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Microgeographic population genetic structure of *Baylisascaris procyonis* (Nematoda: Ascaroidae) in Western Michigan indicates the Grand River is a barrier to gene flow.

Christina A. Sarkissian and Douglas H. Graham
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ABSTRACT

We developed eight polymorphic microsatellites from the parasitic nematode *Baylisascaris procyonis*.

Amplification of these loci in a sample of 74 worms collected from 10 raccoons in Western Michigan

revealed significant population structure. Bayesian clustering indicates two subpopulations, one on either

side of the Grand River which bisects the region sampled. Estimates of F_{ST}, and results from AMOVA

and isolation by distance, further corroborate a scenario whereby the river is acting as a barrier to gene

flow, a rather unusual finding given the high vagility of raccoons and microgeographic scale of the

analysis. We describe one possible mechanism for how this pattern of structure could have become

established.

INTRODUCTION

Baylisascaris procyonis, a.k.a. raccoon roundworm, is a gastrointestinal nematode for which raccoons serve as definitive hosts. They become infected either via the fecal-oral route by ingesting embryonated eggs at latrine sites or by consuming an infected intermediate host. More than 100 animal species, including humans, are known to act as intermediate or 'dead end' hosts, in whose tissues and organs the larval stages of *B. procyonis* migrate causing debilitating, often fatal disease (Kazacos, 2001). The zoonotic importance of this parasite is increasingly being recognized for its impact on wildlife populations (Page, 2013), zoological gardens (Thompson et al., 2008; Ball et al., 1998), commercial poultry operations (Sorvillo et al., 2002), domestic animals (Rudmann et al., 1996; Kazacos, 2006), and humans (Wise et al., 2005). Given that raccoons adapt well to living in close proximity to humans, and their populations are expanding in size and range in North America (Troyer et al., 2014), as well as in Europe and Asia (Popiolek et al., 2011; Hayama et al., 2007), a better understanding of the population dynamics of *B. procyonis* would assist public health and wildlife management efforts to control this parasite.

To this end, molecular tools have the potential to greatly improve our understanding of the genetic architecture and microevolution of *B. procyonis* populations. Ribosomal DNA (rDNA), their internal transcribed spacers (ITS), and mitochondrial cytochrome oxidase genes have provided genetic markers for diagnosis, species discrimination, phylogenetic analysis, and detecting variation between *B. procyonis* populations (Nadler and Hudspeth, 1998: Nadler and Hudspeth, 2000; Blizzard et al., 2010). However, for the purpose of inferring microevolutionary events, these markers have several limitations. Population genetic inferences based solely on mtDNA can be misleading due to incomplete lineage sorting, introgression via hybridization (Anderson, 2001), and non-neutral evolution (Ballard and Whitlock, 2004). Low polymorphism limits the utility of rDNA, and the presence of variation both within and between repeat arrays can complicate interpretation of ITS sequence data (Vilas et al., 2005).

Microsatellites by contrast offer a number of advantages, high development cost notwithstanding. These bi-parentally inherited, co-dominant markers consist of small motifs of one to six nucleotides repeated in tandem. They are abundant, often highly polymorphic, easily typed, selectively neutral (but see Haasl and Payseur, 2013), and are well distributed throughout the genome. They have been employed as genetic markers at many scales of analysis—genome mapping, forensics, parentage analysis, phylogeography—and their high mutation rates make them excellent tools for assaying evolutionary forces acting at the population level (Abdul-Muneer, 2014; Barker, 2002).

The past decade has seen microsatellites developed for the population genetic analysis of a number of parasitic helminth species, e.g. *Ascaris lumbricoides* (Betson et al., 2014), *Schistosoma haematobium* (Glenn et al., 2013; Gower et al., 2011), *Trichinella spirallis* (La Rosa et al., 2012), *Fasciola hepatica* (Vilas et al., 2012), *Dirofilaria immitis* (Belanger et al., 2011), *Opisthorchis viverrini* (Laoprom et al., 2010), *Trichostrongylus tenuis* (Johnson et al., 2006), *Teladorsagia circumcincta* and *Haemonchus contortus* (Silvestre et al., 2009). Our objectives in the current study were to add *B. procyonis* to this list by 1) developing a panel of polymorphic microsatellite markers for this species, and 2) employing these to get a first glimpse of its population structure at a microgeographic level.

