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Microsatellite and Mitochondrial DNA Sequence Analysis of Three Isolated Minnesota Populations of the Bullsnake (*Pituophis catenifer sayi*)

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Abstract.—**Because of a reduction in population size and fragmentation of its grassland habitat, the Bullsnake (*Pituophis catenifer sayi*) is listed by the Minnesota Department of Natural Resources as a Species of Special Concern and a Species in Greatest Conservation Need. The geographic isolation of small populations can substantially affect genetic diversity through genetic drift and inbreeding. This study examined the genetic variation of Bullsnakes at three isolated sites in eastern Minnesota, USA, through analysis of six nuclear microsatellite loci as well as direct sequencing of the 16S and COX1 mitochondrial regions to determine haplotype variation and frequency. The three populations differ in microsatellite allele frequencies and presence of private alleles, and each population contains unique mitochondrial haplotypes. Microsatellite allele frequencies and mitochondrial haplotypes suggest that the three populations were historically part of a large single range, but that recent habitat fragmentation has created three genetically isolated populations. The evidence for a significant reduction in genetic diversity, however, is less clear. Although some of the microsatellite loci show low heterozygosity in two of the three sites, the three populations also show considerable allelic variation, private alleles, and mitochondrial haplotypes, and the majority of loci are in Hardy-Weinberg equilibrium. This study provides a valuable reference point to which future sampling and DNA analysis can be compared. Monitoring genetic variation in these and other isolated Minnesota Bullsnake populations will be useful in developing an effective conservation management plan.**

Key Words.—Colubridae; conservation; genetic diversity; habitat fragmentation

Introduction

Pituophis catenifer sayi, commonly known as the Bullsnake (Fig. 1), is a member of the most speciose family of snakes in the world, Colubridae. This charismatic snake is the top ophidian predator throughout much of its range, which extends from Canada to Mexico and the Pacific coast to the central Midwest of the USA (Powell et al. 2016). An actively foraging, large, predatory snake, Bullsnakes have large home ranges and occur in fairly low densities (Kapfer et al. 2008b; Moriarty and Hall 2014). In a 3-y study of Bullsnakes in southwestern Wisconsin, USA, home range sizes averaged 41 ha for males and 23 ha for females (Kapfer et al. 2008a). In studies where Bullsnake densities were calculated, there is typically fewer than one individual per ha (Fitch and Echelle 2006). In Idaho, USA, Diller and Johnson (1988) estimated 1.3 Bullsnakes per ha and Parker and Brown (1980) measured 0.32 individuals per ha in a Utah, USA, population. The wide-ranging activity of Bullsnakes means that they are especially sensitive to habitat fragmentation. Much of the natural habitat has been lost due to conversion to agriculture or development for housing and other human activities (Martino et al. 2012). One survey from Iowa, USA, estimates that Bullsnake numbers have been reduced by as much as 91% in most of their range over an approximately 80-y span (Christiansen 1998).

The non-venomous Bullsnake is the largest snake in Minnesota, reaching lengths of over 180 cm, and is

Figure 1. Adult Bullsnake (*Pituophis catenifer sayi*) from Minnesota, USA. (Photographed by Carol Hall).

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mostly found in the central and southeastern portion of the state, especially along the Mississippi, Minnesota, and St. Croix rivers (Moriarty and Hall 2014). Bullsnakes in the upper Midwest of the U.S. occupy open habitat such as sand prairies, oak savannahs, hillside prairies, bluff country hillsides, and altered habitats (pastures and old fields) when they border undisturbed prairie areas. In parts of Minnesota, sandy soil habitats are important for hibernation, nesting, and the presence of burrowing rodents as a food source (LeClere 2013; Moriarty and Hall 2014). The Minnesota Department of Natural Resources (MDNR) lists the Bullsnake as a Species of Special Concern and a Species in Greatest Conservation Need because of the loss and ongoing fragmentation of grassland habitats by agricultural and residential development, and the accompanying increases in road traffic through these areas (MDNR 2013).

