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Short communication

Characterization of nine microsatellite loci for Dicrocoelium dendriticum, an emerging liver fluke of ungulates in North America, and their use to detect clonemates and random mating

Bradley J. van Paridon [a,*], Cameron P. Goater [a], John S. Gilleard [b], Charles D. Criscione [c]

[a] Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, T1 K 3M4, Canada
[b] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada
[c] Department of Biology, Texas A&M University, College Station, TX 77843, USA

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ABSTRACT

This study characterizes polymorphic microsatellite loci from adults of the liver fluke *Dicrocoelium dendriticum* sampled from a population of sympatric beef cattle and wapiti in a region of emergence in southern Alberta, Canada. We also scrutinized the markers to validate their use in studying the population genetics of this complex life cycle parasite. Among the nine loci described, four deviated significantly from Hardy Weinberg Equilibrium (HWE) due to technical artefacts. The remaining five loci were in HWE. These five provided sufficient resolution to identify clonemates produced from the obligate asexual reproduction phase of the life cycle in snails and to assess the impact of non-random transmission of clonemates on measures of F_{IS}, F_{ST} and genotypic disequilibrium. Excluding clonemates, we show that the sub-population of worms was in HWE, that average F_{IS} within hosts was 0.003 ($p = 0.4922$) and that there was no population genetic structure among hosts $F_{ST} = 0.001$ ($p = 0.3243$). These markers will be useful for studies of *Dicrocoelium dendriticum* ecology, transmission, and evolution.

Parasites are a diverse group of organisms with well-known negative impacts on the health of human, animal, and plant populations. They also present many challenges to researchers as questions concerning diagnosis, transmission, host specificity, host-parasite interactions, and the identification of cryptic species are often difficult to answer via egg counts and morphological analyses alone [1,2]. Since their discovery, polymorphic genetic markers have been increasingly used to indirectly and in some cases, directly, infer these aspects of parasite ecology and evolution [1]. In particular, due to their co-dominance and tendency for high polymorphism, microsatellite markers have a wide variety of applications in population genetic studies including investigating the genetic diversity and differentiation of populations and distinguishing individuals via multilocus genotypes (MLGs) [3]. This utility is especially pertinent among trematode parasites where asexual reproduction in the first intermediate host and subsequent clonal transmission can influence the underlying pattern of parasite genetic structure among successive hosts in the life cycle [4,5]. Identification of clonemates (individuals that are the product of an asexual reproductive event of a progenitor individual) is necessary to make correct inference on trematode transmission via population genetics [4]. Microsatellites can be used to identify individuals with identical MLGs and subsequent statistical tests can be employed to test if these individuals are indeed clonemates [6,7]. To date, studies examining fluke transmission via genetic markers have been conducted on aquatic or semi-aquatic systems [5]. Our understanding of trematode transmission (as inferred through population genetics) in a completely terrestrial environment is nonexistent, in part, because microsatellites have yet to be developed for a trematode with a completely terrestrial life cycle.

Here we present the development of microsatellite markers for the lancet liver fluke *Dicrocoelium dendriticum* (Trematoda: Dicrocoeliidae). This parasite is found throughout Europe, North Africa, Asia and North America [8]. *Dicrocoelium dendriticum* has a three host life cycle that is completely terrestrial [9]. Snails (where there is asexual reproduction) are first intermediate hosts, ants are second intermediate hosts, and several mammals can serve as the definitive host (where there is obligate sexual reproduction) [8]. The lancet liver fluke is famous for its ability to alter the behavior of ants. A single metacercaria encysted in the ant ganglia causes the ant to cling to vegetation, thus making them available for accidental

* Corresponding author.
E-mail addresses: bradley.vanparidon@uleth.ca (B.J. van Paridon), ccriscione@tamu.edu (C.D. Criscione).

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ingestion by grazing mammals [8]. Once metacercariae are ingested by definitive hosts, juveniles emerge and migrate to the bile ducts where they develop to into adults. Infection in a range of ungulate definitive hosts is associated with anemia, edema emaciation, cirrhosis of the liver and in high-intensity infections, photosensitization [10,11]. *Dicrocoelium dendriticum* is an invasive species in North America, first described on the eastern coast of the continent in the 1950s [12]. Since then, sporadic reports have found it as far as the western coast of North America [13,14]. In 1990, it was first reported in Cypress Hills Provincial Park, Alberta, Canada where it can now be found in sympatric elk, cattle, and deer [15].

