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Microevolutionary Patterns and Molecular Markers: The Genetics of Geographic Variation in *Ascaris suum*[1]

S. A. Nadler[2]

Abstract: Molecular markers have been used only rarely to characterize the population genetic structure of nematodes. Published studies have suggested that different taxa may show distinct genetic architectures. Isoenzyme and RAPD markers have been used to investigate geographic variation of *Ascaris suum* at the level of infrapopulations (nematodes within individual hosts), within localities, and among geographic regions. Independent estimates of genetic differentiation among population samples based on isoenzyme and RAPD data showed similar patterns and substantial correlation. Heterozygote deficiencies within infrapopulations and large values for inbreeding coefficients among infrapopulations suggested that the composition of these populations was not consistent with a model of random recruitment from a large panmictic pool of life-cycle stages. Both isoenzyme and RAPD markers revealed moderate levels of genetic differentiation among samples representing infrapopulations and localities. Of total gene diversity, 9.4% (isoenzyme) and 9.2% (RAPD) was partitioned among infrapopulations. Geographic localities accounted for 7.8% (isoenzyme) and 6.2% (RAPD) of total diversity. Only infrapopulations from the same farm had low levels of differentiation.

Key words: *Ascaris suum,* ecology, genetics, geographic variation, isoenzymes, microevolution, RAPD.

In microevolutionary studies, molecular markers are of great potential utility for revealing intraspecific geographic variation among population samples. Observed differences in allelic frequencies for nuclear loci, or differences in the geographic distribution of mitochondrial DNA haplotypes, have been used to estimate levels of genetic differentiation within and among populations of various organisms (3). For nuclear genes, assessments of genotypes for single individuals are needed to evaluate the breeding structure of populations. Allelic frequency distributions also may be used to estimate genetic differentiation among populations, provided that the alleles are nearly neutral with respect to natural selection. Unfortunately, the small size of many nematodes has been an obstacle to determining genotypes for single individuals using classical protein electrophoretic methods. As a result, there are relatively few published studies that have used genetic markers to characterize the architecture of nematode populations. New approaches employing the polymerase chain reaction (PCR) can overcome many of the limitations due to small individual size. However, certain popular PCR-based techniques such as RAPDs or random amplified polymorphic DNA (37), a technique with established utility for systematic studies (14), may cause novel methodological (14) and analytical (8) problems in population-level studies. For example, although breeding studies for a variety of different organisms have demonstrated that most polymorphic RAPDs have a Mendelian pattern of inheritance (17,18,37), these same studies revealed that patterns of expression of RAPD markers are almost always consistent with dominance. Unfortunately, certain properties of population structure, such as the amount of inbreeding within subpopulations, cannot be assessed using dominant markers. In addition, artifactual RAPD products have been produced in some instances (10,29), and detailed duplication of reaction parameters is required to ensure the reliability of individual markers (7,10, 37).

Many basic population parameters re-

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[2] Associate Professor, Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115-2861. Present address: Department of Nematology, University of California, Davis, CA 95616-8668.

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main to be defined for nematodes. For example, what group of individuals represents the deme or random mating population, and how do the corresponding neighborhood area and size differ among taxa? How do ecological factors of individual species, such as mechanisms of dispersal, levels of host specificity, and general life history features, impact on the genetic structure of populations? Characteristics of species such as the mating system and population-level attributes such as effective population size will affect certain aspects of demes, including the likelihood of random genetic drift. It has been proposed that populations of parasitic nematodes (and other parasites generally) will be characterized by high levels of inbreeding, low intrapopulation genetic variability (e.g., polymorphism and heterozygosity), and high levels of interpopulation differentiation due to genetic drift, founder effects, and the influence of patch dynamics (28). Unfortunately, these predictions rarely have been tested empirically. Herein, I discuss a comparative analysis of isoenzyme and RAPD genetic markers in midwestern population samples of the swine parasite *Ascaris suum* (24). These isoenzyme data have been used to infer patterns of genetic structure within and among population samples, and as a benchmark for comparing results obtained with RAPD markers.

