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Microsatellites in the Sand Lizard (*Lacerta agilis*): Description, Variation, Inheritance, and Applicability

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*We developed microsatellite markers for the sand lizard (*Lacerta agilis*) to enable investigations of the genetic variability within and among populations with a heterogeneous spatial distribution in Sweden. The populations, which could not be characterized by variation in allozymes or mitochondrial DNA, had a substantial level of variability in microsatellite loci. However, the variability in Swedish populations was limited compared to a large, outbred Hungarian population. In the sand lizard, the number of $(GT/CA)_n$ repeats was approximately three times higher than that for $(CT/GA)_n$. The number of repeats and the frequency of microsatellites were within the range reported for other species. Three of nine microsatellite loci showed alleles that could not be amplified, which is in agreement with recent reports describing microsatellite "null alleles" as a common occurrence. We discuss the caution which this calls for when calculating paternity probabilities and when estimating between-population allelic differentiation. A potential problem with different mutation rates for alleles within the same locus is discussed.*

KEY WORDS: sand lizard; *Lacerta agilis;* microsatellites; genetic variation.

INTRODUCTION

Microsatellite loci are the most polymorphic DNA markers available to date in eukaryotic species (Slatkin, 1995). A high mutation rate in a number of short-

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repeat regions constitutes the foundation for their extremely high level of polymorphism in most species. Apart from having the potential for showing a high level of variability, microsatellite techniques also require very small amounts of tissue, allowing the use of blood from juveniles of low body mass and the release of the animals immediately after blood sampling. This makes them ideal tools for studies of genetic variability in small and inbred populations when conventional markers, such as allozymes and mitochondrial DNA, fail this purpose.

During the past decade, DNA fingerprinting (Jeffreys *et al.,* 1985) and amplification of microsatellite loci (Tautz, 1989) have greatly increased the potential of detecting high levels of genetic variability compared to previous generations of conventional methods. Mini- and microsatellite loci show a high mutation rate (Jeffreys *et al.,* 1994; Weber and Wong, 1993) and thereby new genetic variation is generated quickly. These genetic markers can be utilized, for example, in analyses of recent population bottlenecks. So far, these techniques have been used in a very limited number of investigations of genetic variability in vertebrates in general and in amphibian and reptilian populations in particular [DNA fingerprinting (Parker and Whiteman, 1993; Aggarwal *et al.,* 1994; Lenk and Joger, 1994; Gray 1995) and amplification of microsatellite loci (Scribner *et al.,* 1994; FitzSimmons *et al.,* 1995; Villarreal *et al.,* 1996)].

Our model organism, the Swedish sand lizard (*Lacerta agilis*), occurs in populations of varying size and degree of isolation on the northern border of the species' distribution range, probably as a relict from the postglacial warmth period (Gislén and Kauri, 1959; Björck, 1995). In southern Sweden, there is a more or less continuous geographic distribution, probably with gene flow between the subpopulations. Farther north, the populations are small (n = 25–300 individuals) and patchily distributed (Gislén and Kauri, 1959; Ahlén and Tjernberg, 1992), and gene flow between the populations is probably very limited or absent. The distribution pattern makes this species an excellent model system for studies of evolutionary events in small and endangered populations, such as the isolated lizard populations (Ahlén and Tjernberg, 1992).

Swedish sand lizard populations show a very low level of genetic variability when estimated by allozyme electrophoresis and restriction fragment length polymorphism (RFLP) of mitochondrial (mt) DNA (Gullberg, unpublished). A higher level of variability was detected using DNA fingerprinting, but still the average similarity within Swedish populations was high (61%) compared to that in a large Hungarian population (19%) from the center of the distribution area (Olsson *et al.,* 1994). Despite a comparatively low level of variability, DNA fingerprinting was successfully used to determine paternity in controlled laboratory breeding experiments, where individuals were selected to maximize the genetic dissimilarity of potential sires (Olsson *et al.,* 1994). In a natural population, paternity of only 48% of the analyzed offspring could be determined using DNA fingerprinting, although the number of putative fathers was limited with the

help of observations of matings (Gullberg *et al.*, 1997). Consequently, studies of evolutionary and conservation genetics in this system calls for higher resolution techniques than DNA fingerprinting. To meet this demand we set out to develop microsatellites and evaluate their applicability in studies of genetic differentiation and for assignment of paternity.

