History of microevolutionary thought in parasitology: The integration of molecular population genetics

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> I never cease to marvel that the DNA and protein markers magically emerging from molecular-genetic analyses in the laboratory can reveal so many otherwise hidden facets about the world of nature.
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> —John C. Avise, *Molecular Markers, Natural History, and Evolution* (2004).

Molecular population genetics has had two major impacts in biology. It has opened the door to many questions in areas such as genetic inheritance modes and mapping, reproductive modes (sexual vs. asexual reproduction), mating systems (random vs. nonrandom), relatedness, population demography (growth/decline) and connectivity (gene flow), inference of selection, phylogeography, and delimitation of species. It has also mixed the disciplines of ecology, evolution, genetics, and molecular biology. As a result, we have a better understanding of microevolution (theory and empirical), and a means (albeit often indirectly) to study the population biology of any living organism. The latter is especially pertinent for parasites which, "because of their small size, location, biology, and behavior, direct observation of their population biology is almost impossible" (de Meeûs et al., 2007).

In the present chapter, I have been tasked with reflecting on the development of microevolutionary concepts in the field of parasitology over the past 100 years, since publication of the first volume of *The Journal of Parasitology* (*JP*). My goal is not to review or make broad generalizations about parasite microevolution, but rather to recap the history of thought and applications of molecular population genetics in parasitology. As chapter authors, we were also asked to pick an early paper from *JP* to highlight its relevance or impact in its respective field. I have selected Steven Nadler's (1995) review, "Microevolution and the genetic structure of parasite populations," because I believe, personally, that it is one of the most under-recognized papers with regards to parasite microevolution. Indeed, I recall early in my graduate career reading his elegant analysis, but filing it away. When I started to write my dissertation, I felt that several of my ideas were novel. However, upon revisiting Nadler (1995), I realized otherwise and remember begrudgingly yelping, "Nadler!" (in much the same way that Jerry Seinfeld would exclaim "Newman!" in the comedy TV show *Seinfeld*). So, why feature an under-recognized paper and why choose a paper from 1995 as opposed to one early in *JP*'s history? To answer these questions, we need to step back in time.

I will first provide a brief history of population genetics in general and then expand the discussion to include parasites. I will then recount the history of some specific topics to show how molecular population genetics has resonated in different aspects of parasite population biology and evolution. To save space, reviews are referenced when possible rather than original publications. I will attempt to cover various protozoans and metazoan

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parasites of animals, but admittedly my own experiences bias the review towards helminths. Stealing the words from a historical account of population genetics by James Crow (1987), a renowned population geneticist, "Space limitations dictate that this review be selective … The choice of subjects is arbitrary; they are topics that I think are interesting and historically important."

A very brief recap of population genetics history

Modern evolutionary theory has its origins with Darwin (1859), who focused on natural selection, but also hinted at the evolutionary mechanisms of inbreeding, gene flow, and drift. But, Darwin did not have correct explanations for two factors that are at the heart of what we now call genetics, namely, the mode of inheritance and mutation, the ultimate source of heritable variation. Although contemporaneous with Darwin's, Gregor Mendel's work on inheritance (segregation and independent assortment) in 1866 was not brought to light until its rediscovery in 1900 (Bowler, 2010). In 1908, two publications would formulate Mendelian segregation at the population level in what we recognize today as the Hardy–Weinberg Law (HWL); however, Weinberg's paper was neglected for 35 years (Crow, 1999). The status of HWL in modern evolutionary theory is well stated by Crow (1987): "Although the principle is trivially simple, it is nevertheless the foundation for theoretical population genetics."

From the 1920s to the 1950s, population genetics was dominated by, and gained its quantitative base from, its founding "fathers," Sewall Wright, Ronald Fisher, and J. B. S. Haldane (Crow, 1987). Their work, of which there was overlap among the three, was prominent in melding Mendel's inheritance principles with Darwin's theory of natural selection (Crow, 1987). By the 1940s, modern evolutionary thought had its start (Bowler, 2010). As a brief flash forward, Wright's work on inbreeding, population structure, and genetic drift has probably had the greatest impact on the field of molecular ecology. I believe this impact is obvious in the near universal use of hierarchical *F*-statistics and analogs, which have their origin with Wright. Crow (1987) noted two newcomers to the discipline in the 1950s. Motoo Kimura, who is known for the neutral theory of molecular evolution, and Gustave Malécot, who actually had several publications in the 1940s that were novel or extended Wright's work (Crow, 1987). Malécot's recognition, however, as with Mendel and Weinberg, came late (Epperson, 1999). Obviously, microevolutionary theory has advanced since the 1950s, but the basics were in place by then. Thus, I shift now to recount the history of some important molecular genetic markers/techniques as these provided the empirical means to test or implement population genetics theory.

In 1966, three papers published genetic diversity data based on gel electrophoresis of proteins (Lewontin, 1991). Much of the initial population genetic applications in the 1960s and 1970s focused on the Classical-Balance debate. Under the "classic" view, genetic variation would be low due to purifying selection, whereas the "balance" view held that variation would be high due to balancing selection, e.g., heterozygote superiority. This discussion morphed into the Neutralist-Selectionist debate, in which Neutralists proposed that most genetic variation was the product of mutation and genetic drift rather than natural selection; see Lewontin (1991) and Avise (2004) for details regarding these disputes). Here, I would emphasize that electrophoresis provided the first methodology for accessing co-dominant genetic data "at many independent loci, chosen without bias with respect to magnitude of genomic variability" (Avise, 2004).

Biologists hit the ground running with electrophoretic genotyping and, in about 10 years, reviews were already meta-analyzing polymorphism and heterozygosity data from plant and animal populations (Avise, 2004). For example, Nevo (1978) examined broad scale patterns of enzyme-based genetic variation across 228 animal species, but, as a prelude to my discussion next, none was parasitic! Lewontin (1991) remarked that electrophoresis "marked the first stage in a new path of evolutionary genetics" and "provided for the first time the possibility of including virtually any organism in the study of evolutionary variation on the basis of a common denominator across species." Was the latter statement true for parasites? As a sizable amount of tissue was needed to obtain multilocus genotypes (MLGs), tiny organisms that could not be cultured clonally were still not fully available for such research.