Assessing Population Structure

Methods for describing population subdivision such as F-statistics are useful for characterizing the breeding structure within subpopulations, and can reveal the potential for interpopulation differentiation by genetic drift for neutral alleles. Wright (38,39) developed a numerical method to analyze systems of mating (ancestor-offspring relationships in pedigrees), such that a single numerical quantity (F) can summarize the correlation of genetic state at a locus. The correlation between uniting gametes is represented by F, such that with random mating F = zero and, with sustained inbreeding beyond that expected given the effective population size, F will be a positive number $\leq$ one. One expected effect of sustained inbreeding is to reduce the individual heterozygosity within populations. The inbreeding coefficient (F_{IS}) describes the reduction in heterozygosity of an individual within its subpopulation compared to that expected in a randomly mating population with the same allelic frequencies. An inbreeding coefficient may be interpreted as the probability that two alleles in a diploid individual are identical by descent (autozygous), or share common ancestry via replication from a single ancestral allele. Two alleles at a single locus in an individual can be identical in nucleotide sequence but different (allozygous) with respect to replication common ancestry. Characterization of inbreeding may be extended beyond a single subpopulation to estimate the probability of autozygosity for alleles selected at random from a sample representing more than one subpopulation. Subdivided populations also may be affected by nonrandom mating, and this type of inbreeding can be quantified at two additional levels: the subpopulation relative to the total population (F_{ST}), and the individual with respect to the total population (F_{IT}). F_{ST} measures the reduction in heterozygosity of a subpopulation due to random genetic drift, whereas the overall inbreeding coefficient (F_{IT}) reflects the reduction in heterozygosity due to nonrandom mating within subpopulations (F_{IS}) plus that due to population subdivision (F_{ST}). Calculations of F-statistics should be corrected for biases that may be introduced by sampling few individuals per subpopulation (33), which is likely to be the case for certain endoparasitic nematodes that typically have small infrapopulations.

Materials and Methods

Ninety-six *A. suum* adults representing seven pig infrapopulations and five general geographic localities were collected for study: Burlington (Bur-1 and Bur-2)

and Cassopolis (Cas-1 and Cas-2), Michigan; Indianapolis (Indian), Indiana; Fulton (Fult) and Hinckley (Hinck), Illinois. The two infrapopulations from Cassopolis were obtained from the same farm; those from Burlington came from different farms. Starch gel electrophoresis and histochemical staining for specific enzymatic loci were performed as described previously (22,24). Phenotypic differences in isoenzyme banding patterns at a specific locus were used to deduce genotypes of individuals and the number of alleles segregating in the population samples. Total nucleic acid preparations extracted from muscle tissue (24) were used as templates for RAPD-PCR. Nine different 10-mer oligonucleotide primers that yielded reliable, prominent (of intense fluorescence), and polymorphic amplification products in screening experiments (24) were used to score 74 *A. suum* individuals in RAPD-PCR.

Version 1.7 of the BIOSYS-1 program (31) was used to calculate isoenzyme allelic frequencies and to test conformance of genotype frequencies to Hardy-Weinberg equilibrium expectations. *F*-statistics were calculated according to the formulas of Nei and Chesser (26) as modified by Van Den Bussche et al. (33). For the isoenzyme data, direct-count heterozygosity and allelic frequencies were used to calculate *F*-statistics. Because RAPD markers display a dominant mode of inheritance (7, 17,18,35,37), genotype and allelic frequencies within populations were calculated based on the observed frequency of the homozygous recessive condition (q^2 = marker absence), using the Hardy-Weinberg (diallelic) equation. Expected heterozygote and inferred allelic frequencies were used to calculate F_{ST}. Individual RAPD markers were excluded from calculations of F_{ST} when any subpopulation in the comparison had no individuals with $q^2 = 0$, because with marker dominance and relatively small sample size, alleles masked in heterozygotes will cause the frequency of the recessive allele to be underestimated. Mean F_{ST} was calculated as $1 - S\ H_S/S\ H_T$; average heterozygosity among subpopulations (H_S) and total heterozygosity (H_T) were determined according to the formulas of Van Den Bussche et al. (33).

