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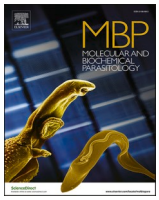

Characterization of 21 microsatellite loci for the precocious, grass-shrimp trematode *Alloglossidium renale*

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ABSTRACT

We developed microsatellite markers to use in studying the population genetics of the trematode *Alloglossidium renale*, a fluke with a precocious life cycle where sexual maturation occurs in a grass shrimp. Among 21 tested loci in a Mississippi population sample, 14 were polymorphic, 12 of which significantly deviated from Hardy-Weinberg Equilibrium (HWE). We estimated identity disequilibrium (ID) to confirm whether the deviations from HWE were due to significant amounts of selfing or due to technical factors. The selfing rate derived from F_{IS} was 86.6%, whereas the selfing rate obtained by ID was 83.9%, indicating that the deviation in HWE was due to a high amount of selfing within the population. These markers will be useful for ecological and evolutionary studies of *A. renale* especially in relation to the interplay of hermaphroditic mating systems, inbreeding depression, and transmission dynamics.

Microsatellite loci have played and continue to play an integral role in molecular ecological and evolutionary parasitology because their co-dominance and high polymorphism enable elucidation of local-scale population genetic patterns [1,2]. In particular, microsatellite markers are suitable for estimating several local-scale population genetic statistics (e.g., deviations from Hardy-Weinberg Equilibrium, relatedness, clonality, etc.), which in turn, can be applied to address the interplay between parasite ecological/life history traits and evolutionary dynamics [1]. For example, the high polymorphism of microsatellites allows differentiation of individuals via multilocus genotypes (MLGs) and testing of whether repeated MLGs represent clonemates (genetically identical individuals). Such clonal data are relevant to understand clonemate transmission in trematodes, which have an asexual developmental phase, as well as the impact of clonal reproduction on digenean mating systems [3,4]. Other applications of microsatellites that have helped advance our understanding of parasite ecological and evolutionary dynamics on a local scale include detecting cryptic structure [5], identifying hybrids [6], and elucidating hermaphroditic mating systems in relation to parasite transmission [7,8].

Here, we developed microsatellite markers for the trematode *Alloglossidium renale*. *Alloglossidium renale* has an obligate 2-host life cycle; the first host is assumed to be a mollusk and the second host is the Mississippi grass shrimp, *Palaemon kadiakensis* [9]. Sexual reproduction for the parasite occurs within one of the two antennal glands of the grass shrimp. A phylogenic study indicates that the ancestral state of the genus *Alloglossidium* was a 3-host life cycle with a catfish as the definitive host [10]. Thus, *A. renale* is considered to reproduce precociously as it develops in what is typically regarded as a second intermediate host. This grass-shrimp fluke has been reported in the Southeastern USA including Alabama, Louisiana, Mississippi, and Texas [9,11,12].

To construct the microsatellite library, three adult flukes collected from Choctaw Road, Louisiana (N 29 51.478 W 90 45.281) were sent to Cornell Life Sciences Core Laboratory Center (Ithaca, NY) to produce a microsatellite library using the methods described in Nail et al. [13]. Library construction and sequencing were conducted by S. Bogdanowicz at the Evolutionary Genetics Core Facility. Microsatellite primers were designed to amplify loci in the size range of 100–400 bp. From a total of 3485 potential microsatellite loci generated for the library, 40 were selected for testing by screening for microsatellite repeats consisting of di-, tri-, and tetranucleotides that repeated between 5 and 15 times. The selected primers were designed to include an 18 bp M13 tag (TGTAAAACGACGGCCAGT) attached to a forward primer on the 5′ end [14] as well as a short tail sequence (GTTTCTT) added to the reverse primer on the 5′ end to reduce polyadenylation [15]. Parasite population samples used to screen the microsatellite loci came from grass shrimp collected by dip net at 3 locations: a standing water body near the Tallahatchie River near Bayou and Whaley Road in Lefore County, Mississippi (N 33.63096280 W 90.10642756) on 24 May 2018; Whisky

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Bay, Louisiana (N 30 23.479 W 91 20.826) on 6 June 2019; and Gus Engeling Wildlife Management Area in Texas (N 31 55.749 W 95 53.279) on 11 April 2015. Henceforth, we refer to these as the Mississippi, Louisiana, or Texas samples.