MATERIALS AND METHODS

Worm collection. In the fall of 2012, 74 adult and juvenile specimens of *B. procyonis* were collected from a ~ 500 km^2 region in Michigan's lower peninsula bisected by the Grand River (Figure 3). These worms were dissected from euthanized raccoons donated by animal control operations, fur trappers, and from road-kills. They were initially stored in 70% ethanol at RT, then transferred to individual tubes and kept at -20C.

DNA extraction. DNA was isolated using the QIAgen DNeasy blood and tissue kit according to the handbook instructions. Concentration and purity were measured using a NanoDrop 2000

spectrophotometer (Thermo Scientific). Only the anterior 1/10 of each worm was digested in order to avoid, in the case of adult females, contamination from sperm and fertilized eggs (Anderson et al., 2003).

Development of microsatellite primers. Two genomic libraries were prepared and enriched for either tetranucleotide [(GATA)$_7$, (GATC)$_7$, and (GACA)$_7$] or dinucleotide [(GT)$_{12}$ and (CT)$_{12}$] repeats using a microsatellite cloning protocol based on Hamilton *et al.* (1999) and Hauswaldt & Glenn (2003), modified as reported earlier (Beheler *et al.* 2004). The library was constructed using a pooled sample of genomic DNA from two male *B. procyonis* worms, collected from fresh (< 1 hr old) road-killed raccoons in Northern Indiana, USA. DNA was extracted from the worms using a standard phenol:chloroform extraction procedure (Green and Sambrook, 2012).

A total of 2000 recombinant clones were sequenced and the sequence data were imported into SEQUENCHER 4.1 (Gene Codes Corporation) for analysis. A subset ($n = 160$) of microsatellite-containing sequences was selected, and primers designed for PCR amplification were developed using PRIMER3 (Rozen & Skaletsky 2000). The 160 microsatellite loci were amplified in 10μL PCRs using a Master-cycler ep gradient (Eppendorf) and 20 ng of template DNA, 0.2 mM of each dNTP, 0.2 μM of each primer, 1 U of *Taq* DNA polymerase (NEB) and 1X Thermopol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100; NEB). The amplification conditions were as follows: 94 ^{o}C for 2 min, then 94 ^{o}C for 30 s, annealing temperature (Table 1) for 30 s, 72 ^{o}C for 30 s for 35 cycles, then 72 ^{o}C for 10 min and a final extension at 60 ^{o}C for 45 min. PCR products were initially screened on 2 % agarose gels stained with ethidium bromide to check for positive amplification. A total of 99 of the 160 loci consistently produced products of the expected size and subsequently were screened for polymorphism in a panel of 23 individuals (i.e. a locus would need to have approximately one allele at a frequency of ≥98% to be scored as monomorphic). The protocols for the above polymorphism screening were the same as those given above, except that 1) in each reaction, the concentration of dTTP was reduced to 1.5 mM and 0.5 mM of chromatide rhodamine green 5dUTP

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(Molecular Probes) was added, and 2) electrophoresis was performed through a 5% polyacrylamide gel on an ABI 377 automated sequencer (Applied Biosystems). Of the 99 primer sets screened, 8 loci were found to be polymorphic (average allelic richness: 3.75; Table 1). After optimizing PCR conditions we screened the 8 polymorphic loci using a screening set of 47 individual worms collected from 22 individual raccoons using the protocol outlined above. We calculated observed heterozygosity (H_O) and expected heterozygosity (H_E) using the software CERVUS 1.0 (Marshall *et al.* 1998).