By the mid-1970s, direct DNA based approaches, such as restriction fragment-length polymorphisms, were being developed. These advances spawned the

field of phylogeography, which in its origin (late 1970s) had more of a phylogenetic base, and hence, a historical approach to studying populations (Avise, 2000). In 1977, Sanger-based DNA sequencing was developed (Nelson et al., 2011), although electrophoresis remained a prominent means to obtain genotypic data through the 1980s.

The next major advance came about in 1985, with the polymerase chain reaction (PCR) (Bartlett and Stirling, 2003). From the perspective of a parasite population geneticist, PCR was truly significant because one could not only target DNA directly (as opposed to the phenotypic expression of a protein), but the technology did not require large quantities of tissue. With the advance of microsatellite markers in 1989, there was now a direct DNA marker that provided the equivalent co-dominant genotypic data of protein electrophoresis. To me, microsatellite scoring was probably the last significant genotyping method developed until the advent of next generation sequencing (NGS) 16 years later in 2005 (Nelson et al., 2011). I will return to NGS in my closing personal reflections.

In summary, I have two comments to close this section. Note how the work of important pioneers, e.g., Mendel, Weinberg, and Malécot, often went unrecognized, sometimes even neglected for long periods. Second, I hope it is clear why I do not highlight a *JP* paper published in 1914 to the early 1920s, i.e., the field of population genetics was just starting!

The snail's pace flow of molecular population genetics into parasitology

Modern microevolutionary thought in parasitology can also be traced back to Darwin (1859), who makes a few statements about parasite traits that could reflect adaptations and co-adaptions to hosts. For example, in talking about a reduced head region in a parasitic barnacle, Darwin (1859) speculated on the potential selective advantage of the loss of morphological features: "Each individual *Proteolepas* would have a better chance of supporting itself, by less nutriment being wasted in developing a structure now become useless." I chose this quote because parasitologists can surely attest to their study organisms' limited morphology, a primary factor that will later contribute to key applications of molecular population genetics. Microevolutionary thought (mostly related to natural selection) in parasitology was therefore present from Darwin's time to the advent of electrophoresis, e.g., for co-evolutionary matching alleles, selection model, see Mode (1958). However, I skip over this part of microevolutionary history in parasitology in order to focus on the integration of molecular population genetics.

The use of electrophoresis in parasite studies began in the late 1960s and early 1970s. Most applications assessed the marker itself and would ask questions such as were all Mendelian genotypes observed, was it a dimer, etc. (e.g., Zee et al., 1970). Or, researchers used banding profiles as more of a phenotypic trait, e.g., strain typing of protozoan parasites (Reeves and Bischoff, 1968), rather than estimate population genetic parameters. Some studies started to examine the distribution of allele frequencies among parasite populations (e.g., Carter and Voller, 1975), but little to no population genetic statistics or tests were used specifically. Thus, the 1970s were largely devoid of parasite population genetics (*sensu stricto*) even though such studies were commonly published on plants and free-living animals (Avise, 2004). The first papers (at least for helminths) that used molecular markers and population genetics theory or statistics to infer something about parasite biology were authored by Beverley-Burton et al. (1977), Beverley-Burton (1978), Vrijenhoek (1978), and Bullini et al. (1978).

However, before discussing these contributions, I feel the need to first underscore Peter Price's (1977, 1980) treatise on parasite evolutionary biology. He was the first to use parasite life history characteristics to make predictions about evolutionary mechanisms. He had to rely heavily upon an inductive process because no parasite population genetic data were available and, being an entomologist, most of his examples were of phytophagous insects. He largely viewed parasites as existing in ephemeral fragmented environments, i.e., the host (Price, 1980). In Price (1980), he stated [from here on, my analogies are in brackets] "For very small organisms [parasites] a wide dispersion of resources [individual hosts] within patches [host populations] and considerable distances between patches makes colonization of new hosts hazardous." Because he saw hermaphroditism and asexuality as common reproductive modes among parasites (Price, 1980), he believed these modes were adaptations to facilitate colonization by individual parasites. As a result, these traits would

cause increased homozygosity via inbreeding and/or bottlenecking.

Although Price (1977) recognized life history variation among parasites, his final conclusion was one-sided when he stated, "The general patterns envisaged for parasitic 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." Overall, I consider many of Price's arguments to be at the macroevolutionary scale, at which hypotheses are best tested within a phylogenetic framework. Nonetheless, the first part of the quote does provide what I view as the first non-natural selection related predictions, i.e., a focus on mating system, genetic drift, and gene flow, in parasites. Price (1977) also readily admitted that his generalizations "need critical evaluation" and outlined several core micorevolutionary questions that required attention. For example,

> In sexually reproducing parasites population structure requires much attention. What is the effective population 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?

He followed by saying, "The genetics of parasite species and races should receive much more attention."

Not until the 1980s do we see a rise in the use of electrophoretic data in population genetic studies of parasites, although numbers of papers still pale in comparison to free-living organisms. Thus, compared to the population genetics literature on free-living plants and animals, there is about a 10-year lag before a synthesis of polymorphism and heterozygosity data in parasites. Nadler (1990) listed 23 helminth species in a review that really is the first to consider parasites in the Neutralist-Selectionist debate, which by this time had started to fade away in the primary literature as it really had no ultimate resolution (see Avise, 2004). A significant insight that stemmed from the Nadler review was that "Most endoparasitic helminth 'populations' or species surveyed have levels of genetic variation similar to those of free-living invertebrates." Though Nadler (1990) does not discuss Price (1977, 1980) specifically, this review may be the first collective hint that Price's predictions may not apply across all parasites. In Nadler's (1990) final comments, he states the obvious: "Parasitologists have rarely used the full potential of biochemical and molecular methods to study the population genetics and phylogeny of helminths." In the same year, a parallel review on molecular population genetics of protozoan parasites was published (Tibayrenc et al., 1990). All references were from the 1980s, thereby illustrating the 10-year lag in population genetics of protozoan parasites as well. The microevolutionary thought of Tibayrenc et al. (1990) was unique from previous population genetic reviews on free-living organisms and of Nadler (1990) in that the attention was on mode of reproduction (clonal vs. sexual). Here, we see the statistical application of HWL and linkage disequilibrium (LD), the nonrandom association of alleles among loci, in order to make inferences on the assorted patterns of reproductive modes found among protozoan parasites.

