	0.0027
207	0	0	0.0025	0
841	0.0053	0.0122	0.0305	0.0275
843	0.016	0.0122	0.0025	0.0027
845	0.0053	0	0	0
Locus d47				
174	0.0053	0.0163	-	-
180	0.0053	0.0081	-	-
182	0.0266	0.0163	-	-
184	0.0372	0.0203	-	-
186	0.0479	0.061	-	-
188	0.2287	0.2439	-	-
190	0.0479	0.0488	-	-
192	0.0479	0.0407	-	-
194	0.0426	0.0488	-	-
196	0.0532	0.0569	-	-
198	0.3245	0.3252	-	-
200	0.0479	0.0488	-	-
202	0.0479	0.0325	-	-
204	0.0053	0.0203	-	-

206	0.0106	0.0041	-	-
208	0.0106	0.0081	-	-
212	0.0106	0	-	-
Locus d04				
158	0	0.0081	0	0
160	0.2979	0.3008	0.2487	0.2665
162	0.1489	0.1585	0.1345	0.1593
164	0.1862	0.1016	0.1066	0.1044
165	0	0	0.0051	0
166	0.1543	0.1341	0.1878	0.1813
168	0.1436	0.1382	0.1193	0.0989
170	0.0266	0.0732	0.0863	0.0797
172	0.0266	0.0488	0.0406	0.0412
174	0.0053	0.0081	0.0152	0.0055
176	0.0053	0.0081	0.0178	0.0192
178	0	0.0041	0.0178	0.0192
180	0	0.0081	0.0102	0.011
182	0	0.0081	0	0.0027
184	0	0	0.0051	0.0055
186	0.0053	0	0	0.0027
188	0	0	0	0.0027
190	0	0	0.0025	0
192	0	0	0.0025	0
Locus d09				
137	0.0053	0	0	0
142	0.0053	0.0163	0.0051	0.0027
144	0.0904	0.0569	0.0711	0.0632
145	0	0	0.0025	0
146	0.0745	0.061	0.0711	0.0687
147	0.0106	0.0081	0	0
148	0.0479	0.0732	0.0838	0.0467
150	0.0426	0.0569	0.0355	0.0275
152	0.0372	0.0488	0.066	0.0577
153	0.0053	0	0.0025	0
154	0.0798	0.0772	0.0508	0.0549
155	0	0	0.0025	0
156	0.0319	0.0488	0.0508	0.0879
158	0.0426	0.0325	0.0279	0.0385
160	0.0585	0.0488	0.033	0.0687
162	0.016	0.0203	0.033	0.044
163	0	0.0041	0	0.0027
164	0.0585	0.0407	0.0457	0.0247
166	0.0426	0.0569	0.0508	0.0247
167	0	0	0.0025	0.0027
168	0.0691	0.0366	0.0635	0.0659

169	0	0	0	0.0027
170	0.0213	0.0447	0.0305	0.0412
172	0.0479	0.0407	0.0381	0.0357
174	0.0319	0.0569	0.033	0.044
176	0.0319	0.0244	0.0482	0.033
178	0.0266	0.0244	0.0279	0.0357
180	0.0319	0.0203	0.0305	0.022
182	0.0319	0.0122	0.0152	0.0247
184	0.0213	0.0285	0.0355	0.0055
186	0.0106	0.0163	0.0152	0.0247
188	0.0106	0.0163	0.0127	0.011
190	0.0053	0.0122	0	0.0192
192	0.0053	0.0081	0.0025	0.0027
194	0.0053	0.0041	0.0051	0.0027
196	0	0	0.0051	0.0027
198	0	0.0041	0.0025	0.0082
202	0	0	0	0.0027
Locus d43				
188	0.0053	0.0081	0.0076	0
190	0.0691	0.0528	0.0178	0.0192
192	0.0106	0.0325	0.0152	0.0137
194	0.1383	0.1667	0.1777	0.1841
196	0.1011	0.1098	0.1371	0.1181
198	0.0851	0.126	0.0939	0.1016
200	0.117	0.1463	0.1421	0.1016
202	0.1649	0.0935	0.099	0.1154
203	0	0	0.0025	0
204	0.0585	0.0772	0.0838	0.0659
205	0	0	0	0.0027
206	0.0426	0.0244	0.0431	0.0412
207	0	0	0.0025	0.0027
208	0.0213	0.0203	0.0254	0.0385
209	0	0	0.0051	0.0027
210	0.0319	0.0081	0.0178	0.022
212	0.0266	0.0244	0.0305	0.022
213	0	0	0	0.0082
214	0.0319	0.0203	0.0178	0.0192
216	0.016	0.0122	0.0152	0.0082
218	0.0053	0.0325	0.0127	0.022
220	0.0106	0.0081	0.0102	0.0275
222	0.0053	0.0163	0.0102	0.0192
224	0.0053	0.0081	0.0051	0.0137
225	0	0	0	0.0027
226	0.0053	0.0041	0.0051	0.0027
228	0.016	0	0.0051	0.0027

230	0.0053	0	0.0076	0.0055
232	0	0	0.0051	0
234	0.0053	0.0081	0.0025	0.0027
236	0.016	0	0	0.0055
238	0.0053	0	0	0.0055
242	0	0	0.0025	0.0027

Table A2. Gene diversity (H_s), number of alleles (A), and F_{IS} by locus

	ALS-2002 (94)[a]			Als-2004 (123)			SIL-2002 (197)			SIL-2004 (182)		
Locus	H_s	A	F_{IS}	H_s	A	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	-	-	-	-	-	-

Note: 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.

[a] The total number of parasites genotyped.