MATERIALS AND METHODS

Construction of the Genomic Library

Sand lizard DNA (10 μg) extracted from blood of a male Romanian–Swedish hybrid from our laboratory collection was digested with restriction enzyme *Sau*3A at 37°C for 3 hr. Fragments smaller than approximately 70 base pairs (bp) were removed with a Chroma Spin + TE 100 column (Clontech) because small fragments were unlikely to contain long (and polymorphic) microsatellites. The remaining fragments were ligated into a *Bam*H1-digested and dephosphorylated vector (p Bluescript II SK+; Stratagene), and transformed into supercompetent XL1-blue cells (Stratagene) according to the suppliers' protocol.

About 2250 recombinant colonies were picked and transferred to LB-AMP-agarose plates. After allowing them to grow for 6–8 hr at 37°C, Hybond-N+ nylon membranes (Amersham) were placed on the plates. The membranes were alkaline-treated (Sambrook *et al.*, 1989) to make the DNA accessible and screening was carried out as described by Estoup and Cornuet (1994) using an equal mix of DIG end-labeled (Boehringer) polynucleotide $(GT)_{10}$ and $(CT)_{10}$. Hybridization was performed overnight at 50°C in 5× SSC, 1% blocking stock solution (Boehringer), 0.1% *N*-lauroylsarcosine, 0.02% SDS, with washing twice in 2× SSC, 0.1% SDS at 50°C for 15 min and at a high stringency in 1× SSC, 0.1% SDS at 50°C for 15 min. The colonies containing microsatellites were visualized by a color reaction using a DIG Nucleic Acid Detection Kit (Boehringer) (Stephen *et al.*, 1990). DNA sequencing was performed on alkaline-denatured plasmid DNA using a Pharmacia T7 sequencing kit with T3 and KS primers.

PCR primers for amplification of the microsatellites were constructed for the sequences flanking the repeat motif element (Tables I and II). The two primers in a primer pair were designed so that they had an equal number of purines and pyrimidines, about the same melting temperature, and a small probability of hybridizing to each other or to themselves. The length of the primers for the 10 loci that were chosen for amplification was between 17 and 24 bp, and they were synthesized by Operon Technology.

Blood was sampled from the corner of the mouth (*v. angularis*), transferred to Eppendorf tubes with a sterile 1× SSC buffer (0.15 *M* NaCl, 0.15 m*M* trisodium citrate, 0.5 m*M* EDTA, pH 7.0), and stored at −70°C. Two methods were used to extract DNA from the blood–buffer mix: Chelex-100 (Bio-Rad)

Table I. Characteristics of the 16 Sand Lizard Microsatellite Clones that Were Sequenced[a]

Clone	Locus	Microsatellite repeat motif	Other repeats	Category
1-119		$(GT/CA)_{16}$	$(TTA)_3$	Perfect
2-23	*La-1*	$(GT/CA)_{19}$	—	Perfect
7-31		$(GT/CA)_9$, $(AT/TA)_6$	$(AT)_3$, $(AT)_4$	Two perfect
7-37		$(GT/CA)_9$	—	Perfect
8-81	*La-2*	$(GT/CA)_{16}$	—	Perfect
10-11	*La-3*	$(GA/CT)_{28}$	—	Perfect
11-45	*La-4*	$(GT/CA)_{19}$	—	Perfect
11-111	*La-6*	$(GT/CA)_{17}$	$AAT(AAAT)_2$	Perfect
12-161	*La-5*	$(GA/CT)_{20}$	—	Perfect
12-177		$(AT/TA)_5$	$(GTT)_4$, $(TCC)_3$	Perfect
13-151	*La-7*	$(GT/CA)_{16}$	—	Perfect
13-161	*La-8*	$(GT/CA)_{14}$–$(GA/CT)_{17}$	$(TA)_3$, $(TA)_3$	Compound
13-177	*La-9*	$(GT/CA)_{12}$	$(GC)_3$	Perfect
14-103		$(GT/CA)_6$		Perfect
15-155	*La-10*	$(GT/CA)_{15}$–$(GA/CT)_{13}$	$(GAGAAA)_2G$ $(AGAGGG)_2(AG)_4$	Compound
18-15		$(GT/CA)_7$	$(GC)_4$, $(AGAC)_4$	Perfect

[a]The number of repeats found in each microsatellite and other repeats close to the microsatellites are given. Microsatellite categories are classified according to Weber (1990). Primers were synthesized for amplification of 10 loci (*La-1*–*La-10*).