The trickle of papers on parasite population genetics continued into the early 1990s and, in addition to electrophoresis, DNA based methods were starting to be used. Since Price (1980), however, no one had revisited the collective expectations of population genetic patterns in relation to parasite ecology and life history. Nadler (1995) stated,

> Although the paucity of available studies makes it premature to develop general conclusions about the genetic structure of parasite populations, it is likely that new technologies [such as PCR] will soon promote groundbreaking research in this area. On the other hand, it is not premature to consider the types of questions that might be addressed in parasite population genetics.

He gave a broad overview (though a focus on metazoan parasites) that related theoretical and empirical population genetics to the extensive variation in parasite lifestyles. Continuing, Nadler wrote,

> The genetic structure of parasite populations will be shaped strongly by ecological factors of individual species, including their demography, life cycle, mechanisms of dispersal, and host specificity. Characteristics of species such as the mating system and population-level attributes such as the effective size will affect certain aspects of demes, including the likelihood of random genetic drift.

In particular, he laid out how different parasite traits would act to increase or decrease genetic structuring (see

Table I in Nadler, 1995). Thus, in comparison to Price (1977, 1980), Nadler's (1995) discourse provided a more holistic perspective in that parasite microevolutionary patterns are predicted to be as multifarious as the life histories of the parasites themselves. While several ideas in Nadler (1995) can be found in earlier or other, current publications, he did a great job of synthesizing the available data, tying them to population genetics theory, presenting novel ideas, and highlighting major gaps.

By establishing specific links between parasite life history and genetic drift, gene flow, and non-random mating, Nadler (1995) also provided a user-friendly framework for the application of molecular population genetics to parasite biology. In retrospect, it seems to me that he garnered less attention that what I would have assumed. For example, a parasite molecular ecology review I co-authored 10 years later (Criscione et al., 2005) has gotten more attention than Nadler (1995) (average citations per year are 16.1 and 4.8, respectively, as of 6 March 2014 in Web of Science). I think [jokingly] I heard Steve grumble "Criscione!" In Criscione and Blouin (2004), I too was guilty of not citing [unintentionally] Nadler's (1995) ideas regarding the influence of life cycle patterns on gene flow. As noted previously, literature neglect seems common in evolutionary biology, so I will blame my lapse on that. On a sincere note, the reason I chose to highlight Nadler (1995) was to give it the credit due for providing a collective microevolutionary perspective of parasites that reflects the diversity of parasites themselves. It is a must read for anyone in the field and of broad interest to those outside.

With a well laid out framework, a list of questions to address, and a genetic toolkit that could be used on any organism, i.e., PCR and microsatellites, one would think that research on parasite population genetics would have skyrocketed. But, it did not. Again, there is an approximate 10-year lag in the use of microsatellites for parasite population genetics compared to free-living species. To illustrate (but see Fig. 1 legend for major caveats), I used a Web of Science search on "microsatellite" and either "fish" or "parasite" (Fig. 1). Notice that in 1999 and 2000, there were ~103 papers/year for "fish" and ~18/year for "parasite". Not until 2009 and 2010 (10 years later) are there ~102 papers/year for "parasite". Sequence data, mainly mitochondrial (mtDNA) data in a phylogeographical framework, are also in extensive use at this time; a similar pattern can be found by replacing "phylogeography" for "microsatellite" in the previously mentioned search (data not shown).

Despite these lags, there has been progress in the application of molecular population genetics to parasite biology from Nadler (1995) onward. It is just much less than one might expect given that parasitism is at least as common as a free-living lifestyle (Dobson et al., 2008). Various topics were being addressed and the next broad scale review to come along agglomerated these topics to emphasize how population genetics was applicable across a range of parasitology disciplines (Criscione et al. 2005). Specifically, we highlighted a hierarchical range of subjects (species identification, phylogeography, host specificity and speciation, population genetic structure, modes of reproduction and transmission patterns, and searching for loci under selection) that could be addressed in parasitology using a molecular ecology approach. By this time, available genetic data made it evident that Price's (1977, 1980) predictions were not applicable across all parasites. Criscione et al. (2005) postulated why:

> Many of Price's examples were phytophagous insects that can have many recurrent generations on a single host plant. In contrast, most animal macroparasites [metazoan parasites] 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 [conspecifics in/on a host] 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 reinfect 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.

The use of population genetics to infer parasite transmission, a topic discussed next, is rooted in these concepts of the parasite deme.

Looking back on the history of the integration of molecular population genetics into parasitology, no one microevolutionary topic seemed to dominate the field