The genetic implications of habitat fragmentation have been documented in many animal species; in small, isolated populations the lack of gene flow can increase the effects of genetic drift and inbreeding, leading to a reduction in genetic diversity (reviewed in Keyghobadi 2007). This loss of diversity is often associated with a reduction in fitness and can threaten the ability of a population to adapt to environmental challenges (Reed et al. 2003). Aside from a single study using nuclear microsatellite markers to examine genetic diversity in a Bullsnake population at their northernmost range in southern Saskatchewan, Canada (Somers et al. 2017), there has been no population studies using molecular genetic markers in this species. Therefore, we aimed to examine the genetic variation in three isolated Bullsnake populations along a 200-km transect in eastern Minnesota, USA, through analysis of microsatellite loci and mitochondrial DNA sequencing. The use of codominant nuclear microsatellite markers is a well-established means of assessing the genetic variation within and between populations (Selkoe and Toonen 2006), and the combined use of microsatellite information and mitochondrial DNA sequencing has proven effective in population genetic analysis in a number of snake species (Lukoschek et al. 2008; Ferchaud et al. 2010). Habitat fragmentation can have the same effect on population structure as a founder event, a small population subject to inbreeding and genetic drift, with a subsequent reduction in the observed heterozygosity. Our identification of informative molecular markers and documentation of population genetic variation provides a useful baseline for the monitoring of these three sites over time, as well as for comparative studies of additional Bullsnake populations.

Materials and Methods

Study sites.—We collected tissue samples and successfully extracted DNA from 54 Bullsnakes at three sites located along an approximately 200-km transect running north to south in eastern Minnesota between 2010 and 2016. We collected 16 samples from the northern site (N), which is located in Chisago County, Minnesota, USA, and consists of dry sand prairie and woodland surrounded by agricultural land, dense forest, and a river 1.5 km away; 15 samples from the middle site (M), which is located in Dakota County, Minnesota, and consists of dry sand prairie bounded by heavily traveled two-lane highway, urban development, and agricultural land, 5.2 km from a large river; and 23 samples from the southern site (S), which is located in Wabasha County, Minnesota, and consists of mostly dry sand prairie surrounded by a major four-lane freeway and agricultural land, large open bodies of water, and 2.4 km from the main channel of a large river. We did not have information available regarding the sex of individuals collected at the northern and middle sites. At the southern site, we determined the sex of 21 of the 23 specimens (12 males, nine females). The straight-line distance between sites was 92 km for N-M and 85 km for M-S. Both the middle site (about 300 ha) and the south site (about 2,250 ha) were separated by large bodies of open water, two and four-lane highways, and agricultural and developed land. The northern site (about 1,000 ha), however, is likely contiguous with Bullsnake populations to the north, east, and west, but not to the middle site population to the south.

DNA extraction.—We clipped two ventral scales from each snake and placed them in vials of 95% ethanol, stored at -20° C, and extracted genomic DNA from the clipped scales using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California, USA). We modified the DNeasy protocol as follows: 5 mg of scales were finely cut and incubated at 56° C for 16–20 h with 360 µL of Buffer ATL and 20 µL of Proteinase K. We added an additional 20 µL of proteinase K and continued incubation at 56° C for 4 h. This procedure typically yielded between 2 and 10 µg of genomic DNA for each tissue sample.

Microsatellite genotyping and statistical analysis.—We examined six nuclear microsatellite loci to gauge the level of genetic diversity in each of the three Minnesota Bullsnake populations. Two of the loci (Piru8 and Piru12) were previously characterized for the Louisiana Pine Snake (*Pituophis ruthveni*) and showed cross-species amplification with the Bullsnake (Kwiatkowski et al. 2010). We identified the remaining four loci (Pica07, Pica08, Pica10, Pica18) in our laboratory using genomic Bullsnake DNA and following the enrichment technique described in Glenn and Schable (2005). We performed the Polymerase Chain Reaction (PCR) using the Qiagen HotStarTaq Kit (Qiagen, Inc.) in a 50 µL reaction volume that contained 25 µL Master Mix, 0.2

Table 1. Analysis of six microsatellite loci in three Bullsnake (*Pituophis catenifer sayi*) populations (N, M, S) in Minnesota, USA. Each microsatellite is described by the repeat motif, the optimum annealing temperature of the PCR primer set (T_a), and the forward and reverse primer sequences. Included for each population is sample size (n), number of alleles (N_A), number of effective alleles (N_E), number of private alleles (N_P), allele size range, expected heterozygosity (H_E), and observed heterozygosity (H_O). Bolded values indicate groups in which H_O is significantly different than H_E as determined by Chi-squared calculation (df = 1, $P < 0.05$).