DNA extraction (Walsh *et al.*, 1991) and salt–chloroform extraction (Mullenbach *et al.*, 1989). Chelex-100 DNA extraction was performed using 3–5 μl of blood–buffer mix, which was added to 1 ml of distilled water, incubated for 15–30 min at room temperature, and centrifuged for 3 min at 10,000*g*. All but 20–30 μl of the supernatant was discarded, and 200 μl of 5% Chelex-100 suspension was added and incubated at 56°C for 15–30 min. After vortexing, the samples were boiled in a water bath for 8 min, vortexed again, and centrifuged for 3 min at 10,000*g*. Finally, 1 μl of the supernatant was used for the PCR.

For the salt–chloroform extraction of DNA, approximately 30 μl of blood was incubated with 2.5 ml of SET buffer (0.15 *M* NaCl, 0.05 *M* Tris–HCl, 1 m*M* EDTA, pH 8.0), 50 μl of 25% SDS (w/v), and 70 μl of proteinase K (10 mg/ml) for 3–4 hr at 55°C. Proteins were removed by precipitation with 1.0 ml of saturated (6 *M*) NaCl, chloroform extraction, and centrifugation. DNA was precipitated with ethanol and dissolved in 0.4–1.5 ml of sterile 0.01 *M* Tris–HCl, pH 8.0, for at least 24 hr. In the PCR, 1 μl of the DNA solution (approximately 60–100 ng of DNA) was used.

Amplification of Microsatellites

All microsatellite amplifications were performed in a 10-μl mixture containing 1 μl of template DNA solution, 1× PCR buffer (following Finnzyme's recommen-

Table II. Primer Pairs for the 10 Loci that Were Tested for Mendelian Segregation Among Offspring with Known Parents[a]

Locus	Primer sequence	Primer length	T (°C)	Allele size range (bp)
La-1	AGG TTT CCT GGC TTG GAG	18	64	86–136
	ATT TGC ACA AAA CAG CAG C	19		
La-2	GCT TAA ATT GGA ACC AGA TTG	21	64–54	179–197
	AAG CAG CCA GAA CAC AGA G	19		
La-3	AGT AGG AGC GAG AAG AAT CAG	21	60	160–194
	GAC ATA TGG CAG AAG AGC AG	20		
La-4	CAT GAG CAA AGC AAT GAG C	19	60	138–160
	TGG AAT GTG TCA TTG AAC TCT G	22		
La-5	TAG ATG CAC TCA GAA TGA CTT C	22	54	Ca. 91
	AAC AGT ATT CTA AGG CTG TTC	21		
La-6	GAC TGG CGC ATT CTA TAA AAC	21	58	269–287
	GCC TTA AAG GGC CAT CAG	18		
La-7	CCT TTG TGG TCT CTT CCA AC	20	—	—
	CCT CAT AGG GTT GTC GTG AG	20		
La-8	AAC CAC TAG CAG AAA TCT CAT TC	23	60	Ca. 165
	GAC CTT GGA ATT TTC ACC TG	20		
La-9	AGA TGC TTT TAT ATA TGC AAC TTC	24	54	110–128
	GTG CCT TCA TTT GTT TAC TTC	21		
La-10	CCC TGA TAA AGC CCC AC	17	54	Ca. 184
	CAC TAG CTG AAA TAA GAA TGA GG	23		

[a]The number of nucleotides in each primer was 17–24. Optimized annealing temperatures [T (°C)] and the range of allele lengths in the investigated populations are given for each locus. For the loci with null alleles, the approximate size is given.