A total of 46 flukes were screened from the 3 population samples with 38 flukes from Mississippi, 4 from Louisiana, and 4 from Texas. To increase our chances of detecting allelic variation, we only used one fluke per host in case there was co-transmission of clonemates or related individuals (e.g., [4]). DNA was extracted in a 25 μl, 5% chelex solution containing 0.2 mg/mL of Proteinase K that was incubated for 2 h at 56 °C and boiled at 100 °C for 8 min before being stored at − 20 °C. Only the anterior portion of the fluke was extracted to avoid 'allelic contamination' of potential sperm donors. After DNA extractions, we preformed whole genome amplification following the manufacturer's protocol (Illustra Ready-to-Go GenomiPhi V3 DNA Amplification kit) and used 3 μl of template DNA and 7 μl of water. PCR amplifications were performed following the methods of Schuelke et al. [14] and were performed in 15 μl reactions consisting of 2 μl of whole genome amplified template, 9.43 μl water, 1.5 μl 10x buffer, 0.9 μl $MgCl_2$ [25 mM stock], 0.3 μl dNTP [10 mM/each stock], 0.24 μl fluorescent-labeled M13 primer (Applied Biosystems: FAM, VIC, NED, or PET) [10uM stock], and 0.24 μl of the M13-tagged forward primer [5 μm stock], 0.24 μl of the reverse primer [10 μm stock], and 0.15 μl of Taq polymerase [5 U/ μl] (Omega Bio-Tek Inc.). The thermocycler profile was 95 °C for 5 min, 31 cycles of 94 °C for 30 s, 56 °C for 45 s, 65 °C for 45 s, followed by 9 cycles of 94 °C for 30 s, 53 °C for 45 s, 65 °C for 45 s and extension at 65 °C for 10 min. Genotyping was conducted at the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT, USA), using a 3730xl 96-Capillary Genetic Analyzer with a 500 LIZ size standard. Genotypes were scored using GENOTYPER 3.7 (Applied Biosystems).

Typically, when evaluating microsatellite markers, Hardy Weinberg Equilibrium (HWE), i.e., testing whether F_{IS} significantly deviates from 0, is assessed to test for mode of inheritance or technical artifacts such as null alleles (e.g., [16]). With *A. renale*, however, we might expect a high level of inbreeding due to the following. An individual *A. renale* can only reside in one of the two antennal glands, which are discrete organs. Thus, parasites infecting the same host can be subdivided. Hulke et al. [12] reported mean intensities per host from 4 different *A. renale* population samples that ranged from 1.85 to 2.58. With relatively low mean intensities along with further subdivision between the 2 antennal glands, there is a high chance that individuals end up in a single infection per gland (see [12]). As such, individuals in single gland infections are forced to self mate, leading to inbreeding. To assess if high F_{IS} values resulted from inbreeding or from technical artifacts, identity disequilibria (ID), i.e., the correlation of heterozygosity among loci, can be used to estimate inbreeding independent of F_{IS} especially since ID is little impacted by null alleles [17]. Congruence between a selfing rate from an ID estimate and from a multilocus F_{IS} estimate (see below) would support the validity of the microsatellite markers.

The 38 individuals from the Mississippi location were used to test HWE and estimate ID. We used GENETIX version 4.05 to estimate gene diversity (H_S) and Weir and Cockerham's [18] estimate of F_{IS} (https://kimura.univ-montp2.fr/genetix/, last accessed 1/30/2023). To test the significance of F_{IS} per locus and the multilocus estimate of F_{IS}, 10,000 randomizations of alleles among individuals were conducted. ID was estimated and tested by resampling single-locus heterozygosities among individuals within the population using 10,000 iterations in the RMES [17]. Genotypic disequilibrium (a surrogate for linkage disequilibrium) was tested between each pair of loci using GENEPOP 4.7.5 (https://genepop.curtin.edu.au, last accessed 1/30/2023) with the parameters as follows: 1000 dememorizations; 100 batches; 1000 iterations. The 8 individuals sampled from Texas or Louisiana were used to simply assess if there was allelic variation among distant locations.

Of the 40 tested loci, 21 gave clear peaks and were unambiguous in scoring. The other 19 (data not shown) were difficult to score (e.g., bad stuttering), showed evidence of duplication, or had poor or null amplification. Among the 21 'good' loci, 17 showed allelic variation within or among locations. Within the Mississippi sample, 14 were polymorphic. Of the 7 monomorphic loci in Mississippi, 3 had allelic variants across locations, 2 had no variation across locations, and 2 were not tested in the other locations (Table 1). For both Texas and Louisiana, we tested 19 of the 21 'good' loci of which 13 were polymorphic in Texas and 5 were polymorphic in Louisiana. However, as there were only 4 individuals tested in both Texas and Louisiana, we recognize that more of the loci could show variation with a larger sample size in these locations. Multilocus genotype data are provided in the Supplementary Data.