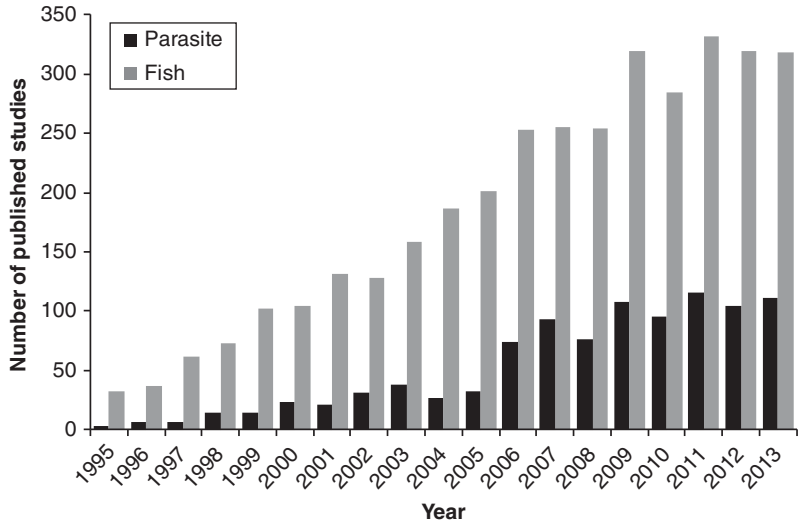

Figure 1 I conducted a Web of Science search on the terms "microsatellite" and either "fish" or "parasite" on March 6, 2014. The search was for each year separately. Extreme caution is advised in strictly interpreting results. All papers may not actually be population genetic studies or may not be using microsatellites in the respective organisms. Indeed, I know the three papers in 1995 under "parasite" are not studies on the population genetics of parasites two are not even on parasites. The figure is mainly of exploratory value and, while the exact numbers are incorrect, I suspect an approximate 10-year lag would be present even if proper scientific scrutiny were used. Keep in mind the search was only for one group of vertebrates as no one uses "free-living" as a keyword. Inclusion of other vertebrates would only increase the discrepancy. Thus, if anything, I suspect the figure gives a gross underestimate of parasites relative to free-living animals. I was not able to do a similar analysis for allozymes as Web of Science does not have abstracts or author provided keywords for most papers prior to 1995; thus, word searches will be less inclusive based on titles alone.

at any single time. Moreover, each topic, like loci in a genome, has had its own historical path; some are parallel, but some diverge. Thus, I shift focus to the history of some of these topics that have resonated over the years in parasite population genetics.

History of a few parasite population genetics topics

How many species?

This question is one of the most fundamental, i.e., a necessary prerequisite before any downstream population genetics study, and maybe oldest questions in parasitology. Because reduced morphology in parasites (something Darwin noticed) can lead to ambiguity in species delimitation, here was an issue for which molecular population genetics could make significant contributions. Thus, it seems natural that the earliest papers (Beverley-Burton et al., 1977; Vrijenhoek, 1978) to use molecular population genetics addressed the potential for what we phrase today as "cryptic species" (morphologically similar, but genetically distinct). The conceptual basis behind this application is eloquently stated by Vrijenhoek (1978),

> Populations diverge genetically as a result of adaptive and random processes. Often, individual gene loci diverge completely, such that distinct electromorphs are diagnostic of different species. Not all independent gene loci are expected to diverge to this extent, however. For example, genetic divergence need not produce concomitant morphological differentiation, resulting in cryptic or sibling species. When cryptic populations are sampled and analyzed as if they were a single randomly mating population (panmictic unit), deficiencies in the number of heterozygous genotypes commonly are observed as compared to the numbers expected by the Hardy-Weinberg equilibrium model [i.e., the Wahlund effect].

The principle of the Wahlund effect was used in several electorphoresis studies in the 1980s and 1990s (e.g., Renaud and Gabrion, 1988; Reversat et al., 1989;

see nematode review by Anderson et al., 1998). A limitation of electrophoresis was that tissue requirements or low allozyme variation often precluded the ability to obtain variable MLGs. Thus, studies often relied on fixed alleles at a single locus between the cryptic groups, i.e., no heterozygotes were found. Sequence data became prominent in the late 1990s onward, especially mtDNA for "molecular prospecting" of cryptic species (Blouin 2002; Vilas et al., 2005). Such studies were largely based on single locus (mtDNA or rDNA) percent divergence or clade designation; hence, the term "prospecting" rather than "delimitation." More recent approaches using MLGs have taken advantage of LD patterns in addition to HWL to identify cryptic species or populations (for examples with sequence data and microsatellites see Criscione and Blouin, 2004; Criscione et al., 2011, respectively). In the past few years, there has been a resurgence of attention given to the biological importance of recognizing cryptic species and the molecular methods that have been used to help identify and delimit (Perez-Ponce de Leon and Nadler, 2010; Nadler and Perez-Ponce de Leon, 2011). Criscione et al. (2005) stated, "The finding of cryptic parasite species has become very common as more phylogeographical and genetic structure studies are carried out on parasites." I suspect this trend will continue.

Hybridization

Curiosity about this topic in parasitology also predates molecular population genetics. Studies were based on laboratory crosses and interest seemed to be driven, in part, by the potential for hybrid sterility to lead to genetic assimilation or replacement of one species over another (e.g., LeRoux, 1954; Southgate et al., 1976; Le Jambre, 1979). By identifying hybrids of horse ascarids, Bullini et al. (1978) demonstrated how molecular markers could be used to assess parasite hybrids in nature (see also Vrijenhoek, 1978). In fact, molecular methods to identify hybrids paralleled cryptic species research due to conceptual similarities. Thus, early studies relied on fixed alleles between parental species, but looked for evidence of heterozygotes. As sequencing became available, nuclear-mtDNA discordance was used as a means to identify potential hybrids in nature. However, this discordance may result from incomplete lineage sorting or historical introgression rather than contemporary hybridization (reviewed in Detwiler and Criscione, 2010).

In the 2000s, the advent of genetic assignment tests, which use MLG data to assess HWL and LD, enabled the identification of contemporary (within ~3 generations) natural hybrids (for helminth examples see Criscione et al., 2007; Steinauer et al., 2008). As shown by the latter two studies, an important epidemiological consideration that stems from evidence of hybridization among two, host-associated parasite species/populations is that cross-transmission among host species must have happened. Hybridization studies on parasites themselves are still relatively few in number. Nonetheless, the current body of studies indicates it may be more common in nature than appreciated (Detwiler and Criscione, 2010). This situation is especially true when considering natural hybridization between clonal lineages of some protozoan parasites (e.g., Zingales et al., 2012), which I discuss next as a separate topic. Thus, more attention is warranted as,

> … hybridization between species or diverged populations could result in the transfer of adaptive traits, promote divergence via reinforcement when hybrids are less fit than parentals, lead to homogenization across the genomes of the interbreeding populations, or promote rapid adaptive diversification via the formation of hybrid species. In relation to host-parasite interactions, such reticulate dynamics are of particular interest because host or parasite hybridization may impact host resistance/susceptibility or parasite infectivity, virulence, transmission, or host specificity.
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> *(Detwiler and Criscione, 2010)*

