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TREMATODE CLONE ABUNDANCE DISTRIBUTIONS: AN ECO-EVOLUTIONARY LINK BETWEEN PARASITE TRANSMISSION AND PARASITE MATING SYSTEMS

Charles D. Criscione[1], **Jenna M. Hulke**[1], **and Cameron P. Goater**[2]

[1] Department of Biology, Texas A&M University, 3258 TAMU, College Station, Texas 77843.
[2] Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, T1K 3M4, Canada.
Correspondence should be sent to Charles D. Criscione at: *ccriscione@bio.tamu.edu*

KEY WORDS

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ABSTRACT

Most trematodes and some cestodes have obligate life history features that include an asexual developmental stage that can produce genetically-identical individuals (clonemates) followed by an adult stage with sexual reproduction. These life history features can influence the evolutionary mechanism of inbreeding in parasites, especially among self-compatible hermaphroditic endoparasites whose mating opportunities are restricted to within hosts. As clonemate mating in hermaphroditic species produces a genetic inbreeding signature identical to that of self-mating, it is important to understand how clonemates are transmitted through their life stages. A handful of prior studies compared clone richness (number of clones) across life cycle stages to infer transmission processes and to characterize clone abundance distributions (CADs) among hosts. Here we illustrate the use of the proportion of clonemate dyads (P_C) within hosts to describe the CADs. P_C has several advantages as an ecological metric in that it is unbiased by sample size, takes into account relative parasite burdens, and has a direct transmission interpretation, i.e., the probability of cotransmitting clonemates. Moreover, P_C is also an evolutionary metric as it can be used to estimate a potential clonemate mating rate. We demonstrate the use of P_C in comparing CADs within and across 2 trematode developmental stages in the lancet fluke *Dicrocoelium dendriticum*. Also, we show how genetic estimates of apparent selfing (true selfing plus clonemate mating) at larval and juvenile stages can be compared to P_C estimated at the adult stage to assess the contribution of clonemate mating to apparent selfing. The eco-evolutionary links presented are generalizable to assess sibling cotransmission as well. Thus, the framework presented herein will facilitate future field-based studies on the transmission and mating systems of parasitic flatworms.

There is a direct eco-evolutionary connection (Pelletier et al., 2009) between the ecological process of parasite transmission and the evolutionary outcomes of parasite mating systems. Transmission plays a central role in shaping both the among-host intensity distribution, i.e., the number of parasites per infected host (Bush et al., 1997), and which individuals, with regard to relatedness, are transmitted together (Criscione et al., 2005). In turn, because adult endoparasites exist in closed mating systems such that individuals cannot mate with parasites in another host, the evolutionary mechanism of inbreeding can be impacted by infection intensities, e.g., hermaphroditic parasite selfing rates may decline with increasing intensities (Detwiler et al., 2017) and/or potential kin mating stemming from cotransmitted, related parasites (Guzinski et al., 2009; Detwiler and Criscione, 2017). For the latter, the degree of relatedness can reach its maximum (100%) in digenean trematodes and some cestodes due to the presence of clonemates, i.e., genetically identical individuals that are the product of asexual reproduction (Whitfield and Evans, 1983). Clonemate mating in hermaphroditic species has the interesting effect of producing a genetic inbreeding signature identical to that of self-mating. Herein we focus on the eco-evolutionary dynamic of clonemate cotransmission and clonemate mating that is possible in hermaphroditic cestodes and digeneans with an emphasis on the latter.

The vast majority of digeneans are hermaphroditic and have complex life cycles where as adults they have obligate sexual reproduction (selfing and/or outcrossing) in a final host to produce genetically distinct larval miracidia (Fig. 1). Subsequently, a miracidium infects a first host (commonly a mollusc) and produces via obligate asexual reproduction numerous genetically identical, diploid larval stages (clonemates): sporocysts to rediae (in some species) to ultimately cercariae that proceed to infect the next host. In addition to potential impacts on the mating system and inbreeding, these clonal dynamics set the stage for various eco-evolutionary feedbacks (Pelletier et al., 2009) related to complex life cycle evolution itself and possible