Among the 38 genotyped individuals in the Mississippi population, we found no repeated MLGs that might indicate clonemates. Of the 14 polymorphic loci, the number of alleles ranged from 2 to 4 and gene diversities ranged from 0.026 to 0.666. The F_{IS} estimates ranged from − 0.014–1 (Table 1); however, 2 loci, R25337 and 5926, stood out as their F_{IS} values, − 0.014 and 0, respectively, did not deviate significantly from 0 ($p > 0.05$). The other 12 loci were all significantly greater than 0 ($p < 0.001$) and had $F_{IS} \geq 0.662$. The multilocus F_{IS} was 0.763 and significantly greater than 0 ($p < 0.001$). The bootstrap 95% CI of the multilocus F_{IS} was 0.633–0.867 (10,000 bootstraps over individuals conducted in GENETIX). The selfing rate (s) obtained through ID ($g_2 = 2.022$) was significantly greater than 0 ($p < 0.001$) where the maximum likelihood value was $s = 83.9\%$ (95% CI: 75.6–89.4%), indicating a highly inbred and likely, primarily selfing population.

Assuming that selfing is the only factor contributing to inbreeding and that the selfing rate has been relatively constant across generations (i.e., there is inbreeding equilibrium) then s can be estimated from the equation $s = 2F_{IS}/(1 + F_{IS})$ [19]. Using this relationship, the multilocus F_{IS} of 0.763 translates to an $s = 86.6\%$ (converting the bootstrap 95% CI of the multilocus F_{IS} to s: 76.9–92.9%). Thus, the selfing rate estimates from F_{IS} and ID were highly congruent and indicated that indeed, *A. renale* is an inbred species. Given this congruence, the non-significant and ~0 F_{IS} values from loci R25337 and 5926 appear to be outliers. Both loci had very low gene diversities (0.052 and 0.026) resulting from highly skewed allele frequencies (R25337: allele 1 = 97.4%, allele 2 = 2.6%, 5926: allele 1 = 98.7%, allele2 = 1.3%). The other 12 loci had a H_S range of 0.102–0.666. When there is inbreeding, such skewed allele frequencies (i.e., low H_S) can result in a very high F_{IS} variance for a locus ([20]; see also supplemental material in [19]. Thus, we hypothesize the 'outlier" appearance of loci R25337 and 5926 is a result of their inherent low H_S.

Genotypic disequilibrium was significant in 11 out of the 91 pairwise comparisons. With an $\alpha = 0.05$, we would expect about 5 to be significant. Thus, there is evidence for overall, but low linkage disequilibrium (exact test $p = 0.005$). While inbreeding does not cause linkage disequilibrium, it is common to find LD in inbred hermaphroditic populations due to demographic factors such as populations that were founded by a single individual (see discussion in [8]).

Overall, both the F_{IS} and ID results indicated that the Mississippi population of *A. renale* is highly inbred and that the high F_{IS} values are not due to technical artifacts. Thus, the microsatellite markers we developed will be useful in assessing the mating system, clonal dynamics, and transmission patterns of this interesting precocious trematode. Future studies will assess if inbreeding is common among different populations of *A. renale* and if the mating system is stable across time. In addition, these microsatellite markers will enable investigation how demographic infection patterns (e.g., proportion of individuals in single infections or mean infection intensities) can shape the mating system and thus, inbreeding in populations of *A. renale*.

Author contributions

C.D.C. and J.M.H. designed the research project. J.M.H. generated the genetic data. J.M.H. and C.D.C. performed the analyses and wrote the manuscript.

Table 1
Characteristics of 21 microsatellite loci isolated from the Mississippi collection of *A. renale* ($n = 38$), M13 dye: fluorescent-labeled M13 primer; Na: number of alleles within the Mississippi population; Na-geo: number of additional alleles found in either Texas or Louisiana (i.e., if the alleles from Texas and Louisiana were found in Mississippi, Na-geo = 0) H_S: gene diversity; F_{IS}: inbreeding coefficient (values significantly greater than 0 are in bold).