dations), a 75 μ*M* concentration each of nucleotides dGTP, dTTP, and dCTP, 6 μ*M* dATP, 0.625 μCi of α-^{35}S-labeled dATP, a 400 n*M* concentration of each primer, 20 μg/ml bovine serum albumin (BSA), and 0.4 U of the DNA polymerase DynaZyme (Finnzyme). The $MgCl_2$ concentration ranged between 1.0 and 2.5 m*M* when optimizing the PCR conditions and 1.0 m*M* $MgCl_2$ was routinely used for loci *La-1, La-2, La-3, La-4, La-6,* and *La-9.* Samples were processed through one denaturing step (3 min at 94°C) followed by 27–32 cycles of 30 sec of denaturing at 94°C, 30 sec at the annealing temperature (Table II), and 30 sec at the elongation temperature, 72°C. The amplification cycles were followed by an elongation step at 72°C for 10 min. For the loci that showed nonamplifying alleles, the annealing temperature was decreased stepwise down to 46°C but no extra alleles were identified. The amplifications were performed using an MJ Research PTC-100 thermocycler. After amplification, 7 μl of the product was mixed with 5 μl of formamide loading dye (bromophenol blue and xylene cyanol), heated to 85°C for 5 min, loaded, with size markers, on 6% polyacrylamide sequencing gels, and run for 4500–8000 Vh, depending upon the length of the amplified fragment (Table II).

Samples from four sand lizard families from our laboratory collection,

including the correct parents and their offspring ($n = 43$), were used to test the Mendelian inheritance of microsatellite alleles. To investigate the level of variability within and among Swedish sand lizard populations, 178 individuals from 10 populations were analyzed and compared with 25 sand lizards from a Hungarian population, representing a large central population (Fig. 1).

To investigate the cross-taxa applicability of the primers designed for the sand lizard, we also tested them on eight individuals of the closely related common lizard (*Lacerta vivipara*) using exactly the methods described for sand lizards.

The number of alleles was determined by direct counting and the expected heterozygosity was calculated assuming Hardy–Weinberg equilibrium. Wright's fixation index (F_{ST}) was calculated according to Weir and Cockerham (1984). The probabilities of false exclusion were calculated according to Jamieson (1994). The identity values, i.e., the probability that two individuals would show the same genotype by chance, were calculated according to Jeffreys *et al.* (1992).

RESULTS

The Genomic Library

The partial genomic library, consisting of 2250 clones, was screened and 128 positive clones were identified. The insert length varied substantially among different clones (200–2000 bp), with an average size of approximately 1000 bp. Sixteen of the positive clones were sequenced and seventeen microsatellites were found. Fourteen of the sixteen clones could not be completely sequenced due to the long insert length (>500 bp). Thirteen microsatellites contained $(GT/CA)_n$ repeats and four microsatellites contained $(CT/GA)_n$ repeats (Table I). Eleven of the $(GT/CA)_n$ repeats were perfect and two were compound as classified according to Weber (1990). Two of the $(CT/GA)_n$ repeats were classified as perfect and two as compound microsatellites. Two perfect $(AT/TA)_n$ repeats were found, but no imperfect repeats.

The average distance between microsatellite loci can be estimated by dividing the total length of screened DNA by the number of microsatellites identified, assuming that the Sau3A restriction site (GATC) and $(GT/CA)_n$ or $(CT/GA)_n$ microsatellite loci have independent distributions (Estoup *et al.*, 1993a). This assumption is widely accepted (references in Thorén *et al.*, 1995). In the sand lizard, the $(GT/CA)_n$ repeat motif occurs in the genome approximately every 22 kilobases (kb), and the $(CT/GA)_n$ motif every 70 kb.

Allelic Segregation and "Null Alleles"

Ten of the sequenced microsatellites were chosen and primer pairs were constructed complementary to the regions flanking the repeated motif (Table I). Six of

