Population genetics of the dice snake (*Natrix tessellata*) in Germany: implications for conservation

DANIELA GUICKING, ANDREA HERZBERG & MICHAEL WINK

Abstract

A molecular study on the population genetics of German dice snakes (*Natrix tessellata*) was carried out to supplement our knowledge on the microevolution and local status of this in Germany critically endangered species. In Germany, the dice snake is restricted to three isolated populations at the rivers Lahn, Mosel, and Nahe. A fourth autochthonous population at the river Elbe went extinct during the 20th century. The dice snake was reintroduced to the river Elbe in 1999 and 2000 with animals originating from the Czech Republic. We analyzed mitochondrial cytochrome *b* sequences and six microsatellite loci of the German and Czech populations and compared these results to data from populations in Austria, Slovenia and Romania. Significant differentiation of the isolated German and Czech populations from southern populations underscores the conservation value of the German dice snakes. Unexpected divergence of the Lahn population from the populations at the rivers Mosel and Nahe raises new questions on the microevolution of the species in Germany. Extremely low within-population variation was detected for all of the isolated populations and is interpreted as the result of founder effects during postglacial colonization and increased genetic drift due to small population sizes, fragmentation and anthropogenically driven population bottlenecks in the past. For the conservation of the species, further population declines and fragmentation must be prevented under all circumstances to reduce further loss of genetic diversity and the risk of inbreeding depression.

Key words: conservation genetics; cytochrome *b*; microsatellite analysis; population differentiation; Squamata: Colubridae: *Natrix tessellata*.

1 Introduction

Several studies have demonstrated the utility of molecular approaches to the conservation of endangered species and snakes in particular (e. g. O'BRIEN 1994, GIBBS et al. 1994, MADSEN et al. 1996, VILLARREAL et al. 1996, GIBBS et al. 1997, ÚJVÁRI et al. 2002). Genetic data provide information on population structure and within-population diversities that often cannot be obtained through traditional demographic studies (AMOS & HOELZEL 1992, MILLIGAN et al. 1994). Data on the genetic structure of populations may reveal evidence for restricted gene flow or genetic isolation that help identifying populations which should receive highest conservation priority (GIBBS et al. 1997, PETIT et al. 1998). Estimates of within-population diversities may reveal recent or ongoing changes in population structure and are of particular interest, because genetic variation is essential for the adaptability of a population and to prevent inbreeding depression (MILLIGAN et al. 1994, BOOY et al. 2000). Populations at the border of the geographic range may contain unique patterns of genetic variation and may therefore be of particular value for maintenance of the long-term evolutionary perspective of a species (LESCIA & ALLENDORF 1995, BOOY et al. 2000).

The dice snake (*Natrix tessellata*) is distributed throughout the western Palearctic from Italy over the Balkans, Near East, and into China. In Germany, it reaches its northwestern distribution limit (GRUSCHWITZ et al. 1999). Today, it is restricted to three isolated populations at the rivers Mosel, Nahe, and Lahn in western Germany. A fourth autochthonous population in eastern Germany, at the river Elbe, became extinct during the 20th century (GRUSCHWITZ et al. 1999). Due to decreasing population sizes and isolated localities, the dice snake is considered to be critically endangered in

Germany (GRUSCHWITZ et al. 1993, GRUSCHWITZ et al. 1999). Anthropogenic alteration of river and shore systems as well as an increasing use of aquatic habitats for recreational purposes are seen as the main factors that led to population decline and fragmentation (GRUSCHWITZ et al. 1993). Population monitoring and conservation efforts have been started more than 25 years ago. Through habitat restoration, establishment of protected areas, a captive breeding program and public education, the situation of the German dice snakes has greatly improved during the last two decades (LENZ & GRUSCHWITZ 1993). Of major importance in this context are the achievements of the "Erprobungs- und Entwicklungsvorhaben" for the conservation of the dice snake in Germany (SCHMIDT & LENZ 2001, HERZBERG & SCHMIDT 2001, LENZ & SCHMIDT 2002). In the framework of this project, the dice snake has been reintroduced to the river Elbe, with animals originating from the rivers Eger and Berounka in the Czech Republic (SCHMIDT & LENZ 2001).