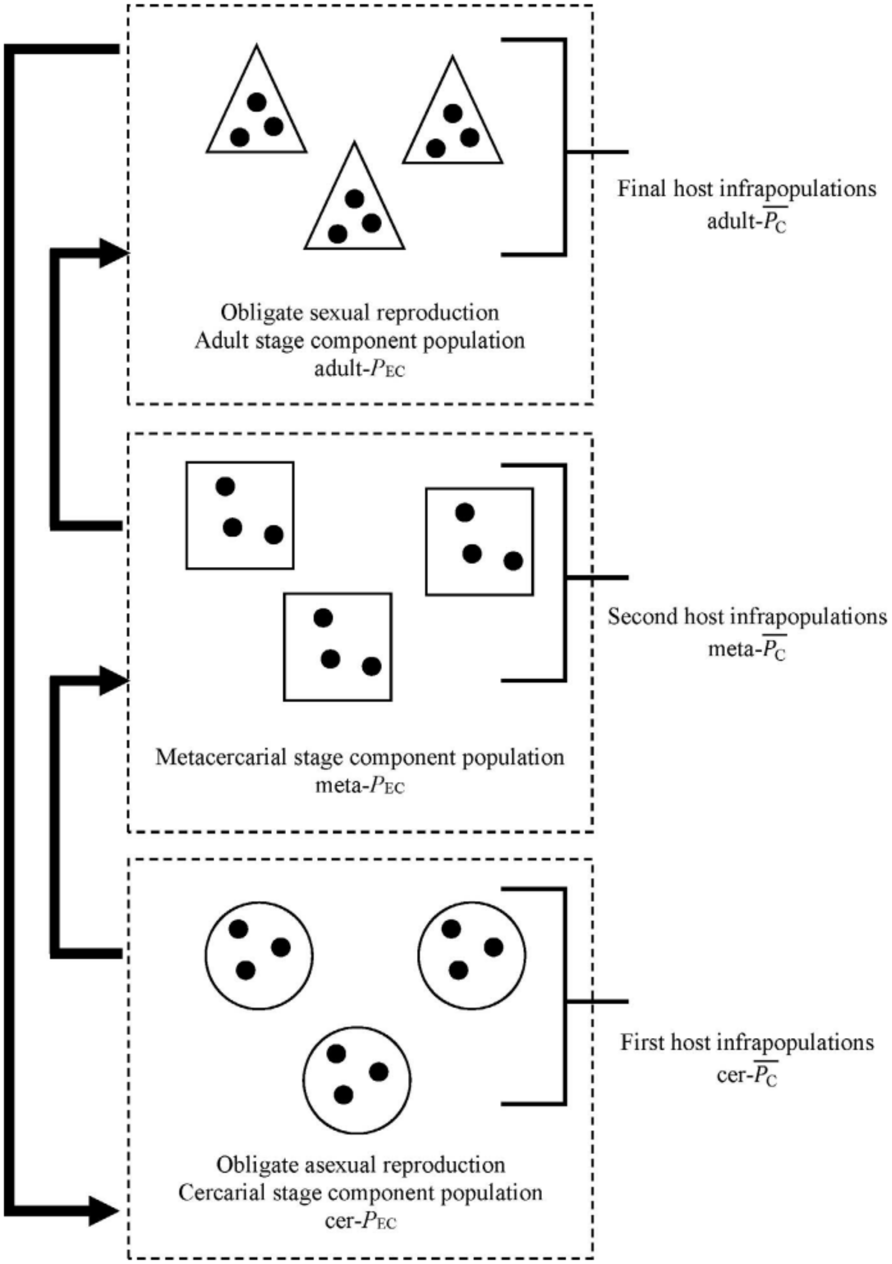

Figure 1. Schematic diagram of a typical 3-host digenean life cycle along with metrics of relevance at each life cycle stage. Black dots represent parasites within a host, i.e., an infrapopulation. P_C (percentage of clonemate dyads within infrapopulations) is calculated for each host and then averaged across infrapopulations. For a given life stage, P_{EC} is the percentage of clonemate dyads that is calculated across all sampled parasites from all sampled infrapopulations. The life cycle of *Dicrocoelium dendriticum* is described in the main text. Triangles represent definitive hosts where trematodes undergo obligate sexual reproduction. Circles represent first intermediate hosts where trematodes undergo obligate asexual reproduction, and squares are second intermediate hosts where trematodes are typically encysted in a resting state.

kin-selected traits such as host-manipulating behaviors, larval division of labor within mollusc hosts, among-clone competition, virulence, and infectivity and transmissibility (Brown et al., 2001; Davies et al., 2002; Gower and Webster, 2005; Rauch et al., 2005; Weinersmith et al., 2014; Gleichsner et al., 2018a, 2018b; Poulin et al., 2019; Klemme and Karvonen, 2019; Criscione et al., 2020). Elucidating the significance of these potential clonal eco-evolutionary dynamics in trematodes requires the characterization of clone abundance distributions (CADs) both across hosts within a developmental stage and across different stages of parasite development in natural environments to determine the extent that clonemates are cotransmitted throughout the life cycle.

Despite an estimated 69,000 species of digeneans (Strona and Fattorini, 2014), there remains a paucity of data on CADs (see Gorton et al., 2012; Louhi et al., 2013; Criscione et al., 2020). In part, the limited data are a reflection of the late and limited application of polymorphic genetic markers to identify trematode clonemates (Criscione, 2016). Nonetheless, 3 seminal studies have made important contributions by comparing CADs across trematode developmental stages (Theron et al., 2004; Rauch et al., 2005; and Keeney et al., 2007). Specifically, these 3 studies assessed the average number of clones per host in the snail first host and subsequent second or final host to estimate the number of infective snails involved in the transmission of larvae into next-stage hosts. In doing so, these studies were able to shed light on long-standing questions relating to the dynamics of clonal transmission and on the processes by which clonemates are distributed within and among their hosts in natural populations (Theron et al., 2004).

Although clone counts give insight into transmission, the metric used in the above foundational studies is 1 of clone richness (akin to 'species richness' in biodiversity and community-level studies in ecology; Chao et al., 2014). As discussed by Chao et al. (2014), the richness index has limitations in that it is sensitive to sample size, and it does not incorporate information on the relative abundance (Chao et al., 2014). In addition, the clone richness value alone does not provide insight into the extent that clonemates are cotransmitted into the same infrapopulation (all the parasites of a given species within an individual host; Bush et al., 1997). It is desirable, therefore, to have a metric that does not suffer from these limitations and that is explicit in its interpretation of cotransmission of clonemates.

The assessment of dyad (paired) kin relationships used by Detwiler and Criscione (2017) provides 1 possible framework. In particular, they compared the percentage of sibling dyads of a tapeworm within gecko host infrapopulations (P_S) to the percentage of sibling dyads over the entire component population (P_{ES}), i.e., all parasites sampled across hosts (Bush et al., 1997). Cotransmission of sibling tapeworms was inferred if the average infrapopulation P_S (denoted $\overline{P_S}$) was greater than P_{ES}. Recently, Criscione et al. (2020) applied this approach to study clonemate cotransmission at a single life stage of a trematode by comparing the percentage of clonemate dyads within infrapopulations (P_C) to the percentage of clonemate dyads over the entire component population (P_{EC}). We propose that P_C provides an alternative metric to clone richness in characterizing CADs.

We first note the broad applicability of P_C in that it is mathematically equivalent to the complement of Hulbert's *Probability of Interspecific Encounter* (*PIE*), i.e., 1 – *PIE* (where *PIE* is eq. 3 in Hurlbert 1971), a metric that carries relative abundance information and is unbiased at small sample sizes (Grivet et al., 2005; Chao et al., 2014). The complement of *PIE* is interrelated to species diversity metrics; specifically, the inverse is a Hill number (Jost, 2007; Chao et al., 2014). However, in our current study, we are not interested in trematode clonal diversity per se. Rather, we continue to use P_C to highlight its dual ecological and evolutionary information content. First, P_C directly reflects a probability of co-occurring clonemates within infrapopulations (probability of clonemates drawn from 2 random trematodes from the same host) and therefore provides information that is explicitly linked to transmission. In fact, 1 – *PIE* has previously been used to infer seed dispersal patterns with the use of the mathematically equivalent metrics called *Probability of Paternal Identity* (*PPI*) and *Probability of Maternal Identity* (*PMI*) (Grivet et al., 2005; Smouse and Robledo-Arnuncio, 2005). An innovative study by Scofield et al. (2012) provided statistical tests of *PMI* to assess how different vertebrate foraging behaviors impacted seed dispersal, i.e., determining if related seeds end up in the same birdseed cache. The latter is analogous to our interest in determining whether clonemates tend to co-occur within hosts. To emphasize, 1 – *PIE*, *PPI*, *PMI*, P_S, and P_C are the same mathematical metric but just differ in the quantified biological entities (i.e., different species, paternal siblings, maternal siblings, full siblings, and clonemates, respectively). We give details about the methods, but in short, the statistical analyses of Scofield et al. (2012) enable a test of whether $\overline{P_C} = P_{EC}$ within a trematode developmental life stage as well as whether both $\overline{P_C}$ and P_{EC} differ between 2 treatments (as would be the case in comparing trematode developmental stages).

The second reason we retain the use of P_C is that this ecological metric of transmission translates directly into an evolutionary metric of interest, i.e., a potential clonemate mating rate, Thus, P_C can also be used to contribute to our understanding of trematode hermaphroditic mating systems. Detwiler and Criscione (2017) showed how $\overline{P_S}$ could be used to estimate a potential kin mating rate, which in turn could be used along with selfing rates (s) to assess total accumulated inbreeding. In conjunction with genetic-based estimates of apparent selfing rates (selfing rate plus clonemate mating rate; s_a) at a larval stage, we here show how $\overline{P_C}$ among final hosts (where trematodes sexually reproduce; Fig. 1) can be used to infer the contribution of clonemate mating to s_a. Hence, P_C itself is a simultaneous eco-evolutionary metric.