All samples used in this study came from ongoing surveys involving sympatric elk and cattle in the Cypress Hills Interprovincial Park, Alberta, Canada (49° 30′N 110° W). Adult worms were recovered via dissections of purchased cattle livers and hunter shot elk following Goater and Colwell [15]. An initial library enriched for microsatellites was constructed using Illumina MiSeq next generation sequencing to identify di-, tri-, tetra- and pentamer repeats throughout the genome of *D.dendriticum*. Library construction and sequencing was conducted by S. Bogdanowicz at the Evolutionary Genetics Core Facility at Cornell University, USA using the methods described in Nali et al. [16]. Potential microsatallite loci with sizes of 150–450 bp were selected for primer design. Testing and optimization of microsatellite loci was performed on a dataset of 66 adult worms collected from 4 elk livers and 3 cattle livers from Cypress Hills Provincial Park. Worms were lysed in lysis buffer and Protinease K (10 mg/ml, New England Biolabs). Lysis buffer contained 50 mM KCL, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.045% Nonidet p-40, 0.45% Tween −20, 0.01% gelatin and dH_2O in 50 ml volumes. Samples were lysed in 50 μl for 98 min at 60 °C followed by 15 min at 94 °C then stored at −20 °C. PCR reactions were carried out in reaction mixtures of 25 μl containing final concentrations of 1X Thermopol reaction buffer (New England Biolabs), 2 mM $MgSO_4$, 100 μM dNTPs, 0.1 μM 6-FAM labelled forward primer and reverse primers, 1.25U Taq DNA polymerase at 5000U/ml (New England Biolabs). Thermo cycling condition were 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min with a final extension of 72 °C for 10 min. Products were then analysed on an Applied Biosystems 3500xl sequencer and sized using Genescan 500 (-250) LIZ size standard (Applied Biosystems) and scored manually using GeneMapper 4.1 (Applied Biosystems).

Gene diversity of each locus (H_s) as well as estimates and tests of F_{IS} and F_{ST} were conducted using FSTAT (Goudet, 1995). Significance of average within-host F_{IS} per locus and F_{ST} among hosts were determined using 10,000 randomizations of alleles among individual worms within hosts and of genotypes among hosts, respectively. Genotypic equilibrium (GD) between pairs of loci was tested in GENEPOP 4.2 (Markov chain parameters: 1000 dememorizations; 100 batches; 1000 iterations) (Rousset, 2008). We also tested for identity disequilibrium (ID), i.e., correlations in heterozygosity among different loci that result from non-random mating such as partial selfing [17]. ID is not affected by scoring artefacts such as null alleles. Thus, comparison of ID with F_{IS} is a useful means of determining if single locus estimates of F_{IS} are artificially elevated due to technical artefacts or are high due to real biological phenomena such as self-mating [17]. ID was tested with 1000 iterations of resampling the single locus genotypes among individuals in the population using RMES [17]. GENCLONE 2.0 was used to identify individuals with the same MLG and test whether or not identical MLGs arose via asexual reproduction [7]. Clonemate testing is determined by calculating P_{sex} which is the probability of observing n copies of a MLG in a sample size N given sexual reproduction (significance determined with $P_{sex} < 0.05$). If the P_{sex} of a multicopy MLG at $n = 2$ is significant, it can be taken that all copies of this MLG are the result of asexual propagation [6].

Our initial library construction yielded 4517 potential microsatellite loci. Of these, 101 loci were tested on a random subset of the dataset ($n = 10$) using unlabeled primers and visualized on 2% agarose gel to confirm amplification and presence of only a single product. Of these, nine loci were found to be polymorphic, reliably amplified in all individuals, and provide unambiguous genotyping profiles (Supplementary Data).

The initial data analysis tested the average within-host F_{IS} per locus using all 9 loci and all 66 individuals. A wide range of F_{IS} values was observed (−0.077–0.526) with 4 loci testing significantly greater than 0 ($p < 0.05$) and 5 loci not significant. Such a large variation in F_{IS} values among loci is atypical for a given mating system and therefore, could be caused by a number of factors such as clonal structure, null alleles, mating system, or cryptic structure [18]. To determine the potential factor(s) that may be causing this variation, we used a multistep approach described below.