To supplement our knowledge on the status of the German dice snakes, a genetic study seemed desirable. Here, we report results on population differentiation and within-population genetic diversities of the isolated German and Czech populations based on an analysis of mitochondrial DNA and six nuclear microsatellite loci. These are compared to data obtained from populations from Austria, Slovenia and Romania. Strong differentiation between the German populations and very low within-population diversities corroborate the necessity of further management efforts and raise new questions about the microevolution and extent of anthropogenic impact on central European dice snakes.

2 Material and Methods

2.1 Origin of samples

A total of 36 German (18 from river Mosel, 7 from river Nahe and 11 from river Lahn) as well as 36 Czech dice snakes were included in the study (Fig. 1). The Czech samples originated from the rivers Eger and Berounka. Eight out of a total of 28 samples from river Eger and all eight samples from river Berounka were obtained from animals that were translocated to the German Elbe river for reintroduction. Further, 29 samples from three populations located within the species' continuous distribution range were included for comparison: seven of these samples originated from the Tulcea region at the mouth of the Danube river in Romania, six from Hrastnik at river Sava in Slovenia, and 16 from Carinthia in Austria (Fig. 1). The Carinthian population is located at the edge of the species' continuous distribution range, whereas the Romanian and Slovenian populations are located more centrally.

Small amounts of blood taken from the caudal vein of living snakes or tissue samples from dead animals served as a source for DNA. Total DNA was isolated following standard proteinase k and phenol/chloroform protocols (SAMBROOK et al. 1989).

2.2 Cytochrome *b* sequencing

The target fragment was amplified by PCR with specific primers situated in the two tRNA genes flanking the cytochrome *b* gene in snakes, and PCR products were sequenced directly on automated sequencers under the conditions given in GUICKING et al. (2002). Complete sequences of the cytochrome *b* gene (1117 nt) were obtained and aligned manually. In all samples the signal for termination of translation was a

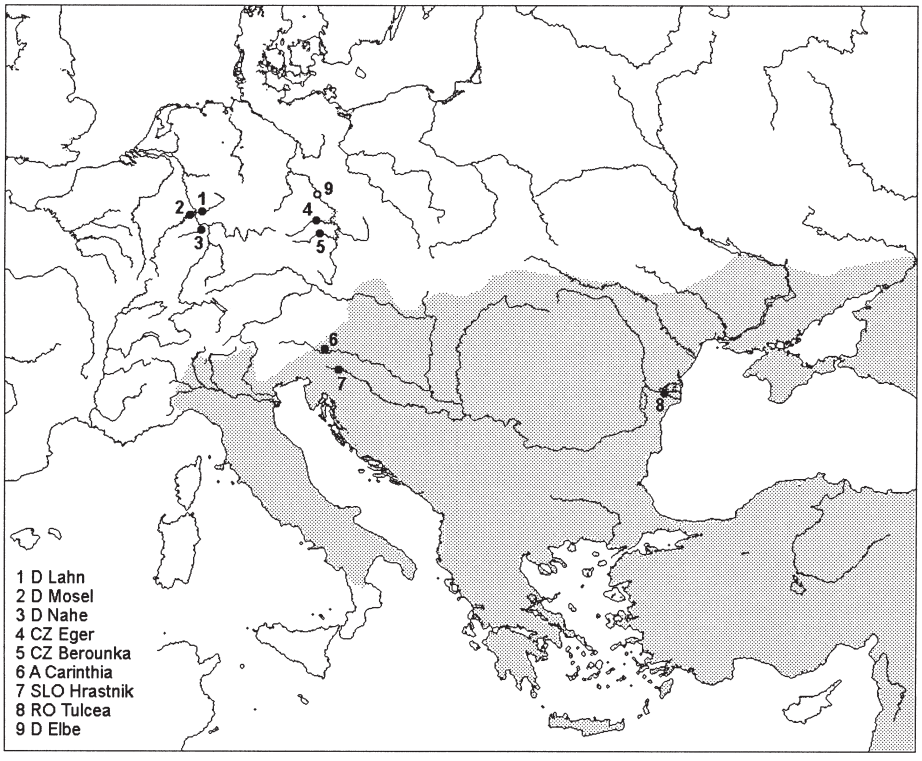

Fig. 1. Geographic location of the eight sample populations. Population 9 refers to the introduced population at the river Elbe in Germany. Individuals from this locality originated from the rivers Eger and Berounka in the Czech Republic (localities 4 and 5). The shaded area represents the distribution range of the dice snake.