To demonstrate the use of P_C in comparing CADs within and across 2 trematode developmental stages as well as its use in elucidating hermaphroditic mating systems, we used clonemate data stemming from previously published studies on the lancet fluke, *Dicrocoelium dendriticum*. How P_C relates to the various stages within a typical trematode life cycle is presented in Figure 1. The lancet fluke has a 3-host life cycle (Krull and Mapes, 1952, 1953). Obligate sexual reproduction among adults occurs in the bile ducts of ungulate final hosts. Eggs, each with a miracidium, pass into the environment and are consumed by terrestrial snails. Obligate asexual reproduction occurs within the snail, leading to the release of packets of cercariae within fluid-filled packages known as 'slime balls' (reviewed by Goater et al., 2014). Slime balls are ingested by ants wherein metacercariae encyst in the ant gaster. One metacercaria, though, is located in the subesophageal ganglion and is responsible for altering the behavior of the ant in a temperature-dependent manner such that the ant attaches to inflorescences (Hohorst and Graefe, 1961; Botnevik et al., 2016). The life cycle is completed when clinging ants are consumed by grazing ungulates. We used the framework of Scofield et al. (2012) to assess trematode clonemate transmission dynamics by comparing $\overline{P_C}$ and P_{EC} in ant second hosts (metacercarial stage), in ungulate final hosts (adult stage) and between the 2 developmental stages. In addition, to assess the contribution of clonemate mating to the mating system, we used genetic-based methods to estimate s_a at the metacercarial stage and compared it to $\overline{P_C}$ estimated at the adult stage.

MATERIALS AND METHODS

Sampling and microsatellite genotyping

Detailed sampling and genotyping methods for the metacercarial and adult data sets are given in Criscione et al. (2020) and van Paridon et al. (2016), respectively. In short, the metacercarial data originated from infected ants collected in 2013 from 2 sites in the Cypress Hills Park in southeastern Alberta (van Paridon et al., 2017): 134 metacercariae from 9 ants were genotyped from 'Staff Camp' site (SC) and 138 metacercariae from 9 ants were genotyped from Trans-Canada Trail site (TC) (Fig. 2A, B). Adult trematodes also came from Cypress Hills Park and were obtained in 2003 from purchased cattle livers or hunter-shot elk (Goater and Colwell, 2007). The adult data set was composed of 63 adult worms across 4 elk and 3 cattle livers (Fig. 2C). All adult and metacercarial samples were genotyped across the 5 microsatellite loci used in Criscione et al. (2020). Because adult and metacercarial sampling was conducted 10 yr apart, we recognize that biological inferences carry the assumption that CADs are

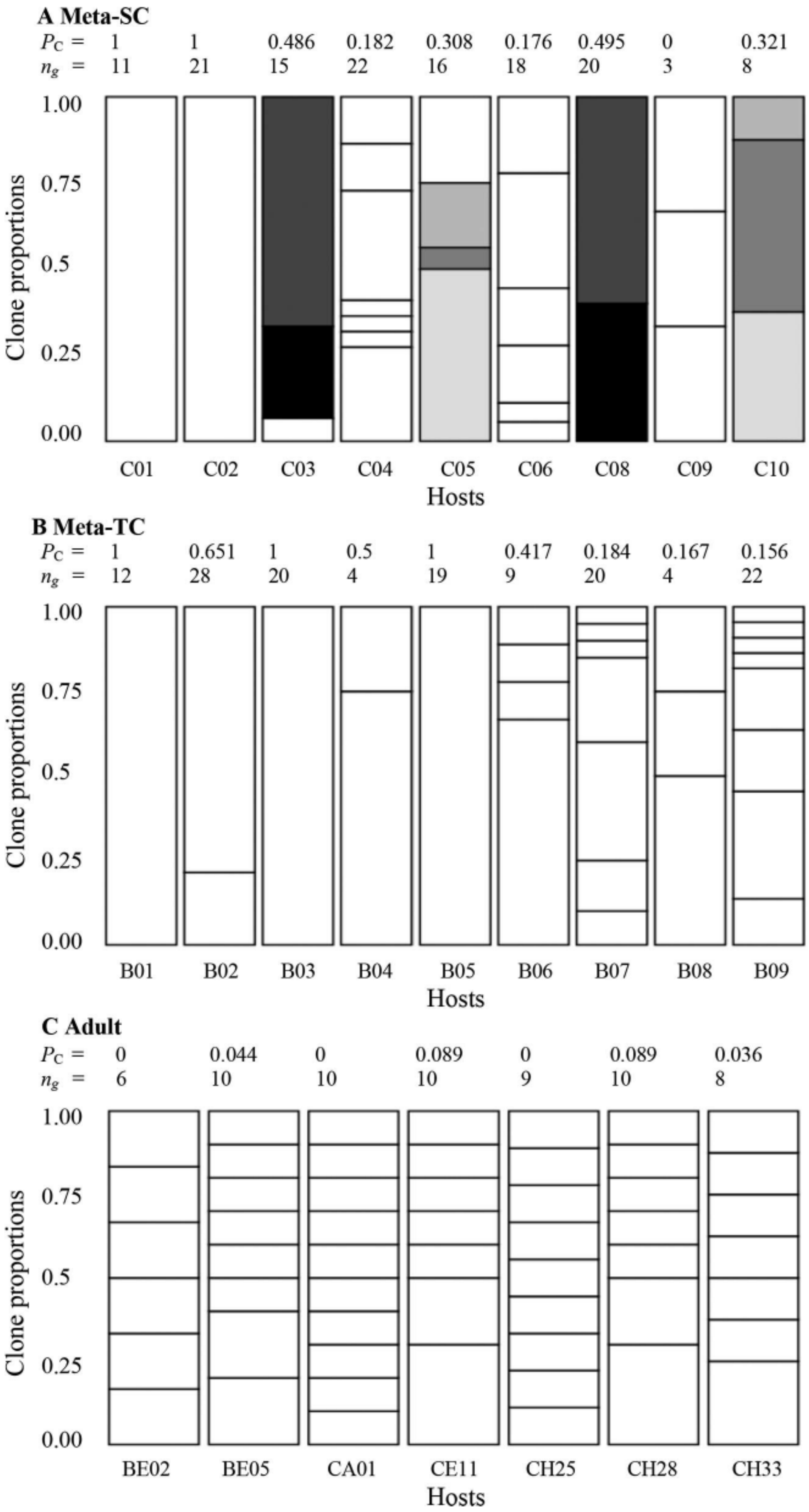

Figure 2. Relative representations of the clone abundance distributions among hosts for the metacercariae in ants at locations SC, Staff Camp (**A**) and TC, Trans-Canada Trail (**B**), and adults in ungulates (**C**). Each bar represents a CAD in an individual host. Each box within a bar represents the proportion of a different clone within that host. White boxes denote different clones across all hosts (i.e., there were 108 different clones across all 3 data sets). Shaded boxes denote clonemates shared between host individuals, which occurred only in the SC location. The number of genotyped individuals per host and P_C of each host is given above each bar.

relatively stable over this time frame. Nonetheless, the main impetus of our study is to demonstrate the utility of P_C in cross-stage comparisons as well as in elucidating the mating system.

Clonemate identification and clonal genetic structure analyses

To identify clonemates, we used P_{sex}, the probability of observing n copies of a multilocus genotype (MLG) in a sample size of N given sexual reproduction (Gregorius, 2005). Detailed methodology for clonemate identification in the metacercarial data set was outlined in Criscione et al. (2020). We repeated these analyses in the adult data set to determine if repeated MLGs were the product of asexual reproduction (i.e., clonemates).