Microsatellite	Repeat Motif	T_a (°C)	Primer Sequence (5' to 3')	Location	n	N_A	N_E	N_P	Allele Range (bp)	H_E	H_O
Pica07	TCTG + CTAT	55	F:GTTGAAACAATCCAGGGCAG R:TAGCAACCCACCACTGGTTAG	N	16	12	6.1	7	297–377	84%	75%
				M	8	6	3.3	5	317–372	69%	88%
				S	23	12	7.2	6	309–382	86%	97%
Pica08	TG	55	F:GAGGGAACGCCAAAAATAGG R:CCCTTCCCTTTTCATTCACA	N	14	8	5.6	2	175–207	82%	64%
				M	15	4	2.9	0	193–207	56%	53%
				S	23	7	4.7	2	175–211	79%	**56%**
Pica10	CA	55	F:TAGCAACCCACCACTGGTTAG R:GGAGTCGACAGTATAGCCTGA	N	15	9	5.3	2	140–172	81%	**53%**
				M	8	6	2.4	3	148–174	67%	50%
				S	22	6	4.3	2	140–164	75%	**55%**
Pica18	TG	55	F:GATCCGACTCAGCAGGTACAG R:GAGAGAAATTCAGGAATGGGACT	N	14	13	7.3	2	171–215	86%	86%
				M	14	6	4.6	1	171–209	78%	64%
				S	23	9	5	1	171–207	80%	78%
Piru8	AAGG	62	F:CTCTGCCCAATTGCTGGATG R:CTGCAGCCAGCTAGTATTTCC	N	16	7	2.8	2	143–175	64%	56%
				M	8	4	1.7	1	141–155	46%	57%
				S	21	8	5.6	3	108–171	82%	81%
Piru12	AGAT	53	F:GTGGGCTACCTGCAAATGG R:ATACCTAAGAGTTGCCCATCC	N	16	6	3.6	1	166–194	72%	**44%**
				M	8	4	2.6	1	178–190	65%	75%
				S	23	7	5	2	174–233	80%	**43%**

μM of each primer, and 10–50 ng of isolated genomic DNA. The PCR profile consisted of an initial 15-min heat activation hold at 95° C, then 34 cycles of 94° C for 30 s, a specified annealing temperature (T_a°C) for 30 s, and 72° C for 60 s. The final cycle was followed by 10 min at 72° C. The repeat motif, PCR annealing temperature, and primer sequences for each locus are reported in Table 1. The PCR products were analyzed on an ABI PRISM 310 DNA Sequencer, and allele sizes were confirmed using GeneScan software. We used these microsatellite genotypes to determine the total number of alleles (N_A), the number of private alleles (N_p), the expected heterozygosity (H_E), and observed heterozygosity (H_O) based on Hardy-Weinberg assumptions. To evaluate differences between H_e and H_O, we computed Chi-square scores and *P*-values in R (R Core Team 2021). In addition, we used GenAlEx v.6.5 (Peakall and Smouse 2012) to determine number of effective alleles (N_E), fixation indices (F_{ST}), and to perform an Analysis of Molecular Variance (AMOVA) test with 999 permutations for pairwise F_{ST} values to compare populations (Ferchaud et al. 2010; Blackhawk et al. 2016). Bonferroni correction was computed to control for multiple comparisons. We estimated the effective population size (N_e) for each Bullsnake population using the unbiased linkage disequilibrium method as implemented in NeEstimator V2.1 and excluded rare alleles with frequencies below 0.05 (Waples 2006; Do et al. 2014).

Mitochondrial DNA analysis.—Because sequencing the entire mitochondrial genome for each of the Bullsnake specimens was not feasible for this study, we analyzed two regions within the mitochondrial genome that we previously determined to contain single nucleotide polymorphisms: 647 bp of the 16S rDNA gene, and 710 bp of the cytochrome oxidase I (COX1) gene. Nucleotide position in the mitochondrial genome was based on the sequenced mitochondrial genome of the Bullsnake (Lele et al. 2016; GenBank reference KU833245). The regions between 989–1,296 and 1,680–2,010 are within the 16S gene; and the region between 7,112–7,822 is within the Cytochrome Oxidase C Subunit 1 (COX1) gene. The primers used for PCR amplification of the 16S and COX1 regions are as follows: 16S-F989 (5'GGTAAGCGTACTGGAAAGTG3'); 16S-R1296 (5'CACCCTTCTCTGTAGCAAAAGA3'); 16S-F1680 (5'GCACCTATCTGCTAGATC3'); 16S-R2010 (5'CAACATAAGACCAGAAGAC3'); COX1-F7112 (5'AGGCTTTGTGGTTTGAGCACA3'); COX1-R7822 (5'CATGGCTAAATCACAGCATC3'). We purified the PCR products using the Invitrogen Purelink Quick PCR