Results

Isoenzyme data: For the seven population samples from individual pigs (infrapopulations), three included loci with statistically significant deviation from Hardy-Weinberg equilibrium expectations by Chi-square and exact probability tests ($P < 0.05$). A fourth infrapopulation showed deviation by two types of Chi-square testing (with and without pooling of rare genotypes). In three infrapopulations (Indian, Hinck, and Cas-1), Peptidase-B (PEP-B, E.C. no. 3.4.11.4) departed from equilibrium expectations. In two infrapopulations (Indian and Cas-1), phosphogluconate dehydrogenase (PGDH, E.C. no. 1.1.1.44) showed significant deviation from equilibrium expectations by both types of Chi-square tests. The Bur-1 infrapopulation also showed significant deviation from Hardy-Weinberg equilibrium expectations at the PGDH locus by Chi-square tests. When Bur-1 and Bur-2 infrapopulations were pooled, PGDH showed significant departure from equilibrium expectations by all three statistical tests. Likewise, analysis of pooled Cas-1 and Cas-2 infrapopulations also showed significant departure for PEP-B in Chi-square tests with pooling.

Average inbreeding coefficients (F_{IS}, Table 1) were high among the seven infrapopulations, the five pooled (by locality) populations, and between pairwise comparisons of infrapopulations, including those from single geographic localities. The mean fixation indices (F_{ST}) for infrapopulations and localities (Table 1) exceeded 0.05 in all cases except the comparison of two infrapopulations from a single farm (Cas-1 and Cas-2). For example, 9.4% of the total allelic variance was distributed among infrapopulations, and 90.6% was found within the infrapopulations. Likewise, 7.8% of the allelic variance

TABLE 1. Average *F*-statistics[a] among populations of *A. suum*.[b]

Statistic	7 Infrapopulations	5 Geographic locations	4 Michigan infrapopulations	Cas-1 vs. Cas-2	Bur-1 vs. Bur-2	Hinck vs. Fult
Isoenzyme F_{IS}[c]	0.224	0.223	0.229	0.312	0.123	0.176
Isoenzyme F_{IT}	0.295	0.283	0.282	0.328	0.194	0.227
Isoenzyme F_{ST}	0.094	0.078	0.070	0.024	0.080	0.062
RAPD F_{ST}[d]	0.092 (6)	0.062 (6)	0.097 (8)	0.044 (13)	0.093 (11)	0.042 (12)

[a] F-statistics summarize the reduction in heterozygosity (relative to expected levels) due to nonrandom mating within subpopulations (F_{IS}), among subdivided populations due to random genetic drift (F_{ST}), and overall due to both of these factors (F_{IT}).
[b] Data, including photographs of representative RAPDs, are found in reference 24.
[c] Isoenzyme statistics are based on three loci.
[d] The number of RAPD markers used for each calculation is shown in parentheses.

was distributed among localities and 92.2% within geographic regions. Alternatively, these *F*-statistics may be interpreted as the proportion of total variance or gene diversity found at a particular level. For example, 90.6% of the total estimated gene diversity was found within infrapopulations. Overall inbreeding coefficients (individual relative to the total population or F_{IT}) were high among infrapopulations and between localities (Table 1).