Population genetic tests of clonal structure in the adult data set entailed analyses of average within-host F_{IS} and among-host F_{ST} (multilocus estimators). Significance was tested in FSTAT (Goudet, 1995) using 10,000 randomizations of alleles among individual flukes within hosts and of genotypes among hosts, respectively. The analyses were conducted on the full adult data set ($n = 63$) and on an adult data set where clones with clonemates are reduced to 1 copy within each host ($n = 54$). If clonemates co-occur within infrapopulations, the expectation is that the average within-host F_{IS} will be lower and among-host F_{ST} will be higher in the data set with clonemates compared to the reduced data set (Prugnolle et al., 2005). The same analyses on the metacercarial data set were previously conducted by Criscione et al. (2020).

There was no evidence of sibling cotransmission in the metacercarial stage (see Criscione et al., 2020), and the clonal structure analyses above provided no evidence of sibling cotransmission in the adult data (see Results). Hence, we did not do additional pedigree reconstruction analyses in the adult data set as was performed on the metacercariae in Criscione et al. (2020).

We tested for genetic structure between adult and metacercarial samples to determine if they were part of the same 'genetic' population. Only 1 representative of each clone was used within each data set as each clone is the product of sexual reproduction. Using FSTAT, we estimated F_{ST} between the adult and metacercarial samples and used 10,000 randomizations of parasite genotypes to test for population differentiation. We also combined the 2 data sets to test for genotypic disequilibrium (tested between all pairs of loci in GENEPOP v4.7.5: Markov chain parameters: 5,000 dememorizations; 5,000 batches; 5,000 iterations; Rousset, 2008), which would be expected if 2 genetically differentiated populations were analyzed as 1.

Clonemate transmission analyses

Metrics and analyses are explained in parallel to the Scofield et al. (2012) framework, which provides a series of sample size-weighted, non-parametric permutation tests. These statistical tests (10,000 permutations for each) were carried out using the R package dispersalDiversity v0.9.9001 (specific functions given in single quotes below; Scofield et al., 2012). We note that the statistical permutation tests of Scofield et al. (2012) were explicitly on the PMI metric though they present their results as true diversity metrics by taking the reciprocal, i.e., a Hill number (Jost, 2007). We do not convert to a Hill number as we are interested in the P_C as a probability as outlined in the introduction. In Scofield et al. (2012), PMI is the probability of drawing 2 seeds from the same maternal source from within a seed pool (see eq. 1, Scofield et al., 2012). The equivalent in our study is P_C, which at a given trematode developmental stage is the probability of drawing 2 clonemates from an infrapopulation (the number of clonemate dyads divided by total possible dyads within a host). Heterogeneity in P_C among infrapopulations at a given developmental stage was tested using the 'alphaDiversityTest' function. In Scofield et al. (2012), R_0 is the probability of drawing 2 seeds across the entire collection from the same maternal source without regard to the source pool (see eq. 3, Scofield et al., 2012). The equivalent of R_0 in our study is P_{EC}, which is the probability of drawing 2 clonemates over the component population sample of a given developmental stage (ignoring host delineations) and represents the expectation based on random chance. In other words, if hosts randomly sample clones and clonemates without replacement from the component population CAD, then $\overline{P_C} = P_{EC}$. The latter can be tested using the permutation-based test ('pairwiseMeanTest' function) of the divergence metric ($\overline{\delta}$) given in Scofield et al. (2012; see their eq. 7b such that a $\overline{\delta} > 0$ indicates that $\overline{P_C} \neq P_{EC}$ (discussed in relation to diversity metrics in Scofield et al. 2012). It follows then that if $\overline{P_C} > P_{EC}$, clonemates are cotransmitted into hosts more often than expected by chance alone.

Scofield et al. (2012) also provided between-treatment statistical tests of PMI and R_0 to determine how bird species with different foraging behaviors impacted seed dispersal patterns. In trematode systems, transmission is directional from the metacercarial stage to the adult stage. Hence, we are interested in how the probability of clonemate co-occurrence might change at the component population level as transmission progresses from the metacercarial stage (meta-P_{EC}) to the adult stage (adult-P_{EC}) as well as changes that might occur within hosts (meta-$\overline{P_C}$ vs adult-$\overline{P_C}$). The function 'alphaContrastTest' was used to test if meta-$\overline{P_C}$ = adult-$\overline{P_C}$. The 'gammaContrastTest' is valid only if the number of hosts sampled is the same between different treatments. Hence, we could compare meta-P_{EC} between the SC and TC locations with this function. We qualitatively assessed if meta-P_{EC} = adult-P_{EC} by using accumulation curves of P_{EC} to compare the value of P_{EC} at the lowest host sample size. Accumulation curves were performed with the 'gammaAccum' function, but the code was modified to calculate P_{EC} rather than its inverse. To generate confidence intervals in the accumulation curves, we performed 10,000 permutations (sampling without replacement). To aid readers in the dispersal diversity functions used above, we provided supplemental files (including our data inputs and modified code) with the commands for the various tests that we ran (Suppl. Files S1, S2).

As a means of exploring the transmission of clonemates across developmental stages, we propose the cross-stage ratio meta-P_{EC}/adult-$\overline{P_C}$. This ratio reflects the random sampling equivalent of the metacercarial component population CAD that is observed within a final host. We refer to this ratio as RSE (random sampling equivalent). In other words, $RSE > 1$ indicates how many times the CAD of the metacercarial component population has to be replicated (each replication adds a set of new clones with the same frequency distribution as that observed) to obtain an equivalent value as that of the adult-$\overline{P_C}$. The biological interpretation would be that given the value of adult-$\overline{P_C}$, it is as if on average a final host randomly sampled (without replacement) from a metacercarial component population CAD that was

X times (where X is the RSE value) the observed metacercarial CAD. We consider RSE to be an approximate means of exploring the transmission process (see empirical justifications in our results) because the relationship is exact only for Simpson's concentration measure (Olszewski, 2004; Jost, 2007) rather than its unbiased estimator, to which both P_{EC} and P_{C} are mathematically equivalent.

Quantifying potential clonemate mating rates

The clonemate mating rate, t_{C}, has the same genetic impact as the selfing rate, s, such that $t_{\mathrm{C}} + s = s_{\mathrm{a}}$, where s_{a} is the apparent selfing rate. However, physically, t_{C} is a component of the total outcrossing rate, t_{T}, where $t_{\mathrm{T}} = 1 - s$. Following Detwiler and Criscione (2017), t_{T} can be broken down such that $t_{\mathrm{T}} = t_{\mathrm{C}} + t_{\mathrm{K}} + t_{\mathrm{U}}$, where t_{U} is the unrelated mating rate and t_{K} is the mating rate between non-clonemate kin (e.g., full and half-siblings as in Detwiler and Criscione, 2017). As noted by Detwiler and Criscione (2017), an advantage of closed mating systems (adult parasites can mate only with others present in the same host) is that possible outcrosses can be quantified. Specifically, P_{C} provides an estimate of the percentage of potential outcrosses that are between clonemates such that $P_{\mathrm{C}} = t_{\mathrm{C}}/(t_{\mathrm{C}} + t_{\mathrm{K}} + t_{\mathrm{U}})$ assuming outcrosses occur randomly within hosts. By substitution and rearrangement, $(1 - s) \times P_{\mathrm{C}} = t_{\mathrm{C}}$. Further, substituting $s_{\mathrm{a}} - s$ for t_{C} enables inference on the contribution of clonemate mating to s_{a} via P_{C} itself. As long as s_{a}, P_{C}, or $s \neq 1$, 1 possible rearrangement we find useful in the context of the current data sets is $s = (s_{\mathrm{a}} - P_{\mathrm{C}})/(1 - P_{\mathrm{C}})$.