Locus	GenBank	Repeat Motif	Primer 5′– 3′	M13 dye	Na	Na-geo	H_S	Size (bp)	F_{IS}
ALRE_r17074	OQ333025	ACA	F: AACCTGGTCAGTCAACTAGAATG R: TAGGACCTGCTTGTCTTCTACAC	FAM	2	2	0.489	225	**0.785**
ALRE_8921	OQ333026	AC	F: CTGGCTGTTTGGTTCGGTAAATG R: ATTTGGTTGACAGTGCATGATGG	VIC	2	1	0.102	190	**1**
ALRE_5947	OQ333027	ACA	F: TGGAGTCAACAGGTAAGAGTTGG R: GTGAACTGTTGAAGGTGAGTTGG	PET	2	1	0.325	294	**0.919**
ALRE_6037	OQ333028	AC	F: TCACTCTAGTTCGGTGATCACAC R: ATTACTGCGCTTTCGATGACAAG	FAM	2	1	0.477	164	**0.779**
ALRE_11453	OQ333029	TG	F: TGGCTTCTTGGTTTATTATGGGTG R: AAGATTGTCCTGAGGTCACAGTC	VIC	2	2	0.384	188	**0.794**
ALRE_3134	OQ333030	AC	F: TAAGCCCTAAACATCTGCTCTCG R: TTGGTGGATAATTCCCTGTCTGG	NED	4	1	0.666	320	**0.724**
ALRE_4875	OQ333031	AC	F: GTTTGAGTCATCTGATTGGCCTG R: TTTGAGTAGGATGAGCCAAGTCC	FAM	3	0	0.529	113	**0.751**
ALRE_2190	OQ333032	TG	F: ATCAATCAATGCTTTCTGCCCAC R: AATCAGTCAATCAACCAGTCAGC	NED	2	3	0.469	202	**0.719**
ALRE_2125	OQ333033	TG	F: ATTTAACAGTTGCAAGCGAAACG R: TGTGCCCTAGTCGTAATATGGAC	VIC	3	0	0.518	237	**0.746**
ALRE_2951	OQ333034	AC	F: GGTTTGATCGTTGTTTGACAAGC R: CAGTTGCTAGGCCATCTTTCAAC	NED	3	0	0.468	142	**0.662**
ALRE_r25337	OQ333035	ACT	F: TGCTCTATCGTTCACTCACTCTG R: TGGATAGACGGACCTGAATTGTC	PET	2	4	0.052	126	-0.014
ALRE_1668	OQ333036	ACAA	F: GACACACAAATGACCTTCAGACG R: TTGTGTCACCTTTGGCTGAAATG	VIC	1	0	0	256	NA
ALRE_200	OQ333037	AC	F: ATTGTTCACGCTGGTGTGTTTAC R: TACTGACTGTTGTCTAGTGCGAC	PET	3	3	0.529	137	**0.801**
ALRE_760	OQ333038	AC	F: AAATGCTTGCTTCCCTGTCTTTG R: ATTCGTGATTGGTGGTTGTGATG	NED	1	NA	0	188	NA
ALRE_4802	OQ333039	AC	F: AACGAGTTTGCACCTTGACATTG R: GATCTGTGTTGTTGTAGAACCGC	FAM	1	1	0	125	NA
ALRE_5576	OQ333040	CTA	F: TTTCTGATATCCGGAGCTCATCC R: CTTGTTCTACGTCTGCTTCTTGC	VIC	1	0	0	218	NA
ALRE_5709	OQ333041	AT	F: AAAGTGTGCACATACCGTTGTTG R: CGAACATTAACCGGTGTAATCCC	PET	1	1	0	153	NA
ALRE_5926	OQ333042	AC	F: TTGTCACACTGCTTTCGTGAATC R: ACAAAGGTGTGCGATTTGTCATG	NED	2	2	0.026	181	0
ALRE_3690	OQ333043	AG	F: TGCAAGAGTAAGTGAAACGTTGTC R: ACTTGGGTAGTATCTGGCCTTTG	FAM	1	1	0	185	NA
ALRE_156	OQ333044	TC	F: CGAATGAACACGTGACAGTAGTC R: AGTTCTGGCATATAAGGTGGTGG	PET	2	0	0.193	308	**1**
ALRE_2616	OQ333045	CAA	F: CGTAGACGCAGCAATTCCTATTC R: ATGCATTTCAGCCAGATTAGCAC	VIC	1	NA	0	202	NA

Declaration of Competing Interest

none.

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