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LIFE CYCLES SHAPE PARASITE EVOLUTION: COMPARATIVE POPULATION GENETICS OF SALMON TREMATODES

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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.

Key words.—Cryptic species, gene flow, genetic structure, mitochondrial DNA, parasite evolution, salmon hosts, trematode.

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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 and 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 United States. 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 and 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 and 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 use the same salmonid hosts and have almost identical geographic

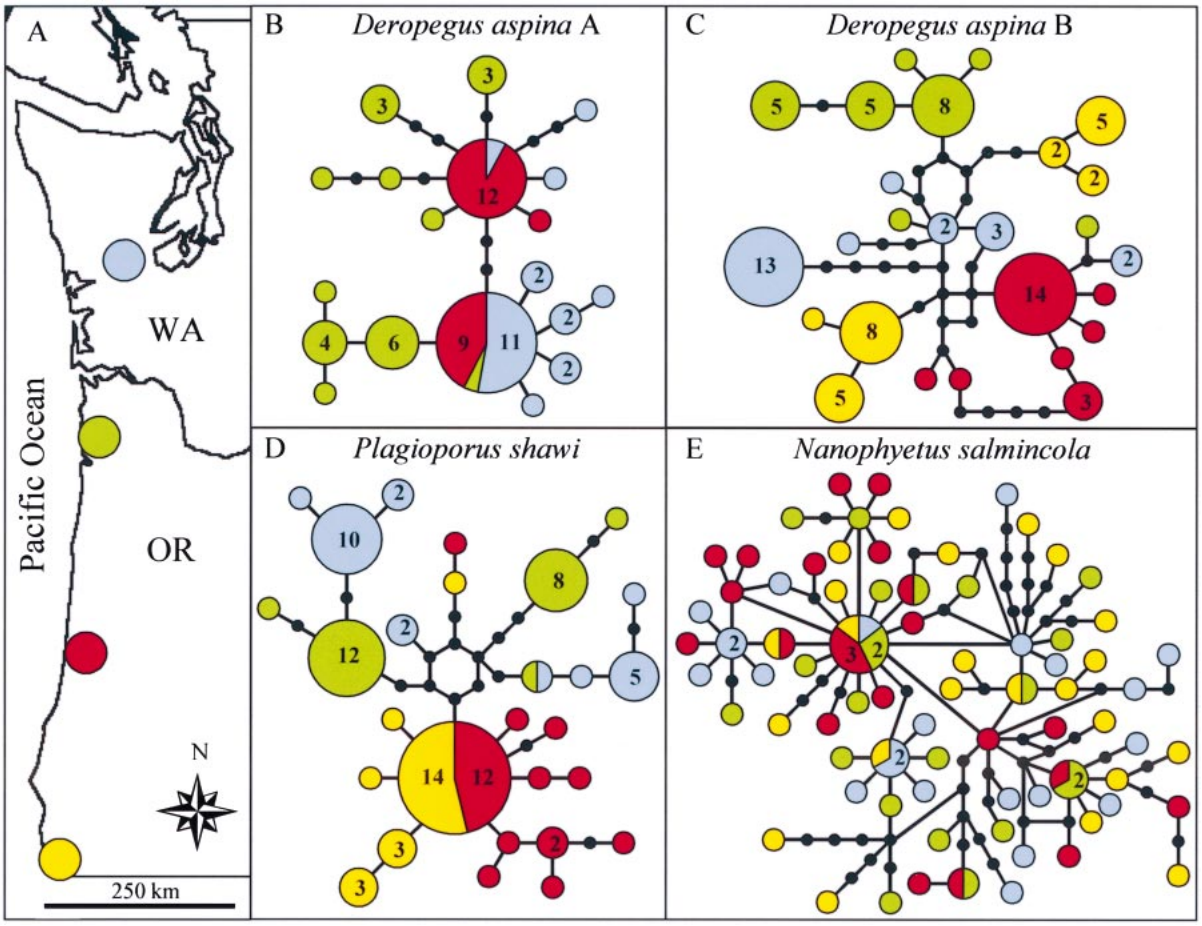

FIG. 1. Sampling locations and ND1 mitochondrial DNA genealogies. (A) Rivers sampled in Washington (WA) and Oregon (OR): Bingham Creek (blue), North Fork Nehalem River (green), West Fork Smith River (red), Winchuck River (yellow). (B–E) Statistical parsimony networks. Each connection is a single mutational step with black circles representing inferred haplotypes. Observed haplotypes are shown as colored circles. Coloring 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*.

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 anadromous 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 (Fig. 1A). Standard parasitological techniques (Pritchard and Kruse 1982) were used to recover the digeneans from the following hosts: *Oncorhynchus mykiss* (steelhead trout), *O. clarki* (cutthroat trout), *O. kisutch* (coho salmon), *O. tshawytscha* (Chinook salmon). To ensure 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 are found 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.

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) 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 (green population, Fig. 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 nonrandom sampling of clones was not a problem in this study. A total of 22 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. Polymerase chain reaction (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, and 1 unit Taq DNA polymerase (Promega, Madison, WI). 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 (for mitochondrial gene order in parasitic flatworms; see Le et al. 2002) 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. Polymerase chain reaction products were purified with the Ultra Clean PCR clean-up Kit (MO BIO Laboratories, Inc., Solana Beach, CA) and then sent to Nevada Genomics Center (Reno, NV) 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). Polymerase chain reaction 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 datasets 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 and 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. 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 2.001 (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 1.13 (Clement et al. 2000). We also used coalescent methods (Beerli and Felsenstein 2001) to obtain estimates of migration. An *N*-island migration model was used in the program Migrate 1.6.9 (Beerli and 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 3.06 (Posada and 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 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 (yellow population, Fig. 1A). However, both haplogroups were sympatric in the other three rivers (Fig. 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 sequences. 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)

TABLE 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 (*Deropegus aspina* A was only found in three 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					
D. aspina A	66	0.172*	0.76	0.0038	0.005
D. 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

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 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. 1B–D). In contrast, the allogenic *N. salmincola* had a very low, nonsignificant Φ_{ST} (0.01; Table 1). Related haplotypes, therefore, are just as likely to exist among as within subpopulations. The lack of geographic structure is evident in the ND1 network of *N. salmincola* (Fig. 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 1 should not be considered absolute numbers, but rather, relative measures (Whitlock and 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 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 1).

It is noteworthy that the four species all have a similar value of specieswide π (Table 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. 1B–E) have about the same total depth, indicating that they all have similar specieswide 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 1). This pattern is again consistent with low gene flow for the autogenic species because reduced migration among subpopulations can substantially increase specieswide N_e beyond the sum of the subpopulation effective sizes (Nei and 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. Although 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 trade-off 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, because 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 and 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 and 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 and Tabashnik 1992; Roper et al. 2003). Therefore, prediction of parasite gene flow from life-cycle patterns will help in evaluating the po-

tential for the evolution and spread of drug resistance in parasites (Anderson and May 1991).

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