PCR amplification. The 5' end of one oligo from each primer pair was ligated with the following universal tag sequence (UTS): 5'-CAGTCGGGCGTCATCA-3'. The choice of whether it was the F or the R primer in each pair that received the UTS was based on which potential combination (i.e. UTS-F and R vs. F and UTS-R) received the highest efficiency rating using NetPrimer (Premiere Biosoft). In addition to the F and R primers, each reaction also contained a third oligo consisting of one of the four dyes in Applied Biosystems' Dye Set 33 (6FAM, VIC, NED, and PET) ligated to the 5' end of the UTS. Microsatellite loci were amplified in a reaction volume of 25 μl with Promega's GoTaq® Core System II using 1μl (~ 10ng) of DNA template and 1 μM of each primer; the quantities of the remaining reactants were per the manufacturer's guidelines. Amplification was carried out using a BioRad T-100™ thermal cycler as follows. After 2 min of initial denaturing at 95C: [95C 30 sec, 60C 1 min, 72C 1 min] (x3), [95C 30 sec, 55C 1 min, 72C 1 min] (x30), 72C 10 min, 4C hold. PCR products were screened on 1 % agarose gels stained with ethidium bromide to check for positive amplification. As a quality control step, following genotyping (below), a randomly chosen subset of samples for each locus was reamplified to verify reliability in allele size. The program MICRO-CHECKER (Oosterhout et al. 2004) was used to screen for technical errors that can occur during PCR amplification, such as stuttering, large allele dropout, and null alleles.

Genotyping. Fragment analysis was performed with an ABI 3130xL genetic analyzer (Life Technologies). Alleles were sized in Geneious 7.1.5 (Biomatters) using the LIZ500 size standard.

Population structure. We used STRUCTURE v. 2.3.4 (Pritchard et al., 2000) to test for evidence of cryptic

population structure or physical barriers to dispersal. Structure uses a Markov chain Monte Carlo

(MCMC) algorithm to minimize Hardy-Weinberg and linkage disequilibrium within each of *K* clusters,

and generates a probability *Q* of an individual belonging to each cluster. We used STRUCTURE to calculate

the likelihood of the data assuming the entire population was composed of *K* = 1 to 10 distinct clusters

using a model that assumes admixture and independent allele frequencies. To identify the most likely

number of clusters *K*, we performed 5 independent runs for each *K* with a burn-in value of 10^5 steps

followed by 10^6 MCMC simulations. The most likely *K* was determined by following the guidelines of

Pritchard et al. (2000), and using the second order rate of change in the likelihood distribution, the ΔK

algorithm of Evanno et al. (2005). Output for ΔK was visualized with Structure Harvester (Earl and

vonHoldt, 2012).

Population heterozygosity was assessed by calculating the number of effective alleles (N_e), mean

observed heterozygosity (H_O), and the mean expected heterozygosity (H_E) under Hardy-Weinberg

equilibrium. Deviation from Hardy-Weinberg equilibrium was assessed at each locus using an exact test

as implemented in GENEPOP (Raymond and Rousset, 1995). Hierarchical partitioning of genetic variation

among and within infrapopulations, and among regions was performed by AMOVA (Excoffier et al.,

1992) as implemented in GENALEX 6.5 (Peakall and Smouse, 2012). Wright's *F*-statistics, as

implemented by Weir and Cockerham (1984), were also estimated within the AMOVA framework since

it allows for statistical testing. With panmixia (F_{ST} = 0) as the null hypothesis, to determine whether an

observed value of F_{ST} was significantly greater than that expected by chance, it was compared against the

outcomes of 999 random permutations; if the observed value was greater than the permuted value 95% or

more of the time, the results were deemed significant at the 5% level.

Isolation by distance was tested for all infrapopulations, and separately for those North and those

South of the Grand River, using Mantel's test of matrix correspondence (Mantel, 1967) applied to a

comparison of all pairwise $F_{ST}/(1-F_{ST})$ values (Rousset, 1997) with all pairwise log-transformed

geographic distances. For each comparison (total, North, and South), statistical significance was tested

via random permutation, as described above, using GENALEX 6.5.

RESULTS

We detected 27 alleles at eight microsatellite loci in 74 individual worms. Estimated variability at the

eight loci is summarized in Table 2. Allele frequencies at two loci (Bp107 and Bp122) showed slight

deviation from Hardy-Weinberg equilibrium; significance on exact tests was $P = 0.038$ and 0.044

respectively. There were no null alleles, stuttering errors or evidence of allelic dropout in any of the

loci, as analyzed by MICRO-CHECKER.

The results from the Bayesian cluster analysis clearly indicate a subdivision of the population into

two genetic groups ($K = 2$; Figures 1 and 2). Interestingly, there appears to be very little admixture

between groups (Figure 2), and the probability of a given worm's belonging to one or the other inferred

cluster corresponds strikingly well to its geography: the two inferred subpopulations lie on either side of

the Grand River (Figure 3).