RAPD data: Nine primers yielded a total of 25 scorable amplified products, and 18 of these PCR products were polymorphic (variable among individuals with respect to presence-absence) in the populations surveyed. The 25 scorable markers ranged in size from 277–2,337 base pairs (bp). Only fixation indices were calculated for the RAPD data because estimation of F_{ST} does not depend directly on the frequency of observed heterozygotes. The number of RAPD markers used to calculate mean F_{ST} ranged from 6 to 13 (Table 1), depending on the number of markers excluded due to $q^2 = 0$ values in the comparison. Estimates of F_{ST} by RAPD and isoenzyme methods yielded similar levels of differentiation in several cases (Table 1), and average values of F_{ST} derived from isoenzyme versus RAPD markers had a correlation coefficient of 0.70. Also notable was the low level of differentiation between infrapopulations from the same farm (Cas-1 and Cas-2) versus the moderate level of differentiation observed between infrapopulations obtained from different farms within the same geographic region (Bur-1 and Bur-2). Coefficients of variation for mean F_{ST} values were, on average, 1.8-fold greater for RAPD than isoenzyme markers.

DISCUSSION

Given the difficulty of performing experimental crosses for many endoparasitic nematodes, confirmation of Mendelian patterns of inheritance cannot be used to determine the suitability of particular RAPD markers for population-level studies. Less informative criteria, such as strong band intensity and assessments of technical repeatability for individual RAPD-PCR products, may be the only basis for choosing particular markers for some species. For these *A. suum* individuals, nearly one-third of surveyed 10-mer primers produced one or more amplification products meeting these criteria (24), and the majority of these reliable markers were polymorphic. For RAPD markers, estimating allele and genotype frequencies using the Hardy-Weinberg equation was compromised when the recessive genotype was absent from a subpopulation; therefore, individual RAPD markers were excluded from calculation of F_{ST} whenever the recessive genotype was not observed. Because of this difficulty, using polymorphic RAPD markers to estimate genotype and allele frequencies may necessitate the use of a large number of different primers. For example, 18 polymorphic markers were scored in these midwestern population samples of *A. suum;* however, calculations of fixation indices were based on 6 to

13 markers, depending on the subpopulations compared.

Unfortunately, properties of population structure that depend upon the ability to directly score the genotype of each individual cannot be assessed with dominant markers such as RAPDs. Thus, although this study demonstrated that RAPDs can be used for estimating differentiation among parasite populations (F_{ST}), these markers cannot be used to quantify patterns of nonrandom mating within subpopulations (F_{IS}). Levels of among-population differentiation estimated from isoenzyme and RAPD data showed the same general patterns, and the correlation analysis demonstrated a substantial relationship between the values obtained from these different markers. However, estimates of average F_{ST} based on RAPD markers had almost twice the coefficient of variation than estimates based on isoenzyme markers. It is possible that this difference reflects greater variability in levels of selective neutrality among the RAPD markers used. However, it is also likely that some of this variability is due to errors introduced by estimating allele and genotype frequencies from the observed frequency of homozygous recessives. This finding suggests that fixation indices calculated from few polymorphic RAPD markers should be interpreted cautiously.

High inbreeding coefficients for infrapopulations of *A. suum* were revealed by the protein electrophoretic data. For individual infrapopulations, loci departing from Hardy-Weinberg equilibrium expectations in statistical tests showed excess homozygosity. Analysis of pooled infrapopulations representing individual geographic regions also showed heterozygote deficiencies. A Wahlund effect (34) probably accounts for the heterozygote deficiency in the pooled Burlington sample because the infrapopulations, which were obtained from different farms, displayed markedly different allelic frequencies (24). A Wahlund-like effect might also explain departures from equilibrium expectations if some infrapopulations are composed of individuals representing distinct recruitment (infection) events.