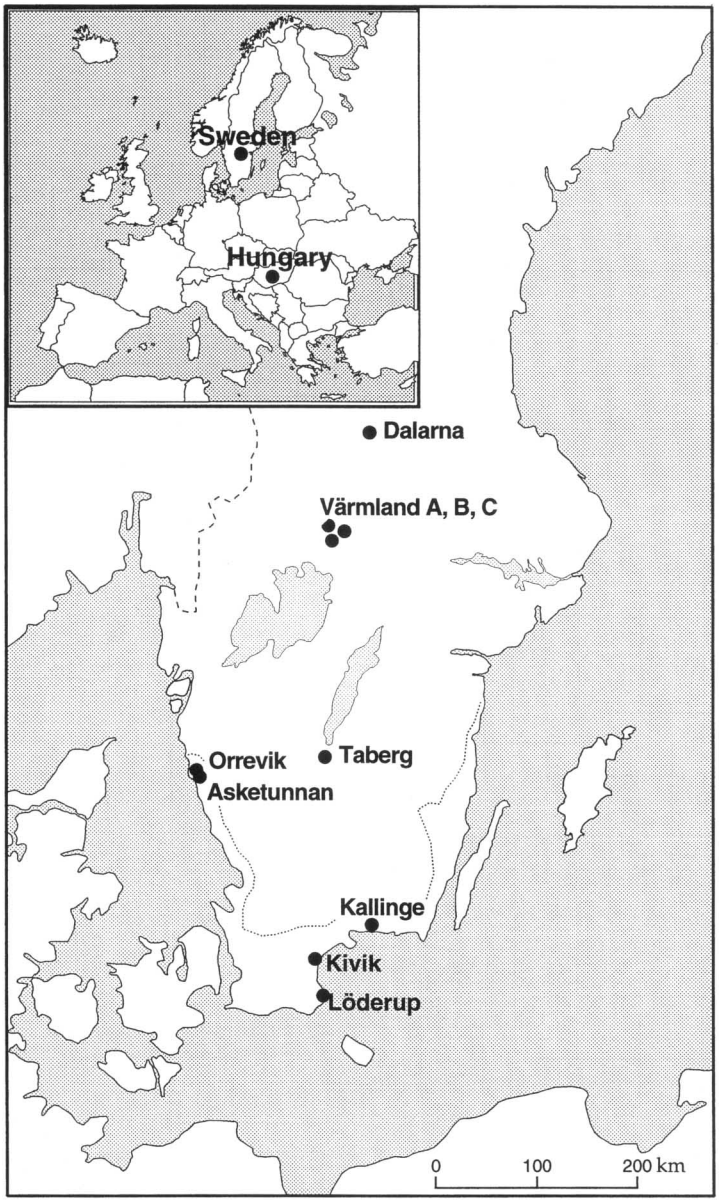

Fig. 1. Map of sampling sites.

these (Table II) showed Mendelian allelic segregation within families. Three loci (*La-5, La-8, La-10*) had segregation patterns that deviated from the Mendelian predictions. A probable explanation is that there were null alleles that could not be amplified due to a mutation within a primer site. The three loci with putative null alleles were subsequently not used in the population analysis. Two of these (*La-8* and *La-10*) were classified as compound repeats. The third locus showing a null allele consists of a $(GA/CT)_n$ repeat. One primer pair (*La-7*) failed to amplify a specific product at any temperature or $MgCl_2$ concentration. Thus, six microsatellite loci were suitable for subsequent use in the population analysis.

Genetic Variability

Locus *La-1* was highly polymorphic, with a total of 16 alleles in the Swedish populations and 14 alleles in the Hungarian population (Table III), and a total of 20 alleles was found among the 203 individuals. The distribution of allelic length was unimodal in the Hungarian population but bimodal in the 10 Swedish populations, which had one common allele (21 repeats) present in several populations and a group of alleles with lengths of 32–46 repeats, but no alleles of intermediate length.

The Hungarian population showed eight, nine, and eight alleles, respectively, in loci *La-2, La-4,* and *La-9,* whereas the Swedish populations in all had five alleles at *La-2* and *La-4* and four alleles at locus *La-9.* All three of these loci had a

Table III. The Number of Alleles, Expected Heterozygosity for Each Locus, and Level of Genetic Differentiation (F_{ST}) in and Between the Hungarian Population (n = 25) and 10 Swedish Populations (n = 178)