Despite the fine geographic scale of this analysis, population structure was significant at several

levels. Our estimate of F_{ST} for the entire population was 0.176 ($P = 0.001$). Mean pairwise F_{ST} values

between infrapopulations North of the Grand River was 0.049 ($P = 0.002$), and for those South of the

Grand it was 0.051 ($P = 0.005$). All pairwise comparisons of F_{ST} between infrapopulations are shown in

Table 3. These values of F_{ST} are largely consistent with a pattern of structure defined by the river:

pairwise comparisons between infrapopulations within regions (i.e. between raccoons on one side of the

river or the other) were mostly nonsignificant, whereas comparisons between regions were all significant

(Table 3). Of 45 possible pairwise comparisons, only six were inconsistent with this pattern (indicated in

grey in Table 3).

The partitioning of variance in our AMOVA was also consistent with this North—South pattern: genetic differences between infrapopulations accounted for only 4% of the total variance, whereas differences between regions (North and South) accounted for over five times this amount (21%; Table 4). Tests of isolation by distance (IBD) tell a similar story. Within each region, significant IBD was detected, whereas between regions, the IBD signal breaks down and is non-significant (Figure 4). Collectively, these results suggest that the Grand River is acting as a barrier to gene flow between sampled populations of *B. procyonis* to its North and those to the South.

DISCUSSION

Here we report the development of eight microsatellite markers for *B. procyonis*, and provide the first description of the population genetics of this species on a local scale. The polymorphism of these markers (mean number of alleles per locus = 3.375) was generally lower than that reported for microsatellites in other parasitic nematodes (e.g. the studies cited in the introduction all report higher mean allele numbers). The observation of reduced microsatellite variability in other organisms has been variously ascribed to low effective population size (Bishop et al., 2009; Hauser et al., 2002); fluctuations in population density (Cimmaruta et al., 2010), low rates of recombination (Payseur and Nachman, 2000), and selection at linked loci (Olafsdottir et al., 2007). The relative influences of these factors in the present study remain to be assessed. Our sample size (n = 74) is more than sufficient to estimate allele richness and frequencies (Hale et al., 2012), although the restricted geographic scale of the sampling might possibly impact this; future studies of *B. procyonis* population genetic structure (PGS) on a broader geographic scale will shed light on this.

PGS in parasitic nematodes runs the gamut from panmixia (Grillo et al., 2007; Johnson et al., 2006; Webster et al., 2007) to moderate (Hawdon et al., 2001) to highly structured (Blouin et al., 1999; Redman et al., 2008). This variation is influenced by ecological factors, differences in nematode life

history, and host biology (Nadler, 1995; Huyse et al., 2005). Nematodes of domestic and companion animals generally exhibit low structure owing to high gene flow resulting from widespread host transport (Blouin et al., 1995; Grillo et al., 2007), whereas in wild host species, factors relating to vagility, territoriality, and ecological barriers will generally determine the degree to which structure is exhibited in their parasite populations. Our results indicate that, at least in the case of Western Michigan, on a microgeographic scale *B. procyonis* exhibits significant PGS, and that this structure correlates strongly with a physiographic feature, the Grand River. Global F_{ST} and pairwise values between infrapopulations separated by the Grand River (i.e. between regions, Table 3) were significant—but not among infrapopulations within regions. With a few exceptions highlighted in grey in Table 3, these estimates are consistent with a pattern of gene flow being impeded by the river. IBD and AMOVA results corroborate this.

Although rivers have been implicated as dispersal barriers to raccoons under certain conditions (Smith et al., 2002), their permeability to dispersal varies depending on their width, whether or not they are frozen (Cullingham et al., 2009), and the presence of bridges (Rosatte et al., 2007). The width of the Grand River in our collection area ranges from over 1,000 feet at several points near its mouth to under 200 feet at a number of points East of Grand Rapids (as measured on Google Earth). It is not inconceivable that raccoons might occasionally swim across this; however, it is more likely that they would walk across it during the winter months when it is frozen, as has been observed in other temperate regions (Cullingham et al., 2009).