Geografische Lage der untersuchten Populationen. Die Population 9 bezieht sich auf die wieder angesiedelte Population an der Elbe in Deutschland. Individuen dieser Population stammen von den Populationen an der Elbe und Berounka in der Tschechischen Republik ab (Fundorte 4 und 5). Das Verbreitungsgebiet der Würfelnatter ist punktiert dargestellt.

terminal T which is post-transcriptionally adenylated to form a functional stop codon. Variable sites of the sequences were determined with the program package Mega 2.0 (KUMAR et al. 2001). As a reference for cytochrome *b* data the sequence of one individual from Bulgaria was used. A minimum spanning network was reconstructed with the program Arlequin 2.0 (SCHNEIDER et al. 2000) to illustrate relationships between the observed haplotypes. The complete cytochrome *b* sequences analyzed in this study were deposited in GenBank under the accession numbers listed in Table 1.

2.3 Microsatellite analysis

For population genetic inferences, six microsatellite loci were analyzed with primers µNt8, µNt7, µNt6, µNt5, µNt3, and µNt2 published by GAUTSCHI et al. (2000). PCR amplification was performed in a 25 µl reaction volume containing 10-50 ng of total

	nucleotides	amino acids	GenBank no.
	2 3 3 3 3 4 4 5 5 6 6 6 7 8 9	1 1 1 2 2 2 2 3	
	9 7 0 1 1 3 7 9 5 8 0 6 8 1 8 5	3 0 6 9 2 3 4 9 1	
	7 9 0 0 8 9 1 0 8 4 0 7 8 8 7 5	3 4 4 5 3 0 0 6 9	
Bulgaria (2)	GAAGGCGGCAACGGTA	VVDELADLT	AY487646
Germany Mosel (10)	• • • • • • • • • • • • • • • •	• • • • • • • • •	AY487646
Germany Nahe (5)	• • • • • • • • • • • • • • • •	• • • • • • • • •	AY487646
Germany Nahe (2)	• G • • • • • • • • • • • • • •	• • • • • • • • •	AY487648
Germany Lahn (11)	• • • • • • • • • • • • • A • •	• • • • • • N • •	AY487653
Czech Rep. Eger (16)	A • • • • • • • • • • • • • • G	I • • • • • • • A	AY487645
Czech Rep. Berounka (6)	A • • • • • • • • • • • • • • G	I • • • • • • • A	AY487645
Romania Tulcea (2)	• • • • • • A • • G • • • • • •	• • • G • • • • •	AY487647
Romania Tulcea (2)	• • • A • T • • • • G • • • • •	• M • • • • • • •	AY487638
Romania Tulcea (4)	• • • A • T • • T • G • • • • •	• M • • • • • • •	AY487639
Slovenia Hrastnik (9)	• • • • • T • • • • G • A • • •	• • • • • T • • •	AY487659
Slovenia Hrastnik (1)	• • T • AT • • • • G • A • • •	• • • • • T • • •	AY487672
Austria Carinthia (17)	• • • • • T • A • • GA • • C •	• • N • I • • P •	AY487644

Tab. 1. Variable sites of cytochrome *b* sequences in the eight study populations of the dice snake. Presented are nucleotide and amino acid positions that show differences to the reference sequence from Bulgaria. Identity to the reference sequence is indicated by a "• ". The number of shared haplotypes in each population is given in brackets. In the last column, the GenBank accession numbers for the corresponding haplotypes are indicated.

Variable Positionen des Cytochrom *b* Gens bei den acht untersuchten Würfelnatter-Populationen. Angegeben sind die im Vergleich zu einer aus Bulgarien stammenden Referenz-Sequenz abweichenden Nukleotide bzw. Aminosäuren. Ein „• " bedeutet Identität mit der Referenzsequenz. Die Anzahl identischer Haplotypen aus einer Population ist jeweils in Klammern angegeben. In der letzten Spalte sind die GenBank Referenznummern der jeweiligen Haplotypen-Sequenzen angegeben.

genomic DNA, 5 pmol each of forward and reverse primer, 0.2 mM of each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 % Triton x-100, 10 mM Tris-HCl (ph 8.5), and 0.75 units of Taq polymerase (Amersham Biosciences). After an initial denaturation step (94 °C, 5 min), 31 cycles (36 for µNt5) were performed at 94 °C for 30 s, the locus-specific annealing temperature for 20 s, and 72 °C for 30 s. A final elongation step at 72 °C for 10 min followed. Optimal annealing temperatures as determined in a gradient PCR were 54 °C for µNt2 and µNt5, and 58 °C for µNt3, µNt6, µNt7, and µNt8.