Mode of reproduction in parasitic protozoa

If there is an area where parasite microevolution has been at the forefront of diploid population genetics (or at least on par), I would have to say it would have to be with respect to inference of asexual versus sexual as a primary means of reproduction. Just as with helminths, electrophoretic studies on protozoans in the 1970s largely focused on taxonomic issues such as strain identification, but there was also interest in trying to determine if strains were associated with ecological/epidemiological variables (e.g., Miles et al., 1977, and references therein). In the 1980s, there began a focus on whether protozoan parasites had genetic exchange (meiosis and recombination) and this came to the forefront with the "clonal theory of parasitic protozoa" proposed by Tibayrenc et al. (1990). It sparked controversy via its inclusion of malarial

parasites, which have an obligate sexual reproductive phase in its mosquito host (Dye, 1991). I will not delve into this debate, but simply state that the review stimulated many molecular population genetic analyses that explored parasitic protozoan reproductive modes in nature. By focusing on the effects clonal reproduction has on segregation and recombination, Tibayrenc et al. (1990) provided a framework to study the microevolution of protozoan parasites. Fascinating patterns that reflect complex reproductive histories involving some lineages with variable levels of genetic exchange, persistence of clonal lineages, and hybridization among these lineages have been observed among protozoan parasites (e.g., Miles et al., 2009; Zingales et al., 2012).

Important questions that remain today are: what drives the variable modes of reproduction and what is the ecological and evolutionary, and hence epidemiological, significance of these lineages (referred to as discrete typing units, DTUs)? For instance, Miles et al. (2009) stated,

> Increasing evidence supports the idea that the 6 *T. cruzi* DTUs are historically and currently associated with distinct ecological niches, with concomitant implications for the epidemiology of Chagas disease. The niches are not fully understood, partly due to limited sampling and genotyping of *T. cruzi* isolates. As to be expected, interaction between niches occurs, changing as ecologies are disturbed, as evidenced by mixed DTU infections in vectors and mammals, including humans.

Interestingly, although a lot of empirical work on clonal reproduction in parasitic protozoans had been carried out since 1990 (see review on *Leishmania* and *Trypanosoma* by Tibayrenc and Ayala, 2013), theoretical work in terms of how population genetic statistics reflected clonal reproduction did not appear until the mid-2000s. This population genetics theory of clonal diploids, which had its origins in parasitology thanks to de Meeûs, Balloux, and colleagues (reviewed in de Meeûs et al., 2006), provided a framework to use measures of LD and inbreeding coefficients to assist in inferring modes of reproduction from population studies.

Comparative approach to studying population genetic structure

"A comparative approach using both sexually and asexually reproducing parasites should be employed to examine the variation inherent in siblings and populations" (Price, 1977). It was recognized early on that comparing parasites with different life histories could be useful for illuminating parasite traits that affect evolutionary mechanisms (see also Nadler, 1995), but 9 years passed before such a study was forthcoming. Bullini et al. (1986) used the comparative approach to determine whether parasite gene diversity is correlated with habitat heterogeneity and found "a significantly higher level of genetic variability exists in multiple-host ascaridoid species relative to single-host ones." The authors argued that selection maintained polymorphism in parasites with complex life cycles, "one allozyme may work better in a certain step of the life cycle (e.g., when the host is a fish), and some other allozyme in another step (e.g., when the host is a marine mammal)." I view their conclusion as a product of the time because studies of this period often used natural selection as an explanation for observed differences in electrophoretic polymorphism (e.g., Nevo, 1978). Nadler (1990) criticized their conclusion on the basis that even direct life cycle parasites may encounter complex environments during within host migrations. I note here that the natural selection argument itself was flawed. In Bullini et al.'s (1986) scenario, selection would have to be for the heterozygote to maintain both alleles; otherwise, there would be directional selection for the allele with the greatest lifetime fitness, i.e., a reduction in diversity. I suspect the observed gene diversity patterns were, in part, driven by differences in effective population size (N_e), a topic I address next. Today, it is recognized that "molecular measures of genetic diversity [neutral diversity] have only a very limited ability to predict quantitative genetic variability [adaptive diversity]" (Reed and Frankham, 2001).

It was 9 years later before another truly comparative paper appeared, but this time the focus was on a non-selective evolutionary mechanism. Blouin et al. (1995) published a seminal paper comparing patterns of genetic structure among trichstrongylid nematodes of domestic hosts and a wild host. Their approach enabled inference of host movement as a major factor affecting parasite gene flow. In the 2000s, comparative studies revealed how host or parasite characteristics, life cycle patterns, or host specificity could impact gene flow among parasite populations (reviewed in Criscione, 2008; Falk and Perkins, 2013). This body of work supports predictions by Nadler (1995). To date, comparative studies have focused on among-population dynamics, but within-population comparisons would be useful

to highlight what factors affect mating systems or N_e (Criscione, 2008). As an aside, to address how organismal traits affect evolutionary mechanisms such as gene flow and N_e, studies must sample natural populations. As natural systems often have confounding factors, it may be difficult to find ideal comparative systems. In my opinion, one of the greatest strengths of using parasites as model systems in evolutionary studies is that their diverse life histories enable comparative studies.

Inference of transmission and epidemiological monitoring

Nadler et al. (1990) noted that genetic differentiation of lice among individual gophers is promoted by "transmission of relatively small number of lice from a female host to her offspring." Inherent in this study, which addressed the scale of the parasite deme, was that patterns of genetic variation informed about transmission. The principal is nicely stated by Sire et al. (2001), "Genetic substructuring at the level of individual hosts from a transmission site would mainly result from the recruitment histories experienced by each of the individual host." A human roundworm study by Anderson et al. (1995) brought population genetics as a means to infer transmission to the forefront of epidemiological studies, "Patterns of fine-scale population structure may provide information on transmission processes … Nonrandom distribution of parasite genotypes [based on neutral markers] could be generated if genetically related infective eggs are clumped in space" (Anderson et al., 1995). A study on malarial parasites 5 years later revealed a correlation between "high levels of self-fertilization [inferred from high LD and low MLG diversity] in populations with low levels of transmission [inferred from low prevalence]" (Anderson et al., 2000).