As presented in the prior section on clonemate transmission, an estimate of P_{C} can be taken from the average value among adult infrapopulations. Here we use a weighted by sample size average designated adult-$w\overline{P_C}$ to account for random variation in reproductive success across all sampled parasites (Detwiler and Criscione, 2017). Next, adult-$w\overline{P_C}$ is compared to a genetic-based estimate of s_{a} taken from a larval or juvenile stage (see mating system analyses below). Specifically, assuming there is no inbreeding depression from offspring development to the larval or juvenile stage, the following mating system interpretations could be drawn. If the numerator $s_{\mathrm{a}} - w\overline{P_C} = 0$, then clonemate mating explains all the apparent selfing. If $s_{\mathrm{a}} - w\overline{P_C} > 0$, then there is some contribution of true selfing or clonemates prefer to mate with 1 another beyond that expected by random outcrossing. If $s_{\mathrm{a}} - w\overline{P_C} < 0$, then clonemates may avoid mating with 1 another. In the discussion, we return to the effect inbreeding depression would have on these inferences.

Mating system analyses

Clonemates are not the product of sexual reproduction within the adult generation but rather are produced in a larval asexual stage after mating has taken place. Thus, to infer the mating system among adults, the metacercarial and adult data sets need to be reduced to 1 representative of each clone. In both cases, the sample size was reduced to 54 clones (see Results). We used 4 methods to infer s_{a} in both the adult and metacercarial datasets. The first method was based on excess homozygosity relative to Hardy-Weinberg equilibrium as quantified by F_{IS} (Weir and Cockerham, 1984), whereas the second method was based on the amount of identity disequilibrium (g_2, the standardized identity disequilibrium; David et al., 2007). F_{IS} was estimated in SPAGEDI v1.5 (Hardy and Vekemans, 2002) and tested as a 1-tailed test with 20,000 permutations of alleles among individuals. F_{IS} confidence intervals were generated by 10,000 bootstraps over individuals in GENETIX v4.05 (Belkhir et al., 2004). The *R* package inbreedR (Stoffel et al., 2016) was used to estimate $g_{2,}$ test its statistical significance (10,000 permutations of single-locus data among individuals), and estimate confidence intervals (10,000 bootstraps over individuals). We found no evidence of inbreeding in the adult data set (see Results), so for the metacercarial data set, we used single-generation formulas (as opposed to inbreeding equilibrium formulas) to convert F_{IS} and g_2 to selfing rates, i.e., s_{a} (see eqs. 21 and 23 for diploids, respectively, in Hardy, 2016).

The other 2 methods used to estimate selfing rates were the Bayesian model-based approaches implemented in INSTRUCT (Gao et al., 2007) and BES (Redelings et al., 2015), both of which model the number of generations back that individuals are selfed, with the former relying on patterns in homozygosity and the latter on patterns in identity disequilibrium. In INSTRUCT, we assumed a single population ($K = 1$) and ran 3 independent chains, each with 1,000,000 iterations in total, 500,000 burn-in, and 10 thinning intervals, under Mode 2 (infer population selfing rates). The Gelman-Rubin statistic for convergence of the chains was good in each analysis (adult and metacercarial data sets separately), and all replicate chains produced nearly identical median and 95% credible intervals for the selfing rates. Results from the chain with the highest posterior median log-likelihood are reported. In addition to the population selfing rate estimate, INSTRUCT also provides for each individual the number of generations (median estimate) back since the last outcross (e.g., 2 generations back indicate 1 generation of selfing). In BES, we used the generic module (f_other option set to 0) to infer the selfing rate where we ran 3 independent chains of 100,000 iterations and used the built-in 'statreport' command to estimate the population selfing rate (median estimate and credible interval set to central) and number of selfed generations of each individual across the chains. The Potential Scale Reduction Factors for the estimated selfing rates in the adult and metacercarial data sets were 1.001 and 0.9998, so in each data set, the different chains had similar posterior distributions. Results from the chain with the highest posterior median log-likelihood are reported.

RESULTS

Clonemate identification and clonal genetic structure analyses

In the adult data set, 55 unique MLGs of 63 genotyped individuals were identified. However, 1 pair of MLGs differed only by 1 allele at 1 locus. After removing the discrepant locus (see method justification in Criscione et al., 2020), this pair had $P_{\mathrm{sex}} < 0.0001$ at $\mathrm{n} = 2$. Thus, the individuals in this pair were considered to be clonemates. In the remaining cases where there was more than 1 copy of an MLG, the $P_{\mathrm{sex}} < 0.0001$ at $\mathrm{n} = 2$. In total, there were 54 clones out of 63 genotyped individuals. Seven clones had clonemates with group sizes of 3, 3, 2, 2, 2, 2, and 2. All clonemates co-occurred within hosts and never between. Figure 2C shows the CAD among final hosts.

Clonemate identification results in the metacercarial data set are given in Criscione et al. (2020). In short, there were 54 clones out of 272 genotyped individuals. Clonemates occurred only

Table I. Clonemate dyad metrics $\overline{P_C}$, average percentage of clonemate dyads within infrapopulations, and P_{EC}, percentage of clonemate dyads over the entire component population, for each data set along with the *RSE*, random sampling equivalent, ratios.

Clonemate dyad metrics	Meta-SC*	Meta-TC*	Meta-combined	Adult
$\overline{P_C}$	44.09	56.38	50.24	3.68
P_{EC}	7.95	8.07	3.99	0.56
RSE (meta-P_{EC}/adult-$\overline{P_C}$)	2.16	2.19	1.08	

* SC, Staff Camp; TC, Trans-Canada Trail.

within sampled locations SC and TC and not between. Within TC, no clonemates were found between ants. Within SC, 1 pair of ants shared 2 sets of clonemates, and another pair shared 3 sets. Overall, 32 of the 54 clones had clonemates. Figures 2A and 2B show the CADs among ants. There were no clonemates between the adult and metacercarial data sets; thus, there were 108 clones in total.

Using the complete adult data set across final hosts (n = 63), average within-host F_{IS} was negative but not significant (–0.022; *P*-value = 0.807). Among-host genetic structure was significant (F_{ST} = 0.016; *P*-value = 0.0022). When the adult data set was reduced to 1 representative of each clone within each host (n = 54), the average within-host F_{IS} increased but remained non-significant (–0.002; *P*-value = 0.568). Among-host structure was reduced and became non-significant (F_{ST} = –0.002; *P*-value = 0.453). These results provided evidence of a clonal genetic structure at the adult stage of *D. dendriticum*. Also, because there was no genetic structure among final hosts once clonemates were reduced to 1 copy, there was no evidence that sibling parasites were cotransmitted beyond what was expected by chance alone.