The amplified products were genotyped on an ALF Express II genetic analyzer using the software ALFwin™ Fragment Analyzer 1.00 (Amersham Biosciences). For external and internal standards, fragments of lengths 75 bp, 99 bp, 156 bp, 193 bp, 199 bp, 260 bp, 317 bp, and 440 bp from the cytochrome *b* gene of *N. tessellata* were obtained through PCR with specially designed primers (D. GUICKING, unpubl.). For each analysis, four fragments were used as internal standards and six fragments as external standards. All results from genotyping were validated by repetition of the analyses. Single fragments were sequenced to determine exact lengths of fragments that served as additional size references and to check for mutations in the microsatellite and flanking regions. Locus- and population-specific allele frequencies are listed in the appendix.

Analysis of microsatellite data was performed using several population genetic software packages. To test for deviation from HARDY-WEINBERG equilibrium (HWE), measures of F_{is} were calculated and tested with the exact HARDY-WEINBERG test of GUO & THOMPSON (1992) as implemented in the program Genepop (RAYMOND & ROUSSET 1995). All loci were tested for two-locus linkage disequilibrium (RAYMOND & ROUSSET 1995). No significant results were obtained in this analysis, confirming that genotypes at one locus were independent of those at the other loci.

Levels of genetic differentiation among populations were assessed by calculating F_{st} and R_{st} values, estimated as theta (WEIR & COCKERHAM 1984) with the program Fstat 2.9.3.2 (GOUDET 1995) and rho with the program Rst Calc 2.2 (GOODMAN 1997). R_{st} is an analogue of F_{st} which has been specifically developed for the analysis of microsatellite data as it takes into account variances in allele size and utilizes a stepwise mutation model (SLATKIN 1995). Statistical significance of pairwise differentiation was tested with the same programs, using a log-likelihod G-Test for F_{st} and a permutation procedure for R_{st} values. We present measures based on both values, because at least one locus (µNt6) showed disjunct distribution of alleles, raising the possibility that mutations at this locus did not follow the stepwise mutation model (see below).

To reconstruct evolutionary relationships, an unrooted Neighbor Joining phylogram was calculated with the program Populations 1.2.28 (http://www.cnrs-gif.fr/pge) based on the CAVALLI-SFORZA and EDWARDS' chord distance (CAVALLI-SFORZA & EDWARDS 1967). This distance measure has been shown most efficient in obtaining the correct tree topology under many conditions (TAKEZAKI & NEI 1996).

Analysis of Molecular Variance (AMOVA, EXCOFFIER et al. 1992) was performed with the program Arlequin 2.0 (SCHNEIDER et al. 2000) to determine the amount of genetic variation attributable to within- and between-population variation both for the German and the Czech populations.

To examine within-population genetic diversities, several indices were calculated and compared between eight populations. Mean number of alleles per locus, allelic richness (PETIT et al. 1998) and NEI's gene diversity (NEI 1987) were calculated with the program Fstat 2.9.3.2 (GOUDET 1995). Mean observed and expected heterozygosities were obtained with Genepop (RAYMOND & ROUSSET 1995). All indices were tested for significant differences between populations using a FRIEDMAN ANOVA and WILCOXON matched pairs tests, following VAN HOOFT et al. (2000).

Where appropriate, a sequential BONFERRONI correction was applied to correct for multiple statistical tests (RICE 1988).

3 Results

3.1 Plausibility of the data

There are a few characteristics inherent to microsatellite data that may lead to false inference and therefore should be checked prior to population genetic analysis, one of these is the potential occurrence of null alleles. To test for the presence of null alleles, all eight populations and six loci were analyzed for deviations from HARDY-WEINBERG equilibrium (HWE) (JARNE & LAGODA 1996), giving a total of 48 tests. In 19 cases, loci within single populations were monomorphic and could therefore not be tested. Of the remaining 29 tests, seven showed significant deviations from HWE. Only one combination (locus µNt2, population CZ Eger) was significant after sequential BONFERRONI correction. All deviations from HWE resulted from heterozygote deficiencies. Referring to the uncorrected F_{is} values, departures from HWE were found in almost

all populations at locus μNt2 and therefore may indicate the presence of null alleles at this locus (PEMBERTON et al. 1995). By sequencing μNt2 fragments from the Czech population of the river Eger, two point mutations were detected. One fragment contained an uninterrupted microsatellite $(GT)_{22}$, whereas the other fragment consisted of a two-fold interrupted microsatellite $(GT)_4TT(GT)_2TT(GT)_{12}$. This gave further evidence that at least in this population identical fragment lengths may not correctly specify identity by descent for two fragments.