This latter observation had two large implications. Biologically, the result showed how clumped transmission could also interact with the mating system as co-transmission of related individuals could lead to biparental inbreeding or selfing (union of malaria gametocytes of a single parental oocyst is self-mating). Epidemiologically, the population genetic patterns resulting from the high inbreeding when there is low transmission are now recognized as a means to monitor if malarial parasite populations decline in response to control (Volkman et al., 2012). Indeed, Nkhoma et al. (2013) provided empirical support for the latter. At this point I am reminded of a comment I received just 5 years ago on my rejected proposal that was aimed at using population genetics to monitor chemotherapy treatment of schistosomes: "Ultimately, it is not genetic diversity that will guide intervention and control programs, but egg counts in stool" (anonymous grant reviewer). I suspect that history will bypass this reviewer.

It is now recognized that molecular population genetics is a necessary tool in epidemiological studies from viruses to schistosomes (Pybus and Rambaut, 2009; Steinauer et al., 2010). For example, in a study on human roundworms Criscione et al. (2010) used evolutionary model-based, genetic assignment methods to identify transmission foci. Subsequent incorporation of these results into landscape genetics analyses revealed epidemiological insights such as temporally stable, focal transmission around households. Although the latter conclusion was not obtainable from worm counts alone, i.e., we cannot directly observe worm dispersal and acquisition, I do not view genetics data as a replacement for infection intensity data. Both provide different information and, thus, are complimentary (Criscione, 2013).

Clonal transmission in trematodes

"Potential complicating effects of parasite genetic structure such as asexual amplification within intermediate hosts (digeneans) … have rarely been investigated" (Nadler, 1995). Though largely specific to trematodes, I chose to discuss the history of this topic as an example to emphasize the role different life parasite life histories may play in impacting their microevolution. Trematode asexual reproduction is fundamentally different than that in many parasitic protozoa because adults have obligate sexual reproduction (except rare parthenogenetic forms). Thus, larval clonal lines produced in the first host do not persist over generations. Although the actual mode of reproduction in the larval propagation stage of mollusk hosts has been of historical interest in parasitology (e.g., Whitfield and Evans, 1983), I am not aware of any studies prior to the early 1990s that addressed fluke clonality with molecular population genetics.

A likely hurdle to be cleared before such work could be done was the need for MLGs, which was not possible with enzyme electrophoresis and small flukes. Indeed, the first study to examine clonal transmission in trematodes was conducted with *Fascioloides magna*, a large deer liver fluke, by Mulvey et al. (1991).

These workers found that clonemates (individuals of the same clone) co-occurred within individual hosts more often than expected, a situation that in turn inflated genetic differentiation among hosts. They also concluded that aggregated clonemates resulted in a reduced observed heterozygosity within hosts, but later simulation modeling would show this conclusion to be wrong. PCR and microsatellites should have opened the door to additional work on fluke clonal transmission, but the next studies were not published until the 2000s (e.g., Prugnolle et al., 2002; Theron et al., 2004; Criscione and Blouin, 2006). Also during this time, Prugnolle et al. (2005) developed a theoretical framework for the population genetics of trematode clonal transmission. They found that a high variance in clonal reproduction did increase genetic differentiation among hosts, but actually created heterozygote excess within hosts (contrast to interpretation of Mulvey et al., 1991). The latter illustrates the need for proper theory to interpret population genetic patterns from organisms with life cycles that "depart from those used in theoretical population genetic models" (Prugnolle et al., 2005). In the same year, the observation of a lack of clonemate aggregation in second hosts led to an interesting hypothesis by Rauch et al. (2005), namely, that complex life cycles evolved to reduce inbreeding by decreasing the chance of clonemates ending up in the same final host (clonemate mating equals self-mating in hermaphrodites). Based on this hypothesis, Rauch et al. (2005) predicted that congeners with truncated life cycles would show greater clonemate aggregation in definitive hosts compared to species with a full life cycle. Gorton et al. (2012) provided a recent review on clonal transmission and proposed that clonal aggregation may be greater in trematodes with semi-terrestrial than fully aquatic life cycles; the latter providing an environment conducive to cercariae dispersal. The hypotheses of Rauch et al. (2005) and Gorton et al. (2012) are based on a limited number of studies, thus more data are needed to understand how clonal transmission may vary among trematodes with different life cycles. As noted by Rauch et al. (2005) and as I discussed previously, a comparative framework would be ideal to test these hypotheses.

Effective population size

Effective population size directly quantifies the evolutionary mechanism of genetic drift. Populations with larger N_e will have greater gene diversities (assuming the same mutation rate). Although Nadler (1990) showed helminths had gene diversities similar to free-living invertebrates, Blouin et al. (1992) provided the first estimate of N_e. "Long-term N_e in these populations [of *Ostertagia ostertagi*] is estimated to be four to eight million individuals. This is a very large number given that long-term N_e is the harmonic mean of N_e in past years" (Blouin et al., 1992). Clearly, parasite populations could be larger than perceived by Price (1977, 1980). So, what have we learned about parasite N_e since the early 1990s? Even though N_e is one of the most important parameters in evolutionary biology, research on what impacts parasite N_e and actual estimates of parasite N_e are mostly recent. Long-term estimates of N_e were made for malarial parasites, but conflicting estimates stem from the use of different sets of loci that may have experienced different selective histories (Hartl, 2004). Prugnolle et al. (2005) modeled how increased selfing or variance in clonal reproductive reduced N_e in trematodes. Criscione and Blouin (2005) applied a subdivided breeders model, which highlighted how "several features of [metazoan] parasite life cycles probably function in concert to reduce N_e below that expected in a single free-living population of equivalent census size." Note this statement is not to be misconstrued (though it already has) as meaning parasite populations will have small N_e. Criscione et al. (2005) showed a positive correlation between nucleotide diversity and mean intensities of some nematodes, but this observation provided a crude approximation of how parasite population parameters might impact N_e. Thus, the result is better viewed as a hypothesis to test than as an established relationship. Because little was (and is still) known about parasite N_e, we also suggested "comparisons of short-term genetic estimates of N_e among parasite populations that differ in key traits would help identify the ecological determinants of N_e" (Criscione et al., 2005). The latter idea will be facilitated by recent developments of genetic, single-sample estimators of contemporary N_e (Wang, 2009; Waples and Do, 2010).