This seasonal variation in river permeability, if considered in light of annual fluctuations in infection prevalence, may explain the PGS we observed. The prevalence of *B. procyonis* infection among raccoons in our study area shows a pronounced seasonality; it exceeds 70% during the late spring and fall, and approaches zero during the winter months (data not shown), a pattern also observed in other temperate regions in the U.S. (Kidder et al., 1989; Kazacos, 2001). Whether this is a function of worm life span, self-curing on the part of raccoons, starvation induced by host torpor, or other factors has yet to

be determined. Each spring, a new generation of *B. procyonis* adults arises, in a manner analogous to the hatching of annual plant seedlings, via infections recruited into the raccoon population from latrine sites (in the case of juveniles) and infected intermediate hosts (in the case of adults). Therefore, the time of year when raccoons are most likely to cross the Grand River is the time when they are least likely to be harboring worms. This would permit gene flow among raccoon populations on either side of the river, but not among populations of *B. procyonis*. Although microsatellites have been developed for the raccoon (Fike et al., 2007), as it was not our initial objective, we did not simultaneously assay genetic structure of the raccoons in this study (i.e. a co-structure analysis *sensu* Criscione (2008)), the results of which would have informed the above hypothesis. A finding of little or no genetic structure in the raccoon population would lend support to our proposed explanation, whereas significant structure would suggest that the Grand River is a barrier to gene flow for both host and parasite in this particular case.

It is worth noting that the prevalence and distribution of infection among intermediate hosts in this region is unknown. In other regions, *larva migrans* has been detected in a number of bird species (Evans, 2002), a host category for which a river would not pose a significant dispersal barrier. To the degree that birds in West Michigan are infected with *B. procyonis*, they are not apparently contributing to gene flow across the Grand River.