Departures from random mating expectations also were suggested by the inbreeding coefficients. The mean inbreeding coefficient among infrapopulations (F_{IS} = 0.22) is high when compared to values reported for other endo- or ectoparasites and free-living organisms. For example, the mean F_{IS} for midwestern *A. suum* was approximately an order of magnitude greater than values reported for geographic populations of the endoparasitic fluke *Fascioloides magna* that have been described as inbred (20,21). Likewise F_{IS} levels for *A. suum* were much greater than reported for infrapopulations of chewing lice (mean F_{IS} = 0.069) parasitizing pocket gophers (23). The inbreeding coefficient indicates that the genetic composition of *A. suum* infrapopulations, whether from a general geographic region or a single farm, is not consistent with a model of random recruitment from a large panmictic pool of life-cycle stages. Since sexual reproduction of *A. suum* occurs only within definitive hosts (pigs), inbreeding may be promoted if sibling or parent-offspring matings occur with a greater-than-expected frequency. The likelihood of matings between siblings would be enhanced if infrapopulations were frequently established from infective eggs representing closely related individuals. For example, infection of a host might result from ingesting eggs derived from a single mating or matings between close relatives, rather than from eggs representing a random sample of the available genotypes. Because *Ascaris* generations are overlapping and the host humoral immune response to infection does not appear to prevent reinfection (6,16), parent-offspring matings also may contribute to the observed F_{IS} values.

Inbreeding (F_{ST}) due to population subdivision among infrapopulations and localities, as inferred from both isoenzyme and RAPD markers, was indicative of moderate genetic differentiation (15). Of the estimated gene diversity, 90.6% (isoenzyme)

and 90.8% (RAPD) was found within infrapopulations. Another interpretation is that for both types of markers approximately 9% of the observed diversity was partitioned among infrapopulations. When these data were partitioned by geographic area, 92.2% (isoenzyme) and 93.8% (RAPD) of the total gene diversity was found within regions. Infrapopulations representing two different farms from a single geographic area also showed moderate genetic differentiation, with 92% (isoenzyme) and 90.7% (RAPD) of the gene diversity at the Burlington locality distributed within the infrapopulations. Likewise, infrapopulations representing southern Michigan revealed differentiation of the same magnitude as observed for all midwestern infrapopulations. In contrast, two infrapopulations from the same farm (Cassopolis) were characterized by low fixation indices. This distribution of genetic differentiation among farms and within geographic regions suggests that only hosts at the same farm have sufficient exchange of *Ascaris* eggs to reduce the effects of genetic drift on allele frequencies.

Random genetic drift among *A. suum* infrapopulations is likely to be promoted by small effective population size and founder effects. The skewed sex-ratio (0.44:1, female bias) observed for these infrapopulations (24), which is also characteristic of other *Ascaris* species (13), significantly reduces the effective population size below the observed census size. Considering only the effects of bias in sex-ratio, the average effective size for these infrapopulations was 12 (24), and the cumulative effect of random genetic drift in populations of this size may lead to significant changes in allelic frequency over relatively few generations (19). Genetic drift facilitated by small effective size also may explain the relatively low levels of isoenzyme heterozygosity reported for certain population samples of *A. suum* (1,5).

Many free-living nematodes and certain endoparasitic species are likely to have much larger effective population sizes than in *Ascaris*. For example, in a study of the bovine endoparasite *Ostertagia ostertagi*, Blouin et al. (4) estimated a long-term effective population size of 4–8 $\times$ 10^6 individuals per geographic population based on the observed diversity of mitochondrial DNA (mtDNA). The large effective size of *O. ostertagi* populations also is supported by studies of infrapopulations. Individual hosts may harbor 10,000 to 100,000 worms at one time (2,36), and the adult sex ratio is approximately equal (32). Importantly, *O. ostertagi* shows high intrapopulation mtDNA diversity and low interpopulation differentiation, with less than 1% of the total gene diversity partitioned among geographic populations (4,9). By contrast, Anderson et al. (1) reported 10-fold lower levels of mtDNA diversity within "population clusters" of *Ascaris*. Thus, these studies are consistent with the expectation that effective population size may influence intrapopulation diversity, and that nematode species characterized by large effective sizes may show minimal differentiation due to genetic drift.