Purification Kit (Invitrogen, Waltham, Massachusetts, USA). DNA Sanger sequencing of the PCR products was performed by Elim Biopharmaceuticals, Inc. (Hayward, California, USA) using forward and reverse primers to confirm base pair identity. We detected nucleotide polymorphisms by comparing against a globally aligned consensus sequence using the Benchling DNA analysis program (https://benchling.com). We used GenAlEx v.6.5 (Peakall and Smouse 2012) to determine haplotype diversity (*h*), and to perform an AMOVA test with 999 permutations for pairwise ΦPT values to compare populations (Ferchaud et al. 2010; Blackhawk et al. 2016). We computed Bonferroni correction to control for multiple comparisons.

Results

The number of total alleles per locus ranged from nine (Piru12) to 24 (Pica07), with each of the three geographic populations displaying polymorphisms for each allele (Appendix). Tests for pairwise population comparisons using microsatellite F_{ST} values indicate that all three populations show significant genetic differences after Bonferroni correction ($P < 0.001$; Table 2). In the majority of microsatellite loci examined, the H_o and H_e values are not statistically different. The observed heterozygosity for the Pica10 and Piru12 loci in the populations at the northern site (N) and the southern site (S), and the Pica08 locus in the population at the S site, however, were significantly different from what was expected assuming HW equilibrium (Table 1). The N_e estimate for each site was: northern 75.6 (95% Confidence Interval = 16.9-infinite); middle 132.9 (4.8-infinite); southern 62.1 (21.2-infinite).

We also determined the genetic variation within a population by direct DNA sequencing of regions within the mitochondrial genome (16S rDNA and COX1). Amplification and sequencing of the DNA samples identified nine 16S/COX1 haplotypes. One common haplotype (C) was found in all three Minnesota populations, while the other eight haplotypes were unique to one of the three geographic locations (Table 3). The N and M sites, as well as the N and S sites have significant genetic differences (Table 2). The M and S sites, however, were not significantly different (Table 2). Haplotype diversity (*h*) calculations (N = 0.578, M = 0.556, S = 0.533), which represent the probability that a haplotype allele is unique within the population, also indicate that the three sites show moderate genetic diversity.

Table 2. Pairwise F_{ST} comparison for the microsatellite data for Bullsnakes (*Pituophis catenifer sayi*) in Minnesota, USA, at three geographic sites (below the diagonal, in bold), and mitochondrial haplotype pairwise ΦPT value comparisons for the three geographic sites (above the diagonal, in *italics*). An asterisk (*) indicates $P < 0.001$ after Bonferroni correction.

	N	M	S
N	0.000	*0.267**	*0.278**
M	**0.087***	< 0.001	*0.140*
S	**0.048***	**0.101***	< 0.001

Discussion

The Bullsnake was once a common species in the upper Midwest of the USA but is now often listed as a Species of Special Concern by state agencies. Much of the grassland habitat that remains is heavily bisected and bordered by agricultural fields and paved roads. Because the reduction of genetic diversity and the accumulation of deleterious mutations in small populations can lead to decreased fitness (Keyghobadi 2007), the determination of the genetic structure of fragmented populations is critical in identifying at-risk populations in need of conservation action. Our study focused on three isolated Bullsnake populations in eastern Minnesota. All three sites contained dry sand prairie, ranging from 300 to over 2,200 ha. Based on the distances separating these sites and the intense agricultural use, housing development, and major roadways that border all three sites, it is unlikely that contemporary gene flow occurs among these populations. In addition, previous studies indicate that Bullsnakes avoid agricultural fields, further inhibiting migration between separated populations (Kapfer et al. 2008b). Although these three populations were once contiguous, they have been physically isolated from each other in the last 100 y because of human land use practices.