There was evidence of a clonal genetic structure at the metacercarial stage as well. For ease of comparison, we re-report the clonal structure results on the metacercariae from Criscione et al. (2020). In the complete metacercarial data set (combining parasites across both sites, n = 272), average within-host F_{IS} was significantly negative (–0.407; *P*-value < 0.001), and there was significant among-host genetic structure (F_{ST} = 0.275; *P*-value < 0.001). In the reduced metacercarial data set where there was 1 representative of each clone within each host (n = 59), the average within-host F_{IS} increased and became non-significant (0.031; *P*-value = 0.15). Among-host structure was reduced and was no longer significant (F_{ST} = 0.015; *P*-value = 0.1).

Using only 1 representative of each clone in both the adult and metacercarial data sets (n = 54 in both), there was no significant genetic structure between the 2 data sets (F_{ST} = 0.002; *P*-value = 0.355). After combining the 2 data sets, all 10 pairs of loci were non-significant in the genotypic disequilibrium tests (all *P*-values > 0.09). Hence, we concluded the metacercarial and adult samples belonged to the same underlying genetic population of *D. dendriticum* in Cypress Hills Park.

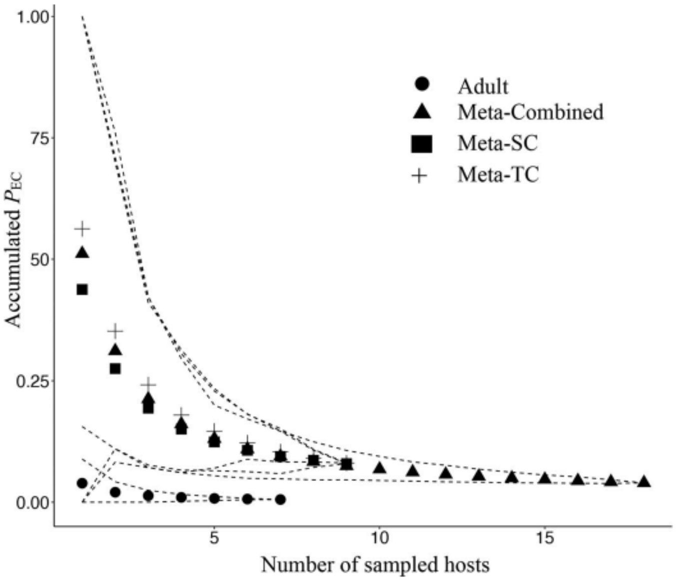

Figure 3. Accumulation curves for P_{EC} as hosts are added to the total sample. Dashed lines are the permutation-based confidence intervals (95%) for the adult and combined metacercarial data sets. Confidence intervals for the TC and SC data sets overlap extensively and fall within the combined metacercarial confidence intervals. There is no overlap in confidence intervals between the adult data and any of the metacercarial data sets.

Clonemate transmission analyses

In agreement with our prior study (Criscione et al., 2020), the Scofield et al. (2012) approach showed a strong signature of clonemate cotransmission at the metacercarial stage. Whether each site is considered separately or if the metacercarial samples are combined, $\bar{\delta}$ was always significantly greater than 0 (in SC: meta-$\bar{\delta}$ = 0.95; in TC: meta-$\bar{\delta}$ = 1; metacercarial combined data set: meta-$\bar{\delta}$= 0.99; *P*-values = 0.0001). Indeed, meta-$\overline{P_C}$ in ant second intermediate hosts was considerably higher (44% in SC, 56% in TC, or 50% when combined; Table I) than meta-P_{EC} (8% in both SC and TC, or 4% when combined; Table I). There was also significant heterogeneity in meta-P_C among ant infrapopulations (*P*-values = 0.0001 for tests within SC, TC, or when combined). This heterogeneity can be seen in Figures 2A and 2B where some ants are composed of a single clone whereas others have a mixture of clones. We also highlight that there was no difference in meta-P_{EC} between the 2 metacercarial sample sites, TC and SC ('gammaContrastTest' function, *P*-value = 0.93), both of which had a value of 8% (Table I). This result is also confirmed in the P_{EC} accumulation curve, which shows an extensive overlap between these 2 samples as hosts are added (Fig. 3). The meta-$\overline{P_C}$ was marginally significant between TC and SC ('alphaContrast-Test' function, *P*-value = 0.027), but we note the test value fell within the 95% confidence interval of simulated test statistics, i.e., the *P*-value is not significant as a 2-tailed analysis. In general, the CADs at the component population level in ants are similar between the TC and SC collection sites. Both sites also have a meta-$\overline{P_C}$ much greater than meta-P_{EC}, but the meta-$\overline{P_C}$ may be slightly higher in TC than SC (Table I).

In the adult data set, adult-$\bar{\delta}$ = 1 (*P*-value = 0.0001) where adult-$\overline{P_C}$ (3.7%) > adult-P_{EC} (0.6%) (Table I). Thus, the adult data also showed significant evidence of clonemate cotransmission. However, there was no significant heterogeneity (*P*-value = 0.69) in adult-P_C among ungulate final hosts.

Comparing across developmental stages at the component population level, the P_{EC} accumulation curves (Fig. 3) showed that at a sample size of 7 hosts none of the estimates for meta-P_{EC}

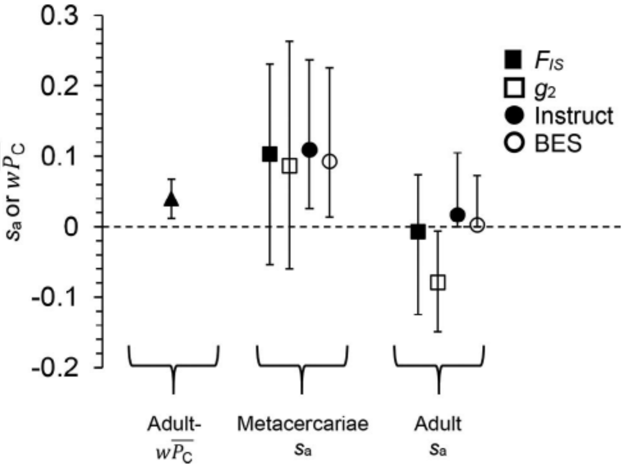

Figure 4. Estimate of the weighted average of the proportion of clonemate dyads among adult infrapopulations, adult-$w\overline{P_C}$ (triangle), and estimates of apparent selfing rates, s_a, at the metcercarial and adult stages (indicated by the 4 genetic-based estimators in key, squares, and circles). Bars are 95% confidence (F_{IS} and g_2) or credible (Bayesian estimators) intervals (see main text).

(9.5% in SC, 10.4% in TC, and 9.4% in the combined metacercarial data) had confidence intervals that overlapped the estimate of adult-P_{EC} (0.6%). Hence, adult-P_{EC} is significantly lower. Comparing across developmental stages at the infrapopulation level, adult-$\overline{P_C}$ (3.7%) was significantly less than the estimates of meta-$\overline{P_C}$ (44% in SC, 56% in TC, or 50% in a combined metacercarial data set; 'alphaContrastTest' function, P-values = 0.0001; Table I). Thus, in comparing the metacercarial data to the adult data, there were significant reductions in the proportion of clonemates that were cotransmitted at both the component population level and within infrapopulations as transmission progressed across the developmental stages from ant second hosts to ungulate final hosts.