Future analyses that include additional microsatellite loci and other nuclear and mitochondrial markers will provide useful context for these results. Additionally, the simultaneous assay of raccoon PGS offers the prospect of separating ecological barriers (i.e. the Grand River) from the possible role of host variables (e.g. demographic structure) in shaping the structure of *B. procyonis* populations in this region. These efforts are ongoing.
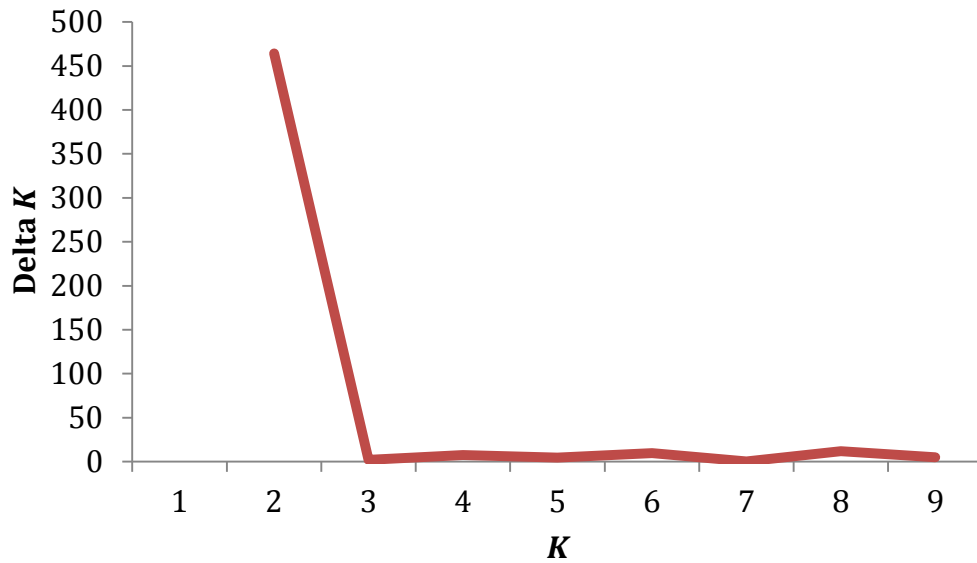
Figure 1. Identification of the best *K* as inferred by STRUCTURE using the ΔK method of Evanno et al. (2005).
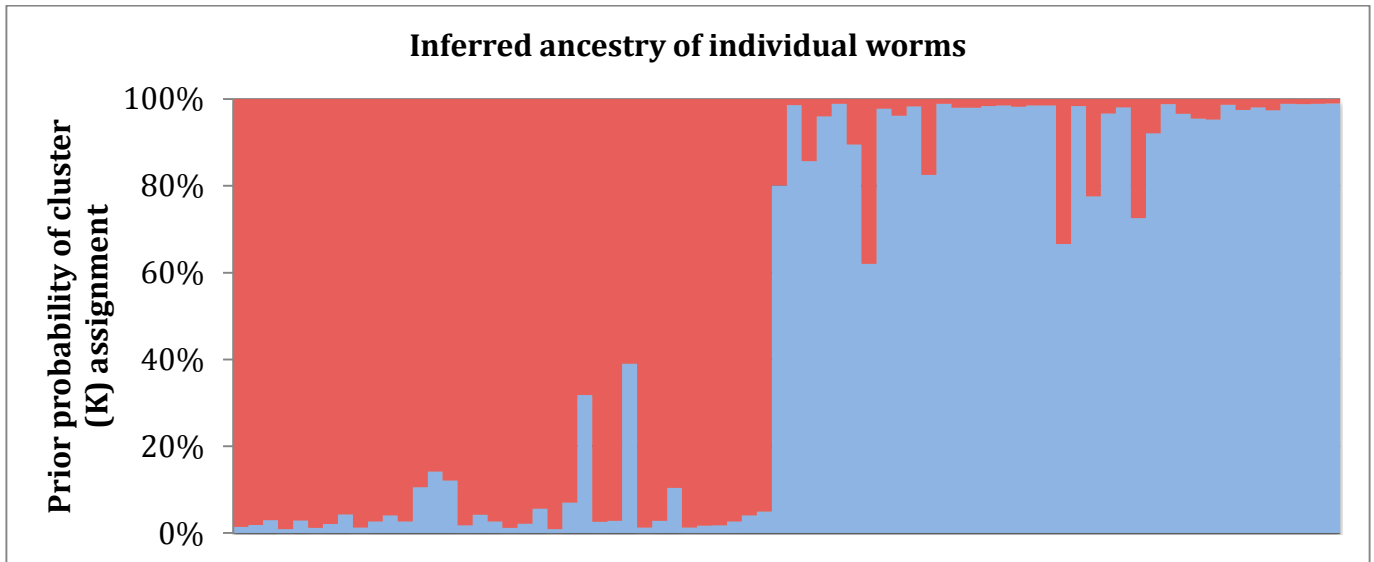
Figure 2. A box plot showing the results of the Bayesian clustering analysis performed by STRUCTURE. Each of the 74 worms is represented by a column partitioned into two colored segments representing the worm's probability of belonging to each of the two inferred genetic clusters. The first 36 columns are from worms collected North of the Grand River (red = North); the remaining 38 samples were collected from South of the Grand River (blue = South).
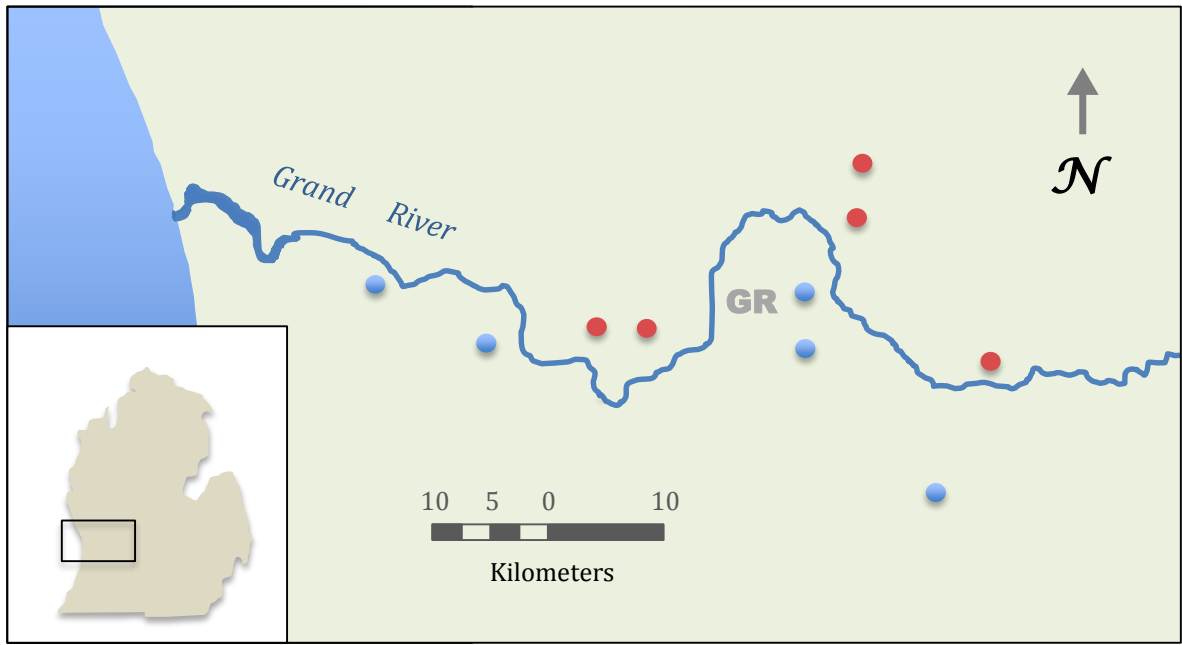
Figure 3. *B. procyonis* sample collection area in Western Michigan. Points represent the locations where raccoons were collected. Color coding reflects the results of Bayesian cluster analysis; red = North of the Grand River; blue = South of the Grand River. GR = Grand Rapids.