First, individuals with missing data were removed resulting in a data set of $n = 58$ individuals. Removal was necessary as P_{sex} cannot be calculated on individuals with missing data. Among these 58 individuals there were 52 unique MLGs where 4 MLGs had more than copy. At this point we note that testing for the significance of clonemates (i.e., calculating P_{sex}) relies on the estimation of allele frequencies from a given population. If there is population structuring, then within subpopulation allele frequencies need to be used to test for clonemates by subpopulation, otherwise P_{sex} will be biased towards significance. However, clonemates themselves could drive among-host structure [4]. Thus, as noted by Criscione et al. [18], the presence of clonemates creates a 'catch-22' as how does one test for clones if there is structure among hosts (not due to clonal transmission) while at the same time addressing if there is structure among hosts that is due to the presence of clonemates? We followed a similar approach used by Criscione et al. [18] by determining the influence of having repeated MLGs in the data set compared to a data set where repeated MLGs were reduced to single copy within individual hosts. Thus, a second dataset was made where repeated MLGs were reduced to one copy within each host ($n = 52$). Genetic structuring among hosts was then tested on both datasets. In the complete dataset ($n = 58$), significant structure among hosts was found (multilocus $F_{ST} = 0.02$ ($p = 0.0001$)), whereas when MLGs were reduced to only unique copies within hosts ($n = 52$), there was no significant among-host structure (multilocus $F_{ST} = 0.003$ ($p = 0.8060$)). This pattern indicates that the presence of clonemates is likely driving the significance of F_{ST}. Therefore, one can conclude that all worms are from the same population, as transmission prior to asexual reproduction is random [4,5].

Next, using the same two data sets above, we ignored the among-host division of worms and analysed the datasets as a single population to test F_{IS} across loci. Whether repeated copies of MLGs were included or not, there remained extensive variation in F_{IS} among loci (−0.032 to 0.514 when $n = 58$, and −0.002 to 0.491 when $n = 52$; 4 loci still tested significantly greater than 0 in both data sets). Table 1 shows the F_{IS} estimates from the reduced data set. In contrast, GD was significant in 15 of 36 pairwise comparisons in the complete data set ($n = 58$), whereas this fell to 1 of 36 (a number expected by chance alone with $\alpha = 0.05$) with repeated copies of MLGs reduced ($n = 52$). The lack of linkage disequilibrium (LD) in the reduced data sets suggests the presence of clonemates is driving the excess LD in the complete data set [19]. Moreover, the lack of LD in the reduced data set suggests that there is no underlying cryptic population structure as population admixture would cause LD.

To determine if technical issues were the cause of variation in F_{IS} values, we tested whether ID was significant in the reduced data set ($n = 52$). $\hat{g}_2$, the estimator of the two-locus heterozygosity disequilibrium [17], was not significantly greater than 0 ($p = 0.774$). Thus, there was no evidence of non-random mating such as might

Table 1
Characteristics of nine polymorphic loci isolated from *D. dendriticum* based on dataset with clones reduced to one copy ($n = 52$) T_a: annealing temperature; Na: number of alleles; H_s: gene diversity; F_{IS}: inbreeding coefficient (values in bold are significantly greater than zero).