The *RSE* (meta-P_{EC}/adult-$\overline{P_C}$ ratio) in relation to either the TC or SC metacercarial sample was ≈ 2 (Table I). In other words, it was as if each final host was taking a random sample from a metacercarial component population that was 2 times the observed metacercarial CAD. As an example of empirical justification, if the CAD of SC is doubled (a new set of clones with the same CAD is added), the value of meta-$P_{EC} = 3.96\%$, which is approximately the value of the adult-$\overline{P_C}$, 3.7%. Indeed, this effect is observed in combining the 2 metacercarial data sets into 1. Because the meta-P_{EC} values were not statistically different between TC and SC and because the 2 sites did not share any clones (see above), combining the 2 was as if the metacercarial component population CAD was doubled. Hence, in comparison to the combined metacercarial data set, the *RSE* (meta-P_{EC}/adult-$\overline{P_C}$) ≈ 1 (Table I).

Potential clonemate mating rate and mating system estimates

The potential clonemate mating rate, adult-$w\overline{P_C}$, was 3.98% (95% CI: 1.2%, 6.73%; based on 10,000 bootstraps over infrapopulations). The genetic estimates of selfing rates are given in Figure 4. Recognize these estimates reflect apparent selfing rates, s_a, because the combination of clonemate mating and selfing could have generated them. At the adult stage, there was no signature of inbreeding based on excess homozygosity (F_{IS} = –0.004; 95% CI: –0.062, 0.037; 1-tailed P-value = 0.581) or identity disequilibrium (g_2 = –0.02; 95%CI: –0.037, –0.001; 1-tailed P-value = 0.974), and, hence, s_a estimates at the adult stage do not differ from 0 (Fig. 4). Congruent with this result, the Bayesian analyses had very low estimates for s_a with 95% credible intervals containing 0 (Fig. 4); all individuals were identified as being the product of outcrossing events.

At the metacercarial stage, however, there were signatures of inbreeding where $F_{IS} = 0.052$ was marginally significant (1-tailed P-value = 0.045; 95% CI: –0.027, 0.115) and $g_2 = 0.022$ was not significant (1-tailed P-value = 0.121; 95% CI: –0.015, 0.064), but in the same direction suggesting possible inbreeding. A combined probability test, Z-method (Whitlock, 2005), of these independent estimators is significant (2-tailed P-value = 0.043). These values of inbreeding translate into single-generation s_a values of 0.103 and 0.087, respectively. Both of the Bayesian model-based methods estimated $s_a \approx 10\%$ where the 95% credible intervals did not contain 0 (Fig. 4). In addition, both INSTRUCT and BES identified 3 individuals (the same individuals) with a median estimate of being the product of a single generation of apparent selfing. All other individuals were estimated to be the product of outcrossing events. Hence, a conservative estimate of the s_a would be 5.6% (3/54) in the metacercarial data set.

DISCUSSION

Dispersal has long been recognized as a critical trait in shaping patterns of genetic diversity, especially how limited dispersal of kin groups could influence identity by descent within populations (Wright, 1946). Recent methods to estimate the relatedness of individuals in populations along with classical ecological or population genetic metrics have enabled finer resolution of patterns of sibling offspring dispersal and factors that influence such dispersal (e.g., Scofield et al., 2012; Jasper et al., 2019). Here we adopted the statistical framework of Scofield et al. (2012) to show how P_C can be used to describe the dispersal of clonemates across life stages, i.e., to describe clonemate transmission itself. Moreover we show an additional application of P_C in that it quantifies a potential evolutionary outcome leading to inbreeding, i.e., clonemate mating.

Clonemate transmission

At both the metacercarial and adult stages, *D. dendriticum* shows a significant signature of clonemates cotransmitted into hosts more often than expected by chance; i.e., $\overline{P_C} > P_{EC}$ at the respective stages. As discussed by Criscione et al. (2020), the low dispersal of terrestrial snails coupled with the fact that cercariae cannot disperse from the exuded slime balls likely promotes a high potential for cotransmission of clonemates into ants that feed on the slime balls. Here we elucidate that subsequently there continues to be clumped transmission of clonemates into final hosts. The latter finding makes sense given that final hosts consume whole ants, which within individuals have a high frequency of clonemate dyads. However, the transmission phase from the second to the final host of *D. dendriticum* results in a dramatic reduction in the frequency of co-occurring clonemates (meta-$\overline{P_C} = 50\%$ >> adult-$\overline{P_C} = 3.7\%$; Table 1). It might seem counterintuitive that there would be a large reduction in adult-$\overline{P_C}$ compared to meta-$\overline{P_C}$. But, as we discuss below, the consumption of multiple ants, each predominantly infected with different clones, in itself will reduce the frequency of co-occurring clonemates within hosts.

The statistical tests of Scofield et al. (2012) also enable a test of heterogeneity in P_C among infrapopulations. At the metacercarial stage, there is heterogeneity in P_C among ants where some ants have clonemates originating from a single miracidium whereas others have a mix of clones (Fig. 2A, 2B). A study of the CAD in snails is needed to ascertain if such heterogeneity is already present at the first host or if it is induced during transmission to the second ant hosts. For example, if snails are predominantly infected with a single miracidium, then ants are somehow being differentially exposed to varying numbers of snail 'slime balls.' At the adult stage, we did not find significant heterogeneity in P_C among final hosts. We did have a small sample size per final host, but the lack of heterogeneity could be because ungulate hosts are sampling numerous ants (discussed next), which may enable a more consistent pattern among hosts.

From a heuristic standpoint, the *RSE* (meta-P_{EC}/adult-$\overline{P_C}$ ratio) sheds light on the transmission process. When combining the 2 metacercarial samples, *RSE* was approximately 1 (Table I). Thus, it is as if each ungulate final host ingests a random sample (without replacement) of clones and their clonemates from the combined (TC and SC) metacercarial component population CAD. Because the combined meta-P_{EC} was obtained from 18 infected ants, another way to think about this is that a final ungulate host ate all 18 ants and there was random survival of clones and clonemates within that final host. The latter interpretation highlights that the percentage of clonemate dyads within infrapopulations can be reduced from 1 life cycle stage to the next (meta-$\overline{P_C}$> adult-$\overline{P_C}$) simply by the collective sampling of multiple prior-stage hosts that harbor different clones. It is important to note that *RSE* reflects 'effective' sampling equivalents and is not necessarily exactly what a final host consumed. The ratios are meant to give perspective on what it would take to achieve similar values across the stages. Additionally, inference of *RSE* is restricted to the sample at hand. The reason is that if hosts with different clones are added to the overall sample, P_{EC} and hence *RSE* will continue to drop.

Mating system

The theoretical work of Prugnolle et al. (2005) showed how a high variance in clonal reproductive success impacted the foundational population genetic statistics within-host F_{IS} and among-host F_{ST}. P_C provides an extension of this work to quantify a potential consequence of clonemate cotransmission, i.e., clonemate mating. While there is a dramatic reduction in the percentage of clonemate dyads within final ungulate hosts relative to ant second hosts (adult-$\overline{P_C}$ << meta-$\overline{P_C}$; Table 1), there remain co-occurring clonemates within final host infrapopulations greater than expected by chance (adult-$\overline{P_C}$ > adult-P_{EC}; Table I). Hence, there is the potential for clonemate mating (adult-$w\overline{P_C} = 3.98\%$, 95% CI 1.2–6.73%) to generate inbreeding that is the equivalent of that generated by self-mating.