	No. of alleles			Heterozygosity[a]			F_{ST}	
Locus	Hungary	Sweden	Total	Hungary	Sweden	Total	Sweden	Total
La-1	14	16	20	0.878	0.595 (0.039)	0.610 (0.046)	0.187	0.181
La-2	8	5	10	0.747	0.450 (0.032)	0.477 (0.037)	0.213	0.254
La-3	6	12	13	0.467	0.643 (0.022)	0.627 (0.023)	0.132	0.150
La-4	9	5	10	0.786	0.255 (0.061)	0.303 (0.081)	0.652	0.581
La-6	3	4	4	0.582	0.396 (0.024)	0.412 (0.025)	0.293	0.300
La-9	8	4	10	0.765	0.365 (0.053)	0.401 (0.062)	0.432	0.376
Average	8.0	7.7	11.1	0.704	0.452 (0.055)	0.475 (0.057)	0.299	0.295

[a]Numbers in parentheses give the variance calculated for all populations.

total of 10 alleles in the pooled material. In contrast to all other loci, locus *La-3* showed a higher number of alleles in the Swedish sand lizards (12 alleles) than in the Hungarian population (6 alleles). The frequency distribution of alleles in this locus was bimodal in both Sweden and Hungary. Locus *La-6* showed the lowest level of variation, with three alleles in the Hungarian population and a total of four in the Swedish populations, and a total of four alleles among the 203 investigated individuals.

The average number of alleles per locus in the six loci was 8.0 in the Hungarian population, 7.7 in all in the Swedish populations, and 11.1 in the pooled sample. Hence, the variability detected with microsatellites was high compared to the variation found with other genetic methods. The expected heterozygosity ranged from 0.467 to 0.878 for the six loci in the Hungarian population and was on average 0.704. The mean expected heterozygosity for the six loci in the Swedish populations ranged from 0.255 to 0.643 and was on average 0.452 (Table III). For all loci, except *La-3,* the heterozygosity was higher in the Hungarian population than in all the investigated Swedish populations. Locus *La-3* showed the highest heterozygosity in Sweden (0.643) and the lowest level of heterozygosity in the Hungarian population (0.467).

The average F_{ST} for the 10 Swedish populations was 0.299 (range, 0.132–0.652; Table III). When the Hungarian population was included in the analysis, the F_{ST} value for the six loci did not change significantly. The F_{ST} values showed a strong subdivision between the Swedish sand lizard populations at all loci (Table III). The total F_{ST} for all populations, including the Hungarian population, was approximately the same as that for the Swedish populations. A more extended analysis of between-population subdivision of the genetic variation will be published elsewhere.

Four of the six loci were successfully amplified also in the common lizard. However, in this species all loci, except for *La-1,* showed alleles not found in the sand lizard, whereas in *La-1* all nine alleles occurred in both species.

Microsatellites as a Tool for Assigning Paternity

The potential for determining paternity in each population is illustrated by the probability of excluding an incorrectly assigned father (or mother), both for each locus separately and for all six loci combined (Table IV), ranging from 0 to 1. Loci *La-1* and *La-3* show the highest discriminative power, but the variation between populations was very high (*La-1* range, 0.089–0.762; *La-3* range, 0.134–0.619). The other four loci were not very informative for paternity tests by themselves but could increase the probability of exclusion of potential fathers in combination with other loci. The highest value for all loci combined was obtained in the Hungarian population (0.990). Seven of the ten Swedish populations showed paternity exclusion values of over 0.8.

Table IV. The Probability of Excluding an Incorrectly Assigned Father (or Mother) for Each Microsatellite Locus and Population and for All Six Loci Combined[a]

Population	Sample size	Locus						
		La-1	*La-2*	*La-3*	*La-4*	*La-6*	*La-9*	All
Hungary	25	0.762	0.530	0.257	0.617	0.310	0.547	0.990
Asketunnan	66	0.680	0.219	0.619	0.049	0.158	0.048	0.927
Orrevik	9	0.089	0.326	0.481	0.050	0.120	0.089	0.757
Taberg	14	0.520	0.361	0.444	0.189	0.229	0.266	0.921
Dalarna	27	0.507	0.128	0.468	0.034	0.187	0.181	0.853
Löderup	12	0.160	0.038	0.399	0.000	0.120	0.000	0.573
Kivik	5	0.272	0.382	0.134	0.000	0.347	0.082	0.766
Kallinge	9	0.474	0.185	0.251	0.229	0.089	0.143	0.806
Värmland-A	21	0.567	0.213	0.594	0.229	0.183	0.299	0.939
Värmland-B	5	0.412	0.134	0.346	0.082	0.082	0.336	0.814
Värmland-C	10	0.343	0.182	0.441	0.456	0.182	0.309	0.908
Average		0.435	0.245	0.403	0.176	0.182	0.209	0.841

[a]Sample size is the number of presumed unrelated individuals that have been investigated for all six loci.