In spite of these potential difficulties, we did not exclude locus μNt2 from further data analysis for three main reasons. First, GAUTSCHI et al. (2000) did not find indication of null alleles at locus μNt2, suggesting that the irregularities we detected may be population specific or driven by chance alone. Secondly, raw data on allelic distribution at locus μNt2 did not show great differences to those at other loci. Thirdly, exclusion of this locus had no major influence on the overall results except that observed patterns were less apparent.

3.2 Population differentiation

To determine the spatial pattern of population differentiation, we followed various approaches. In a first estimation, population differentiation was evaluated simply on the similarity of cytochrome *b* sequences. This revealed identical haplotypes for all samples from the two Czech populations, whereas the German populations comprised three different haplotypes (Tab. 1). At the rivers Mosel and Nahe the same haplotype was detected as in the reference animal from Bulgaria. A second haplotype was found only in the Nahe population. The Lahn population seemed to be homogenous for an own haplotype. German dice snakes were differentiated from Czech dice snakes by two or three nucleotide and amino acid substitutions (Tab. 1). The minimum spanning network in Figure 2 depicts the phylogenetic relationships between the observed cytochrome *b* haplotypes.

We calculated overall and pairwise F_{st} and R_{st} values based on microsatellite data to quantify population differentiation. Overall F_{st} values were greater than 0.20 for all six loci, indicating strong differentiation between the eight sample populations (HARTL & CLARK 1997). Pairwise F_{st} and R_{st} values are given in Table 2. No significant difference between the results from both parameters was detected by a MANTEL test (correlation coefficient 0.89, $p < 0.001$, program Arlequin 2.0, SCHNEIDER et al. 2000). Significance of pairwise F_{st} and R_{st} values after sequential BONFERRONI correction was detected for most population pairs with only three exceptions. No differentiation was found between the two Czech populations, and differentiation between the German populations of Mosel and Nahe was only weak. Differentiation between the populations of Tulcea (Romania) and Hrastnik (Slovenia) was only significant according to F_{st} but not to R_{st} values (Tab. 2).

AMOVA ascribed 44.2 % of the observed genetic variation in German dice snakes to among-population differences and 55.8 % to within-population differences, based on F_{st} values. Based on R_{st} values, 99 % of the variation was ascribed to among-population differentiation. The difference between F_{st} and R_{st} based values could be attributed to an extreme variation of allele lengths at locus μNt6 between the Lahn population on the one hand and the Mosel and Nahe populations on the other. The locus μNt6 refers to a compound microsatellite $(CT)_n(GT)_m$ that includes long alleles with both parts of the microsatellite present and short alleles, in which the entire first part of the microsatellite is missing. Therefore, in this case R_{st} seems less appropriate than F_{st} for population genetic inference. In a comparison of the more closely related