In the adult data set, none of the individuals were identified as the product of a selfing event nor was there any overall evidence of inbreeding. In contrast, there were marginal signatures of inbreeding that translate to $s_a \approx 9$–10% in the metacercarial data set (Fig. 4). Indeed, the Bayesian methods identified 3 individuals ($s_a = 5.6\%$ as a conservative estimate) that were the product of apparent selfing. The genetic estimates of s_a and the ecological estimate of adult-$w\overline{P_C}$ overlapped in 84% confidence intervals (File S3), which approximates a lack of significance at $\alpha = 0.05$ (MacGregor-Fors and Payton, 2013). Hence, the mating system inference is that the potential clonemate mating rate accounts for all the apparent selfing (i.e., there is no true selfing). The latter assumes no inbreeding depression from offspring development to the metacercarial stage. If there was inbreeding depression, the effect would be to reduce s_a in the larval/juvenile stage relative to the actual mating rates where there would be an equal percent reduction in both t_C and s. Hence, $s_a - w\overline{P_C} < 0$ could mean there was inbreeding depression. If $s_a - w\overline{P_C} > 0$, one could still interpret some role for true selfing, though s would be underestimated. Likewise, because s will be underestimated, if $s_a - w\overline{P_C} = 0$, one might falsely conclude no true selfing. We do not have progeny-array estimates of selfing rates, so we cannot rule out the latter. However, natural infection intensities of ungulates in Cypress Hills Park are very high (mean intensities typically range in the hundreds; Goater and Colwell, 2007), which, in turn, would enable ample outcrossing opportunities. So, in general, we hypothesize there is little to no selfing among adults.

Comparisons of selfing rates across life stages can also be used to infer inbreeding depression (Ritland, 1990; Detwiler and Criscione, 2017). In the metacercarial stage, 3 of 54 individuals were identified as products of apparent selfing. At the adult stage, all individuals out of the 54 were identified as being outcrossed. However, sampling error cannot be ruled as a Fisher's exact test is not significant (P-value = 0.24, 2-tailed). Six metacercariae would have needed to be identified as the product of apparent selfing events to achieve significance.

Broader relevance

Although the vast majority of parasitic flatworms are hermaphroditic, we still have little knowledge on actual mating systems, i.e., estimates of selfing and kin-mating rates, and much less on the causes (e.g., transmission process) and consequences (e.g., inbreeding depression) of their mating systems (see discussions in Detwiler et al., 2017; Detwiler and Criscione, 2017; Caballero and Criscione, 2019). The fascinating peculiarities of parasite life histories, life cycle patterns, and reproductive modes provide comparative opportunities to understand how their ecology might shape their mating systems and, hence, the role of inbreeding in their evolution. For example, some trematode species within the families Leucochloridiidae or Microphallidae have truncated life cycles where metacercariae remain in their mollusc first intermediate host (Schell, 1985). As bird final hosts consume these infected first intermediate hosts, there is little chance for clonemates to disperse among different birds. Thus, while few clonemates of *D. dendriticum* co-occur in final hosts, these other trematodes have life cycles that may be more conducive for cotransmission of clonemates into their final hosts and possible subsequent inbreeding.

Nevertheless, it is some of the same parasite characteristics that have made parasites inherently difficult to study with regard to their mating systems. Here we have shown how a single metric, P_C, enables simultaneous inference on the transmission process and mating dynamics from field-collected samples. Moreover the eco-evolutionary links presented here are not restricted to clonemate transmission and mating. Indeed, the P_S (originally labeled P_K) metric of Detwiler and Criscione (2017) is the same mathematically and can be used under the same framework to test for sibling cotransmission and provide potential kin-mating rates. Thus, the framework presented herein will facilitate future field-based studies on the mating systems of parasitic flatworms.

It is also pertinent to highlight that the clonal dynamics of digeneans have potential parallels among cnidarians. For example, the life cycle of the jellyfish *Aurelia aurita* has an obligate sexual reproductive stage (hermaphroditic adult medusa) and an obligate asexual stage (polyps that produce ephyra larva) (Müller and Leitz, 2002). There are 2 differences to that of trematodes that could impact downstream levels of inbreeding: medusa do not exist in closed mating systems and fertilization is external (broadcast spawning) for the jellyfish. Nonetheless, asexual reproduction enables the possibility of clonemate mating. Other cnidarians (e.g., various corals) may not have an asexual larval stage, but the adult polyps can bud from parent polyps, again setting the stage for clonemate mating. In the brooding coral *Seriatopora hystrix*, Sherman (2008) found variation in (apparent) selfing rates (0.47: range 2–99%) among six progeny-array families and acknowledged that 'levels of clonality within a population (and hence the opportunity for matings between ramets [clonemates] belonging to the same genet [clone])' could be 1 cause for this variation. Thus, the spatial arrangement of clonemate corals will be of relevance. For instance, in a cryptic species of *Seriatopora hystrix* (ShA), Warner et al. (2016) mapped and genotyped colonies across 256 m^2 area (see shaded area in their supplemental figure S2) and found 8 pairs of colony clonemates out of 126 genotyped. Thus, in this sampled area $P_{EC} = 0.001$. The open habitat and continuous distribution precludes an estimate of P_C (see Vekemans and Hardy, 2004 or Jasper et al., 2022 for potential methods in continuous habitats), but there was qualitative evidence that clonemate dyads occurred closer to 1 another than expected by chance alone (clonemate pairs were on average only 0.32 m apart with a max of 0.56 m). Overall there was no evidence of inbreeding at the adult stage ($F_{IS} = -0.023$). However, progeny array data across 13 families showed 9 families with all outcrossed offspring, but 5 families had between 2% and 23% (apparent) selfed offspring (Warner et al., 2016). It would be of interest to see if such rates correlate to spatial proximity to clonemates to assess the potential role of clonemate mating in coral inbreeding.

Broadly, studies of diverse taxa are bringing to light the ecological and life history traits that impact inbreeding (or lack thereof) in natural populations. The role of selfing in hermaphroditic plant mating systems is well studied (Goodwillie et al., 2005), and the relationship between offspring dispersal and mating systems has a history in plant literature as well (e.g., low seed dispersal can lead to biparental inbreeding; Vekemans and Hardy, 2004). A recent meta-analysis of marine invertebrates shows that 'inbreeding is at least as prevalent in sessile and sedentary marine organisms as in terrestrial seed plants' (Olsen et al., 2020). In addition, Olsen et al. (2020) found the life history and ecological traits of marine invertebrates are associated with levels of inbreeding. For example, hermaphroditic species show higher values of F_{IS} relative to dioecious species, and relationships between development and sperm transfer traits to F_{IS} values indicated a role for dispersal in shaping mating systems. Although the data are still relatively limited, similar eco-evolutionary dynamics with regard to parasite dispersal (i.e., transmission) and inbreeding are emerging among studies on parasitic flatworms (see discussions in Detwiler et al., 2017; Detwiler and Criscione, 2017; Caballero and Criscione, 2019). Our current study of a fluke with a complete terrestrial life cycle adds to this the growing literature on parasitic flatworms mating systems, especially bringing to light the interplay between clonemate cotransmission and clonemate mating.

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