We found a significant positive relationship between the average number of repeats and the number of alleles observed at the six investigated loci ($P = 0.0051$, Spearman's coefficient of rank correlation; Fig. 2), indicating that loci with a shorter length have a lower number of alleles.

DISCUSSION

Perfect motifs were the most common microsatellite repeat sequence in the sand lizard genome [88.2%, including one $(AT/TA)_6$ repeat]. The $(GT/CA)_n$ motif

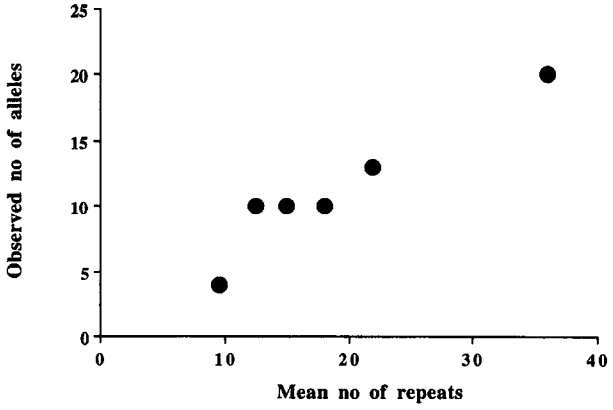

Fig. 2. The correlation between the average number of repeats for different alleles and the number of alleles that were identified among all lizards investigated.

occurs 3.2 times more often than the $(CT/GA)_n$ motif, which is in accordance with values for other vertebrates; for example, in humans $(GT/CA)_n$ repeats occur every 30 kb and $(CT/GA)_n$ every 113 kb (Stallings *et al.*, 1991), and in Brown trout, every 23 and 76 kb, respectively (Estoup *et al.*, 1993b).

No imperfect repeats were found in the sand lizard. In other investigated species, imperfect repeats are abundant; in humans the frequency of imperfect $(GT/CA)_n$ repeats is 25% (Weber, 1990), and the equivalent estimate in *Salmo salar* is 20% (Slettan *et al.*, 1993). In marine turtles, compound repeats were most frequent (58.3%), followed by perfect (29.2%) and imperfect repeats (12.5%) (FitzSimmons *et al.*, 1995). The lack of imperfect repeats in the sand lizard genome could be explained by the relatively small number of loci investigated (17 loci) compared to the other species (24 in the marine turtle, 45 in salmon, and 112 in humans) but may also reflect a true taxonomic difference between the species.

We have previously tried other methods to reveal genetic variability in Swedish sand lizards using mitochondrial DNA and minisatellites, which revealed low levels of variability (Olsson *et al.*, 1994; Gullberg *et al.*, unpublished). In contrast, the microsatellites reveal a high level of variation in both the Swedish and the Hungarian populations, similar to that found in other outbred species (Beckman and Weber, 1992; Estoup *et al.*, 1993b; Taylor *et al.*, 1994; Allen *et al.*, 1995; Primmer *et al.*, 1995). We found an average of 7.7 and 8.0 alleles per locus over the six loci in the entire Swedish and Hungarian populations, respectively. However, the level of variability fluctuated considerably between loci, with the number of alleles ranging from 4 to 20 and the heterozygosity index from 0.255 to 0.878. The difference in variability between loci offers a possibility to select microsatellites with an optimal resolution to address our two main problems: (i) analysis of paternity in natural populations and (ii) microevolutionary processes in the species. Fortunately, the microsatellite loci revealed in our investigation fulfil the requirements for both tasks, with a considerable number of variable loci and other loci with a presumably lower mutation rate, with few new alleles, which confounds the common ancestry between the populations.