Figure 4. Pairwise geographic distance plotted against genetic distance for all 10 infrapopulations (Total), those North of the Grand River (North), and those South of the Grand River (South). *P* values refer to the Mantel test of significance ($\alpha = 0.05$) after 999 random permuations. Isolation by distance was detected within each subpopulation (North and South), but not within the total (Total) population.

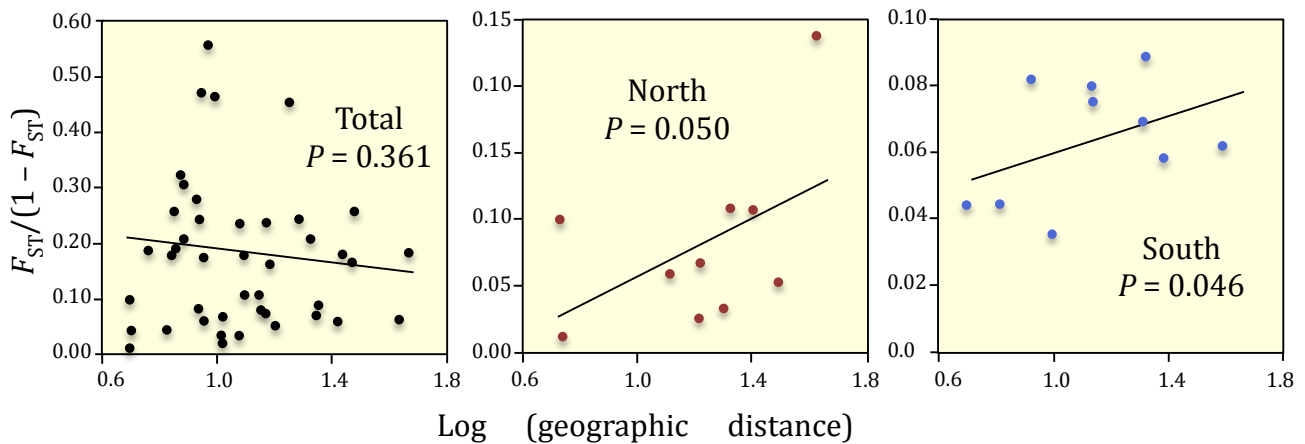**Table 1.** Characterization of eight polymorphic microsatellite loci developed for *Baylisascaris procyonis* in a screening set of 47 individual worms. Reported are the numbers of individuals successfully genotyped at each locus (N), the annealing temperature of the primers (T_M) allelic richness (A), as well as the observed (H_O) and expected (H_E) heterozygosities. No locus deviated significantly from Hardy-Weinberg equilibrium expectations.