Using non-invasive tissue sample collection and DNA analysis, we characterized nuclear microsatellite loci to determine if the three geographically isolated populations represent genetically differentiated groups. Populations in fragmented habitats often demonstrate significant differentiation in microsatellite allele frequencies, and F statistics (F_{ST}) are a common measure used to compare genetic diversity within and among populations (Weir and Cockerham 1984). If there is no barrier to interbreeding between two populations, the F_{ST} value would be zero. Conversely, an F_{ST} value of one indicates that the two populations do not share any genetic similarity. Based on Hartl and Clark (1997), an F_{ST} value between 0.05 and 0.15 is considered to indicate moderate genetic differentiation. Tests for pairwise population comparisons using our microsatellite frequencies produced F_{ST} values between 0.048–0.101, indicating that all three populations had discernable genetic differences. Our values are similar to a previous study that reported F_{ST} values between 0.045–0.156 in three genetically distinct populations in Saskatchewan, Canada (Somers et al. 2017). Overall, our results reveal

Table 3. Mitochondrial haplotypes and frequency as determined by the combined DNA sequence of 1,357 bp of the 16S and COX1 regions in the three Bullsnake (*Pituophis catenifer sayi*) populations in Minnesota, USA. The BS haplotype is used as the reference genome and is from the published Bullsnake mitochondrial sequence from a specimen collected in Payne County, Oklahoma, USA (Lele et al. 2016). Single nucleotide polymorphisms (SNPs) are indicated with the mitochondrial nucleotide designation. The C haplotype is the common haplotype found at all three geographic sites in Minnesota. The remaining eight haplotypes are unique to their specific geographic location (N1-3, M1-2, S1-3).

	16S		COX1						N	M	S
	SNP 1061	SNP 1886	SNP 7267	SNP 7279	SNP 7647	SNP 7668	SNP 7702	SNP 7768			
BS	A	G	G	C	C	T	T	T			
C	G	-	-	A	-	-	-	C	27%	57%	60%
N1	-	A	-	-	-	-	-	C	60%		
N2	-	A	-	A	-	-	-	C	7%		
N3	-	A	-	-	-	-	C	C	7%		
M1	G	C	-	A	-	-	-	C		36%	
M2	G	-	-	A	-	C	-	C		7%	
S1	G	-	-	-	-	-	-	C			30%
S2	G	-	A	A	-	-	-	C			4%
S3	G	-	-	A	T	-	-	C			4%

that the three Bullsnake populations contain a variety of common alleles indicating a formerly overlapping range, as well as numerous private alleles reinforcing the genetic differentiation of the three populations.

Although isolated populations can be genetically distinct, it still remains to be shown that there is a concomitant loss of genetic diversity. One useful indicator of the loss of genetic diversity is the comparison of observed heterozygosity (H_o) to expected heterozygosity (H_e) in the microsatellite allele frequencies assuming Hardy-Weinberg conditions. An isolated population with low density would be susceptible to inbreeding and genetic drift, and this would be manifested in a reduction in observed heterozygosity. We found that the observed heterozygosity for two of the microsatellite markers in the populations at the northern site (N) and the southern site (S), and one locus in the population at the S site, showed significant deviation from the expected heterozygosity. This decrease in the observed heterozygosity is likely the result of population fragmentation with associated inbreeding and genetic drift, a situation also associated with population bottlenecks.

In addition to nuclear microsatellite analysis, we examined the DNA sequence of two regions within the mitochondrial genome that were previously determined to contain polymorphisms: the 16S rDNA gene, and the cytochrome oxidase I (COX1) gene. The PCR amplification and DNA sequencing of the genomic samples from the three populations revealed the presence of nine unique 16S/COX1 haplotypes. All three populations harbor a common haplotype, and it is the most frequent haplotype in sites M and S. This observation suggests that the common haplotype was associated with the founder population in this larger region of eastern Minnesota. Each population also contains two or three unique mitochondrial haplotypes that could be the result of mutational changes in the genome since population isolation, or the loss of haplotypes that were present in the original population.

The unique genetic structure of the three eastern Minnesota sites is not surprising given the geographic isolation and lack of gene flow between the fragmented populations. Although we detected an appreciable amount genetic diversity at each site, it is worth noting that two of the sites (N and S) are showing some early signs of decline in heterozygosity. The continued loss and fragmentation of Bullsnake habitat in the Midwest underscores the importance of understanding the genetic diversity in separated populations to design an effective conservation plan. Maintaining this genetic diversity in the face of climate change and habitat loss is critical if the species is to adapt to future environmental perturbations. Our assessment of the genetic structure in three Minnesota populations serves as a valuable baseline for future analyses, both in terms of monitoring these particular populations and in examining other Bullsnake populations that may be at risk.

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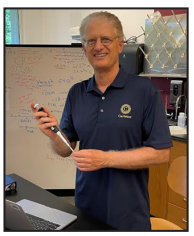
Carol Hall has been with the Minnesota Department of Natural Resources as the lead Herpetologist for the Minnesota Biological Survey since 1991. She received her B.S. in Wildlife Management and Biology from the University of Wisconsin - Stevens Point, USA (1980), and her M.S. in Wildlife Conservation from the University of Minnesota, Minneapolis, USA (1995). Her work has focused on statewide surveys and conservation efforts that target rare herpetofauna of Minnesota. (Photographed by Amy Westmark).