The difference in heterozygosity, not only between populations for the same locus but also between loci, suggests that when a defined combination of microsatellites is used to determine paternity, the probability of excluding a male may vary between different populations. Two loci (*La-1* and *La-3*) were the most variable, with an average paternity exclusion index higher than 0.4, but in some populations other loci were better suited for paternity determinations. Thus, allele frequency data from one population cannot be directly applied to another population for parentage assignment. Information on the allele frequency distribution must thus be obtained for each population where paternity or parentage is to be examined. In the sand lizard, the central large Hungarian population showed an expected high microsatellite variability (average $H_{exp} = 0.70$), resulting in a high paternity exclusion value (0.99) when all six loci were combined. If all six loci are combined, the mean probability that two individuals would show the same

genotype by chance is very low (1.2×10^{-6}). The small Swedish populations generally had a lower level of variation (average $H_{exp} = 0.45$; range, 0.21–0.61) and a corresponding lower level of paternity exclusion power (average, 0.82; range, 0.57–0.95). The mean probability that two individuals would show the same genotype by chance was 5.9×10^{-3} but varied considerably between populations (4.4×10^{-3}–7.6×10^{-5}). Contrary to our data for DNA fingerprinting, the level of microsatellite variation is high enough to assign paternity, even in the Swedish populations.

The F_{ST} values for population differentiation indicate a strong subdivision ($F_{ST} = 0.30$; Table III), not only between Swedish and Hungarian sand lizards, but also between Swedish populations within the region which has been characterized as a continuous population (Gislén and Kauri, 1959). The level of differentiation between the Swedish populations is remarkably high compared to that found in other species using microsatellite variation (Scribner *et al.,* 1994; Taylor *et al.,* 1994; Allen *et al.,* 1995). The main explanation for this finding probably is an absence of gene flow, even between populations within the same region, and the small size of most populations, allowing stochastic allele changes to take place. Thus, our results indicate that migration between populations may be much less frequent than expected from previous understanding of the sand lizard's population structure.

The occurrence of null alleles may confound investigations of natural populations where the opportunity to check for nonamplifying alleles using a family material is limited. Three (*La-5, La-8,* and *La-10*) of nine amplifying loci in the sand lizard showed nonamplifying alleles, which suggests that "null alleles" could be common (e.g., Chakraborty *et al.,* 1992; Callen *et al.,* 1993; Koorey *et al.,* 1993; Ede and Crawford, 1995; Paetkau and Strobeck, 1995; Pemberton *et al.,* 1995; Primmer *et al.,* 1995). Amplification of these alleles was equally unsuccessful when the priming stringency was lowered but redesigned primers, avoiding mutation sites, could perhaps reveal additional alleles in these three loci.

According to Weber (1990), the level of polymorphism of perfect repeat sequences is a function of repeat length and determined primarily by mutation rates. A positive correlation between the mean number of repeats and the number of alleles or variance in repeat counts has been reported (Ostrander *et al.,* 1993; FitzSimmons *et al.,* 1995; Goldstein and Clark, 1995), suggesting that the mutation rate indeed increases with the number of repeats. A similar phenomenon has been described for minisatellites (Gray and Jeffreys, 1991), for which the evolution may be divided into different phases, characterized by the amount of allelic variability and different mutation rates. The "dwell" phase is characterized by a high number of repeats, hypervariability, and a high mutation rate. Finally, deletions produce short alleles which gradually cause a loss of hypervariability during the following "decay" phase. The variability is drained because the short

alleles show a lower mutation rate than the longer alleles and may act as traps. Thus, mutation rates for homologous microsatellite loci may differ not only between taxa, but also between alleles, which certainly will complicate the analysis when microsatellite variation is used to reconstruct historical population events. Two populations with a high frequency of short alleles do not necessarily have to be closely related. The short alleles may simply be common in both populations due to a similarity in evolutionary phase, rather than close ancestry. The effect of mutation-rate variations between alleles on population genetic parameters will be difficult to assess. However, through an analysis of the differences between populations in the distribution of allelic lengths, indications of nonhomologous similarities can be achieved, and mistakes can probably be avoided when microevolutionary scenarios are reconstructed.

Because four of six loci were successfully amplified also in the common lizard, it is conceivable that our sand lizard primers could be useful for amplification of microsatellite loci in other reptiles as well, especially among the Lacertidae. This is consistent with Jarne and Lagoda's recent review (1996), which suggests that cross-taxa applicability of microsatellites should be common.

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