Locus	GenBank Accession Number	N	T_m	Primer sequence	Micro-satellite motif	Expected Product size	A	H_o	H_e
BP_104	--	37	62.1	F: AACCTCTGGAATAATCGTTGG	AC_{16}	175	3	0.16	0.24
				R: AATTCGACCGGATATGTAAGC					
BP_107	--	45	63.4	F: TTGACCTTCACGAATATAGGAATG	AG_{13}	116	5	0.33	0.62
				R: ATACGCCTCTGGTGGATCTG					
BP_109	--	45	63.3	F: ATTTGCCCCTTGAAAATTAGG	AG_{12}	287	4	0.24	0.47
				R: TGTTCGCATACACTTGCACTC					
BP_114	--	45	60.0	F: CAATCAATCGCCATGAAG	CA_5CT_{25}	130	2	0.13	0.35
				R: TGCAATATCACCTATTCACTCAC					
BP_117	--	44	61.6	F: GTTCTTCCTCGTTTCCAGTTC	AG_{12}	275	4	0.48	0.54
				R: CTTAACGATGAGTACACGATTATAGG					
BP_122	--	46	57.0	F: AGTTCATTATGCTCTTCAATTC	AG_{42}	160	3	0.17	0.56
				R: TGAGAACGCTATATTGATGG					
BP_131	--	41	61.5	F: ACATAATGAGGGCATCAAATTC	$GT_{20}\ldots GT_4$	190	3	0.27	0.56
				R: TTTCAACAAGTATGATTTCAATGAG					
BP_138	--	43	59.8	F: TATCTCATTCCTCAGCAGTCTT	CT_{18}	112	6	0.37	0.50
				R: TTCGAATGTTCACTGAATAAGAG					

Table 2. Estimated variability at eight microsatellite loci for *B. procyonis* in Western Michigan. N: number of samples; A: number of alleles; Ne: effective number of alleles, 1/(sum of *i*th allele^2); H_O: observed heterozygosity, H_E: expected heterozygosity; *P*: exact test probability of departure from Hardy-Weinberg equilibrium (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Locus	N	A	Ne	H_O	H_E	*P*
Bp104	73	3	1.953	0.411	0.488	0.142
Bp107	74	3	2.343	0.432	0.573	0.038*
Bp109	66	3	2.833	0.727	0.647	0.170
Bp114	74	3	2.755	0.581	0.637	0.088
Bp117	54	4	3.278	0.685	0.695	0.364
Bp122	71	4	3.442	0.521	0.709	0.044*
Bp131	74	4	2.223	0.689	0.550	0.126
Bp138	74	3	2.505	0.568	0.601	0.107
Mean	70	3.375	2.666	0.577	0.613	
SE	2.486	0.183	0.182	0.042	0.026	

Table 3. Pairwise comparison of F_{ST} values between all infrapopulations. R4 = Raccoon 4, R6 = Raccoon 6, etc… Red = within-region comparison, North; blue = within-region comparison, South; yellow = between region comparison. Values below diagonal are F_{ST}; those above are *P* values based on 999 permutations. Values in grey buck expectation at *P* = 0.05, see Discussion.

		North					South				
		R4	R6	R8	R15	R16	R3	R12	R13	R9	R17
North	R4	--	0.300	0.392	0.430	0.016	0.001	0.006	0.002	0.001	0.001
	R6	0.032	--	0.165	0.327	0.114	0.001	0.005	0.001	0.001	0.001
	R8	0.006	0.037	--	0.185	0.024	0.001	0.001	0.001	0.001	0.001
	R15	0.000	0.008	0.018	--	0.005	0.001	0.001	0.001	0.001	0.001
	R16	0.188	0.049	0.067	0.089	--	0.001	0.001	0.001	0.001	0.001
South	R3	0.251	0.180	0.236	0.212	0.204	--	0.395	0.084	0.063	0.009
	R12	0.264	0.154	0.290	0.219	0.205	0.003	--	0.086	0.047	0.163
	R13	0.311	0.177	0.279	0.205	0.212	0.035	0.065	--	0.010	0.149
	R9	0.352	0.261	0.328	0.269	0.292	0.035	0.091	0.079	--	0.053
	R17	0.365	0.246	0.337	0.249	0.281	0.074	0.045	0.028	0.056	--

Table 4. Results of AMOVA.

	df	SS	MS	Est. Variance	%
Among regions	1	48.283	48.283	0.606	21%
Among hosts	8	30.146	3.768	0.116	4%
Within hosts	136	290.845	2.139	2.139	75%
Total	145	369.274		2.860	100%

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