Locus	GenBank	Repeat Motif	Primer 5′-3′	T_a(°C)	Na	H_s	Size Range (bp)	F_{IS}
DdMs21	KU094045	AAC	F:AACCTTGCCGTCTTCATTGATAC R:CGCTTTGCTCTGATTTCAAATGG	54	11	0.779	311–366	0.086
DdMs28	KU094044	ATCT	F:GATCACTCAGAGCGCTTTAAGTC R:CAACAATGCACTTTCGTCACTTC	54	9	0.811	344–366	−0.068
DdMs43	KU094043	AAC	F:TTGACTAGAGCCACCACAATTAG R:CCCACCCGTCCTTGTTAATAATC	54	7	0.621	463–481	**0.442**
DdMs44	KU094042	AAC	F:TGGAAGCCACGATTAAACTTAGCR:TATGGTGCCAGAATTGAAACGAG	54	20	0.919	248–346	**0.414**
DdMs60	KU094041	AAC	F:TGTGGAAACTTATCAAACACTGC R:AACTACTGACCGATTTGCTTGTC	54	11	0.805	243–288	−0.027
DdMs70	KU094040	AC	F:TAAAGCAACACCATGAGTAGCACR:TAGCAACGTAACGAGAACCAATG	54	16	0.895	145–196	0.055
DdMs89	KU094039	AC	F:TTTCTGAACTCCGAACAACAGG R:TGCCATAGTGCCAACAGAATATC	54	9	0.793	169–185	**0.491**
DdMs93	KU094038	AC	F:CAAGTAAGGCGTTTCAGTTCCTC R:GCTCCAGTATCAAATTCGTGAC	54	13	0.686	180–237	**0.244**
DdMs95	KU094037	AG	F:CATCGCGAAGTAACTTGATTAGC R:AACTACACTGTTCGTACACTGGG	54	9	0.653	285–301	−0.002

occur with self-mating. Because ID is not biased by technical errors such as null amplification and because there was no evidence of cryptic structure (i.e., no LD), we were left with the conclusion that the significant F_{IS} values for the 4 loci DdMs43, DdMs44, DdMs89 and DdMs93 (Table 1) were caused by technical artefacts.

Now that we identified the variation in F_{IS} values was likely due to technical issues, we wanted to re-examine the data to interpret the biology of *D. dendriticum*. Thus, we repeated the above analyses using only the five loci (DdMs21, DdMs28, DdMs60, DdMs70 and DdMs95) that were in Hardy-Weinberg Equilibrium (HWE). Using only these five loci, we had a data set with no missing data of $n = 63$ individuals among which there were 55 unique MLGs where all 6 MLGs present in more than one copy had $P_{sex} < 0.05$. In re-examining structure among hosts, when all clonemates are included ($n = 63$), the average F_{IS} within hosts was 0 ($p = 0.6876$) and there was significant structure among hosts, $F_{ST} = 0.015$ ($p = 0.0025$). When only one individual of a set of clonemates was retained within a host ($n = 55$), average F_{IS} within hosts remained low at 0.003 ($p = 0.4922$), but structure among hosts disappeared, $F_{ST} = 0.001$ ($p = 0.3243$). In all cases where there were clonemates, the clonemates were in the same host individual. Thus, our results confirm the theoretical predictions of Prugnolle et al. [4] that when there is little dispersion of clonemates after asexual reproduction in the first host (i.e., there is clumped clonal transmission), there will be significant genetic structure among subsequent hosts in the life cycle. In looking at the influence of clonemates on LD, we see that 8 of 10 pairwise comparisons had significant GD ($p < 0.05$) when including clonemates ($n = 63$; treating the data set as one population). If only one representative of each clonemate is retained ($n = 55$), then none of the pairwise comparisons were significant. Thus, again, sampling of clonemates will lead to LD.

Given the above results, we conclude that there is random mating among the adult population of flukes and random transmission of flukes prior to infection of the snail first host. After asexual reproduction in the snail host, however, there is some significant clonal transmission such that clonemates end up in the same definitive host more often than expected by chance alone. This pattern of clonal transmission implies that clonemates originating from snails would also be common in metacercariae subpopulations in ants. This prediction remains to be tested. Moreover, although we found that some loci may have technical issues, we ended up with five polymorphic loci that alone had enough power (likely due to their high polymorphism) to statistically detect the presence of clonemates. All nine polymorphic microsatellite loci are reported for *D. dendriticum*. These new markers will be valuable to study the population genetics, life cycle and global spread of this emerging trematode. In particular, these loci could be used in assignment tests to potential identify the source population(s) of the invasion in North America. From an ecological perspective, these markers will enable us to address a long standing question of kin selection regarding the appearance of altruistic self-sacrificing behavior by the single worm that infects ant brains and alters their behavior. Lastly, whenever there is a large amount of variation in F_{IS} among loci, one should be suspect [18,20]. Our study highlights how new markers need to be thoroughly scrutinized as both technical (e.g., null alleles) and biological factors can cause deviations from HWE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2016.05.003.

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