A paradigm of parasite population structure is that these organisms should have small populations with high levels of interpopulation differentiation due to genetic drift, founder effects, and the influence of patch dynamics (28). Given the range of ecological diversity that is characteristic of parasitic nematodes, a broad spectrum of genetic architectures is likely to be revealed as more empirical studies are undertaken. Published studies already have revealed differences in genetic structure among species and, as in other organisms (3), species with life histories conducive to the geographic movement of individuals or dissemination of life-cycle stages tend to show less population structure than species with lower vagility. For example, Paggi et al. (27) and Nascetti et al. (25) have used multilocus protein-electrophoretic data to show that species of ascaridoid nematodes using seal definitive hosts (and fish or invertebrate intermediate and paratenic hosts) have low amounts

of genetic structuring across broad geographic ranges of the Arctic-Atlantic Boreal region. For example, geographic population samples of two *Contracaecum* species had average F_{ST} values of 0.042 and 0.046 respectively, over geographic distances spanning more than 5,000 km (25). Likewise, three species in the *Pseudoterranova decipiens* complex that share the same definitive hosts as the *Contracaecum* species showed average F_{ST} values ranging from 0.021 to 0.059 (27). These studies are particularly noteworthy in that species in these two complexes are known to have the same general life-cycle patterns and would seem to share many of the same intermediate, paratenic, and definitive hosts. The distribution of 94% to 98% of the total estimated gene diversity within all geographic localities is consistent with the hypothesis of Nascetti et al. (25) that migration of the definitive hosts (seals) in combination with the development and transport of juveniles in fish and invertebrates serves as a highly effective mechanism of gene flow over large geographic distances.

Studies of mitochondrial-DNA phylogeography in *O. ostertagi* (4,9) also have revealed extremely low levels of genetic differentiation among localities. Using an estimate of mitochondrial genetic diversity that is analogous to F_{ST}, Blouin et al. (4) reported that, on average, less than 1% of the total gene diversity was partitioned between geographic populations. For *O. ostertagi*, gene flow among the localities appears to be high and may be mediated by the transport of cattle throughout the United States. Interestingly, despite this inference of high levels of gene flow, genetically based geographic differences in the timing of developmental arrest (hypobiosis) have been demonstrated for temperate and subtropical populations of *O. ostertagi* (11,12,30). This difference in the timing of hypobiosis between populations from the northern and southern United States likely is maintained by strong selection pressure in the presence of high gene flow (4).

The current study of isoenzyme and RAPD markers has revealed more subdivision in midwestern A. suum populations than described for *O. ostertagi* (4), even though both nematodes are parasites of livestock and are subjected to frequent movement with their hosts. Clearly, the transport of livestock will complicate interpretation of population structure for certain endoparasites, particularly because such movements are not likely to be uniform over space or time. However, one potentially important difference between these species is that the effective size of *O. ostertagi* infrapopulations is apparently many orders of magnitude larger than in *A. suum*. Thus, these distinct patterns of genetic structure may, in part, be attributed to differences in the amount of genetic drift between populations of these two species.

Final Comments

The development of PCR-based DNA markers has provided new opportunities for assessing the genotypes of small nematodes and investigating population genetic structure. Published studies employing traditional biochemical or DNA-based markers on individual endoparasitic nematodes have revealed differences in population genetic structure among species. Future studies of genetic structure in endoparasites may benefit from a comparative approach in which several species of nematodes that co-occur in populations of a single host species are collected and investigated simultaneously. Designing testable hypotheses to assess the relationships between life history features of nematodes and patterns of population genetic structure will remain challenging. Phylogenetic methods would appear to be one of the most promising approaches for assessing the relationship between life history features and population genetic structure. For example, a model system would permit the comparative analysis of genetic structure for sister-species of nematodes

that coexist in the same host but differ in one or few life history features.

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