Criscione (2013) advocated that these single-sample estimators should also enable N_e estimation as a tool to monitor control programs. As these methods are beginning to be applied, it is important to note for history yet to pass that inference of N_e is dependent on what is sampled. For example, if parasite eggs from a definitive host are used, then a subcomponent of N_e,

i.e., the effective number of breeders (N_b) within that host, is estimated. Steinauer et al. (2013) estimated a range from the tens to low hundreds for the N_bs of *Schistosoma mansoni* in individual people and concluded that estimation of infrapopulation N_bs could be a useful means to "to depict relative worm burdens in patients." Moreover, they noted it would be incorrect to combine larval (miracidia) samples across individual human hosts to generate one genetic estimate (as in Gower et al., 2013). The reason this latter sampling is incorrect is because LD generated by combining sibling groups (which exist in schistosome egg/miracidial samples; Steinauer et al., 2013) across different hosts would be in excess of that caused by breeders in a host. In turn, this would create artifactual estimates based the LD-method (Waples and Do, 2010). More appropriately the N_bs would be used in the subdivided breeders model to estimate N_e (Criscione and Blouin, 2005). Criscione (2013) recently highlighted how life history could also influence interpretation of N_e genetic estimates. In particular, I discussed how long-lived eggs (a trait that creates a "seed-bank" effect as noted by Nadler, 1995) of *Ascaris lumbricoides* leads to overlapping generations in definitive hosts. With adult *A. lumbricoides*, I estimated subpopulation N_es of about 100, which was in accord with the low intensity-low nucleotide diversity relationship given in Criscione et al. (2005).

Hermaphroditic mating systems

Even though hermaphroditism is ubiquitous throughout the Neodermata (parasitic flatworms), which is estimated to have over 130,000 species (Strona and Fattorini, 2014), we know virtually nothing about their primary mating systems, i.e., whether they self-mate or outcross. Evolutionary significance of self-mating stems from the fact that it is the most extreme form of inbreeding, a situation that in turn magnifies the effect of drift, alters selection efficiency, and affects population levels of genetic diversity. In addition, inbreeding or outbreeding depression can be manifested in a single generation of mating. There was research on flatworm self-mating in the 1960s, about the same time as the advent of electrophoretic methods (see references in Nollen, 1971). However, progeny genotyping to estimate adult selfing-rates was not used until two decades later (Trouve et al., 1996). Again, I attribute the slow development to the tissue requirements of electrophoretic methods.

These early studies relied on radiolabeled sperm to assess self-insemination (reviewed in Nollen, 1983), but oddly there were no explicit links to inbreeding. Rather, focus was on whether cross-fertilization was required "for normal development of the life cycle" (Nollen, 1971). For instance, there was no mention of inbreeding depression by Nollen (1971), but he clearly compared several fitness traits between inbred and outbred lines. As another example, Fried and Harris (1971) found that flukes raised alone took longer to produce eggs than when worms occurred in pairs, but stated, "no explanation is available to explain the lag in development and the significant reduction in numbers of fully developed eggs in single-worm infections." If only they had a time machine to read up on the evolutionary hypotheses of delayed selfing (Escobar et al., 2011).

I do not have space to reference a complete history but, as a backdrop, it is fundamental to know that most evolutionary theory and empirical work on hermaphroditism has stemmed from plant research (see Goodwillie et al., 2005). Theory, progeny-array methods to estimate primary selfing-rates from field samples, and empirical work blossomed in the plant literature during the 1970s and 1980s based on electrophoretic analysis. In reference to the evolution of the primary mating system, Schemske and Lande (1985) reviewed selfing rates of 55 plant species and, in Goodwillie et al. (2005), this number expanded to 345, all based on progeny-array data. Work on animals lagged behind. Jarne (1995) listed 55 animal species and Jarne and Auld (2006) had 142 based on progeny-array and indirect population estimates (selfing rate calculated from F_{IS}). How many of these species were parasites? There were 2 and 14 parasitic species, respectively. Even in relation to other evolutionary topics of hermaphroditic animal mating systems such as sex allocation, sex role, and inbreeding depression, parasites (mostly based on *Schistocephalus solidus*) make up only 2–5% of the studies in reviews; most of the others employ snails (Scharer, 2009; Anthes et al., 2006; Escobar et al., 2011).

In general, molecular population genetics work on flatworm mating systems has been sparse and largely inferred from indirect population estimates (Jarne and Auld, 2006; Gorton et al., 2012). Thus, no consensus mating patterns at the population level have emerged. Gorton et al. (2012) hypothesized that aquatic transmission may promote more outcrossing and, therefore,

panmixia, than terrestrial life cycle patterns where clumped transmission may promote more bi-parental inbreeding. However, more work is needed to see what drives flatworm mating systems. Our knowledge regarding the primary mating system is even less extensive. In fact, there are only four flatworm species for which we have estimates of the primary mating system (Trouve et al., 1996; 1999; Luscher and Milinski, 2003; Schelkle et al., 2012; Rieger et al. 2013). All these studies show that mixed-mating (outcrossing and selfing) is possible, but they are laboratory studies and only work by Trouve and colleagues report individual worm selfing rates. To date, we do not have any direct estimates of the primary mating system for any hermaphroditic parasite in nature.