Matthew Rand is a Professor in the Department of Biology at Carleton College, Northfield, Minnesota, USA. He received his Bachelor's degree in Zoology at Oregon State University, Corvallis, USA, and his Ph.D. in Environmental Population and Organismic Biology at the University of Colorado, Boulder, USA. His research interests include reptile reproductive physiology. He is pictured holding an axanthic morph of the Bullsnake. (Photographed by Stephan Zweifel).



Stephan Zweifel is a Professor in the Department of Biology at Carleton College, Northfield, Minnesota, USA. He received his Bachelor's degree in Biology from the University of California, Davis, USA, and his Ph.D. in Genetics at the University of Washington, Seattle, USA. His research interests include conservation genetics and molecular mechanisms of fungal drug resistance. He is thrilled that this Bullsnake project began as a case study in the Genetics laboratory course at Carleton. (Photographed by Matthew Rand).

Appendix. Observed allele sizes (PCR product length), and frequencies, for the six microsatellite loci in the three isolated populations of Bullsnakes (*Pituophis catenifer sayi*) in Minnesota, USA. (The variation in sample size between loci at the same site reflects the minor differences in success rate of PCR amplification and genotype assignment.)

Group	n	Allele size (bp)																							
Pica07		297	309	317	321	325	327	329	332	333	336	337	340	345	346	349	350	353	355	357	363	372	373	377	382
N	16	0.031	0.313	0.000	0.000	0.063	0.000	0.188	0.000	0.000	0.000	0.031	0.000	0.000	0.094	0.000	0.031	0.031	0.031	0.094	0.000	0.000	0.063	0.031	0.000
M	8	0.000	0.000	0.500	0.000	0.000	0.000	0.063	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.188	0.063	0.000	0.000	0.000
S	23	0.000	0.239	0.000	0.130	0.022	0.022	0.043	0.043	0.065	0.000	0.000	0.043	0.087	0.000	0.000	0.022	0.196	0.000	0.000	0.000	0.000	0.000	0.000	0.087
Pica08		175	193	195	197	199	201	205	207	209	211														
N	14	0.179	0.036	0.179	0.143	0.286	0.071	0.036	0.071	0.000	0.000														
M	15	0.000	0.433	0.133	0.000	0.067	0.000	0.000	0.367	0.000	0.000														
S	23	0.304	0.022	0.000	0.043	0.217	0.000	0.000	0.196	0.043	0.174														
Pica10		140	144	148	150	152	154	158	160	162	164	170	172	174											
N	15	0.067	0.000	0.000	0.067	0.000	0.367	0.100	0.067	0.067	0.133	0.067	0.067	0.000											
M	8	0.000	0.000	0.063	0.500	0.063	0.250	0.000	0.000	0.000	0.000	0.000	0.063	0.063											
S	22	0.205	0.091	0.000	0.386	0.000	0.091	0.000	0.136	0.000	0.091	0.000	0.000	0.000											
Pica18		171	179	181	187	189	191	193	195	197	201	203	205	207	209	215									
N	14	0.107	0.286	0.000	0.143	0.036	0.071	0.000	0.071	0.036	0.071	0.036	0.036	0.036	0.036	0.036									
M	14	0.214	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.214	0.000	0.250	0.250	0.036	0.000									
S	23	0.348	0.043	0.000	0.022	0.022	0.000	0.109	0.174	0.000	0.022	0.152	0.000	0.109	0.000	0.000									
Piru8		108	141	143	147	151	155	159	163	167	171	175													
N	16	0.000	0.000	0.094	0.563	0.125	0.031	0.031	0.000	0.094	0.000	0.063													
M	8	0.000	0.071	0.143	0.714	0.000	0.071	0.000	0.000	0.000	0.000	0.000													
S	21	0.024	0.000	0.024	0.238	0.000	0.143	0.119	0.095	0.262	0.095	0.000													
Piru12		166	174	178	182	186	190	194	202	233															
N	16	0.094	0.031	0.000	0.156	0.281	0.406	0.031	0.000	0.000															
M	8	0.000	0.000	0.375	0.063	0.125	0.438	0.000	0.000	0.000															
S	23	0.000	0.109	0.000	0.130	0.326	0.152	0.196	0.022	0.065															