Mode of inheritance and genetic mapping

Genetics started with Mendel and, with this last topic, I will have come full circle. Generating genetic crosses in parasites is not an easy task. Difficulties include life cycle maintenance, controlled crosses, access to purebred phenotypes, and large numbers of progeny. Thus, classic crosses examining the mode of inheritance of parasite phenotypes are historically rare. With the exception of work on lice coloration (Busvine, 1946), I have not found any examples that predate the 1960s. Via several cross-based studies in the 1970s, electrophoretic markers in combination with drug-resistant phenotypic markers enabled proof of recombination in malarial parasites (reviewed in Walliker, 1983). The next step was to use these data to generate genetic linkage maps and locate genes that underlie parasite phenotypes. Indeed, the first protozoan linkage map was for *Plasmodium falciparum* (Su et al., 1999). This map has subsequently been used to map phenotypes associated with drug resistance, pathogenesis, and mosquito infectivity (reviewed in Volkman et al., 2012). Ten years later, the first linkage map for a helminth, *Schistosoma mansoni*, was generated (Criscione et al., 2009). This map has subsequently been used to correct genome assembly errors and map a drug-resistant phenotype (Protasio et al., 2012; Valentim et al., 2013). The population level extension of linkage mapping includes association-based studies and genome scans for regions of recent selection, i.e., population genomics studies. If there was another area where parasitology has led the field in population genetics (as with the clonal research), it would be with the population genomics studies on human malaria. In addition to revealing much about malaria biology, e.g., population history, local transmission, and inference of selection, population genomics studies of malaria have served as excellent examples for genome evolution in general. This literature is too extensive to review here, so, for an excellent review, I refer readers to Volkman et al. (2012).

In addition to mapping, crosses can test two assumptions that underlie all population genetics studies, i.e., Mendelian segregation and independent assortment. For example, de Meeûs et al. (2004) used tick crosses to show that size-based allelic dropout affected microsatellite scoring and Detwiler and Criscione (2011) used nature-provided tapeworm crosses to identify duplicated microsatellite loci, "by taking advantage of the fact that hosts represent closed mating systems for endoparasites, we were able to exploit natural crosses to test Mendelian inheritance."

A few personal thoughts

It was a learning experience for me to write this historical account. I came across papers I was not familiar with and re-affiliated myself with some classics. Unfortunately, there was not room to discuss them all. I also apologize to those who work on protozoan and non-helminth metazoans since I am certain that I have not adequately covered the history of molecular population genetics for these organisms. However, I suspect general patterns parallel those of the helminths.

So after recounting the history of molecular population genetics in parasitology, the succeeding question is: what next? At the end of Criscione et al. (2005), I stated, "Parasite molecular ecology is still in its infancy." In my opinion, molecular population genetics of protozoan and metazoan parasites is still a young sub-discipline. Perhaps we are now at the toddler stage. Yes, we have gained a lot of knowledge from studies on malarial parasites and other medically important species. But, in terms of our general knowledge of parasite microevolution, we have only scratched the tip of parasite biodiversity and life history. Nadler (1995) noted, "Given the range of ecological diversity that is characteristic of parasites, a broad spectrum of genetic architectures is likely to be revealed as more empirical studies are undertaken." For many of the

topics I discussed previously, only a few species have been examined. Thus, the full gamut of parasite life history has yet to be explored. These gaps must be filled before there is enough data to determine if broad scale population genetic patterns or generalities are present across parasite life histories.

I also feel that more theoretical work needed is needed to tie traditional epidemiological models with population genetics. The purpose would be to determine which genetic statistics are most useful for inferring transmission and designing or monitoring control programs. Parasite life history may also dictate what, and how, parasite life stages are sampled. Simulations may also be needed to see how sampling might impact inference (e.g., Steinauer et al., 2013).

It is cliché to say these days that NGS will greatly change molecular population genetics, but I cannot deny this to be true; e.g., the Wellcome Trust Sanger Institute currently has draft genomes for 50 helminth species. Clearly, the topics I covered above will be enhanced by possession of genome-wide data. Nonetheless, to prevent artifactual results, utilization of NGS will require accurate genome assemblies. Moreover, several population genetic analyses assume independence among markers, thereby necessitating estimates of recombination rates across the assembly. NGS will also facilitate additional topics such as searching for signatures of adaptive evolution. We already see this with human malaria (Volkman et al., 2012). Insight into the evolution of the genome itself is now a hot topic. For example, in a review on how mating systems could impact genome evolution Glémin and Galtier (2012) state, "A major current molecular evolution challenge is to link comparative genomic patterns to species' biology and ecology." With over four decades of research on the primary mating systems of hermaphroditic plants in nature, there is a biological context with which NGS data can facilitate genome evolution studies in plants. In contrast, there are only four species of hermaphroditic parasites with primary mating system data, all of which are lab-based. My point is that the limitations of applying NGS data to parasites will not be the technology itself, but rather the biology of the parasites themselves. Granted, NGS data may be able to elucidate the biology, but whether NGS data are necessary or overkill versus other genotyping methods will depend on the nature of the question.

Acknowledgments

I would like to end on a personal note by thanking those who have shaped my own academic history. I have been fortunate to learn from two of the pioneers in parasite molecular population genetics: Mike Blouin, my Ph.D. advisor, and Tim Anderson, my postdoc advisor. They are both still active in applying modern molecular methods to study parasite population biology. Of course, I would have never gotten into parasitology in the first place if it were not for my M.Sc. advisor, Bill Font. As a joke, I now blamably exclaim "Font!" In the true spirit of population genetics I am proud to say that I can trace my academic pedigree to a pioneer in North American Parasitology, Henry Baldwin Ward. Ward is my academic great-great grandfather on my M.S. side (William Font-Kenneth Corkum-Harry Bennett-Henry Ward). Interestingly, this relationship makes Sewell Wright (one of the fathers of population genetics) my great-grand uncle as he was a M.Sc. student of Ward, an interesting fact I first learned from reading Nadler (1995). Now, if only I could trace either of my other advisors' lineages back to Wright, then I could calculate my own academic inbreeding coefficient!

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