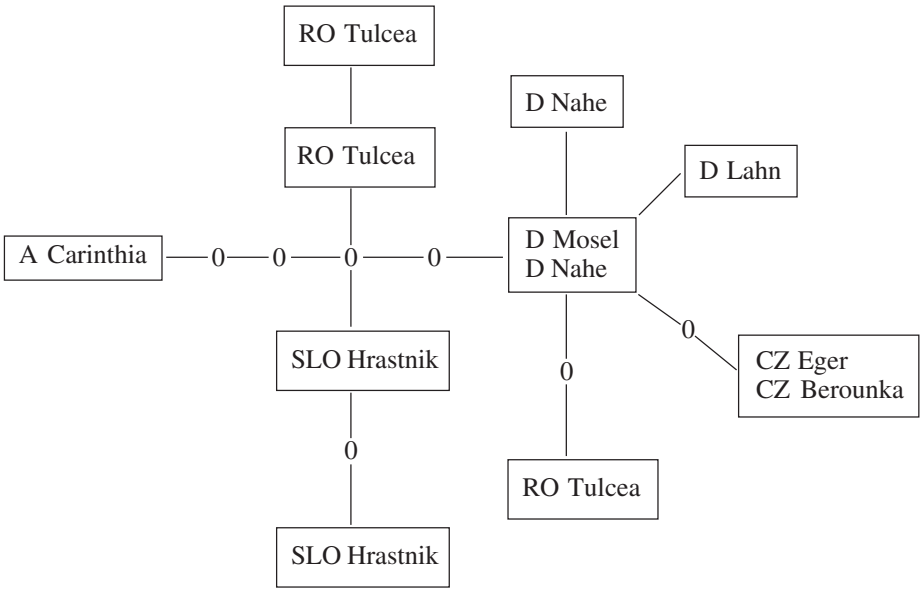

Fig. 2. Minimum spanning network of the observed cytochrome *b* haplotypes detected in the eight European *N. tessellata* populations. Each line represents one mutational change, and zeros indicate missing intermediate haplotypes, which have to be assumed in order to connect the network.

Minimum Spanning Netzwerk der in den acht europäischen *N. tessellata* Populationen nachgewiesenen Cytochrom *b* Haplotypen. Jede Linie stellt eine Mutation dar und Nullen bezeichnen nicht nachgewiesene intermediäre Haplotypen, die zur Verbindung des Netzwerks notwendig sind.

	D Lahn	D Mosel	D Nahe	CZ Eger	CZ Berounka	A Carinthia	SLO Hrastnik	RO Tulcea
D Lahn		0.7384***	0.7353***	0.6758***	0.6563***	0.4339***	0.5911***	0.3879**
D Mosel	0.4600**		0.266(*)	0.858***	0.8457***	0.6575***	0.6505***	0.4824***
D Nahe	0.5211**	0.1080^{ns}		0.8594***	0.8462***	0.6672***	0.6532***	0.4785**
CZ Eger	0.6413**	0.6567**	0.7001**		-0.0106^{ns}	0.5476***	0.5956***	0.3653***
CZ Berounka	0.6198**	0.6733**	0.7809**	-0.0309^{ns}		0.5518***	0.6007***	0.3698**
A Carinthia	0.4033**	0.4696**	0.4696**	0.5397**	0.4777**		0.278***	0.1372**
SLO Hrastnik	0.4473**	0.5816**	0.5484**	0.5649**	0.4576**	0.2667**		0.4173^{ns}
RO Tulcea	0.3896**	0.5136**	0.4789**	0.5254**	0.3988**	0.2914**	0.1253**	

Tab. 2. Pairwise genetic distances between the sample populations as described by R_{st} (above diagonal) and F_{st} values (below diagonal), based on six microsatellite loci. Statistical significance of differentiation between population pairs was calculated with a log-likelihood G-test for F_{st} and with a permutation procedure for R_{st} values. Parentheses indicate that significance was not valid after sequential BONFERRONI correction. Negative values mean that within-population variance was bigger than among-population variance, indicating no differentiation. Levels of statistical significance are indicated as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and n.s. = not significant.

Paarweise genetische Distanzen zwischen den untersuchten Populationen basierend auf sechs Mikrosatelliten-Loci, angegeben als R_{st}- (über der Diagonalen) und F_{st}-Werte (unterhalb der Diagonalen). Statistische Signifikanz der Populationsdifferenzierung wurde mit dem log-likelihood G-test für die F_{st}-Werte und mit einem Permutationsverfahren für die R_{st}-Werte berechnet. Klammern zeigen an, dass der Wert nach einer sequentiellen BONFERRONI-Korrektur nicht mehr signifikant ist. Negative Werte bedeuten, dass die Variation innerhalb der Populationen größer als die zwischen den Populationen ist und damit keine Populationsdifferenzierung vorliegt. Die Irrtumswahrscheinlichkeiten sind angegeben als * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, und n.s. = nicht signifikant.

populations from Mosel and Nahe, only 13 % of the observed variation resulted from population differentiation, irrespective of the distance value used. For the two Czech populations, AMOVA ascribed all observed variation to within-population diversity.

A neighbour joining tree derived from microsatellite data and based on CAVALLI-SFORZA and EDWARDS' chord distance (Fig. 3) clearly supports the former results: (1) no clear differentiation between the two Czech populations, (2) close relationship between the German populations from the rivers Mosel and Nahe, and (3) a stronger differentiation of these two populations from the third German population at the river Lahn. Also, the distinction of both Czech and German populations to each other as well as to the populations of the continuous distribution range is supported (Fig. 3).

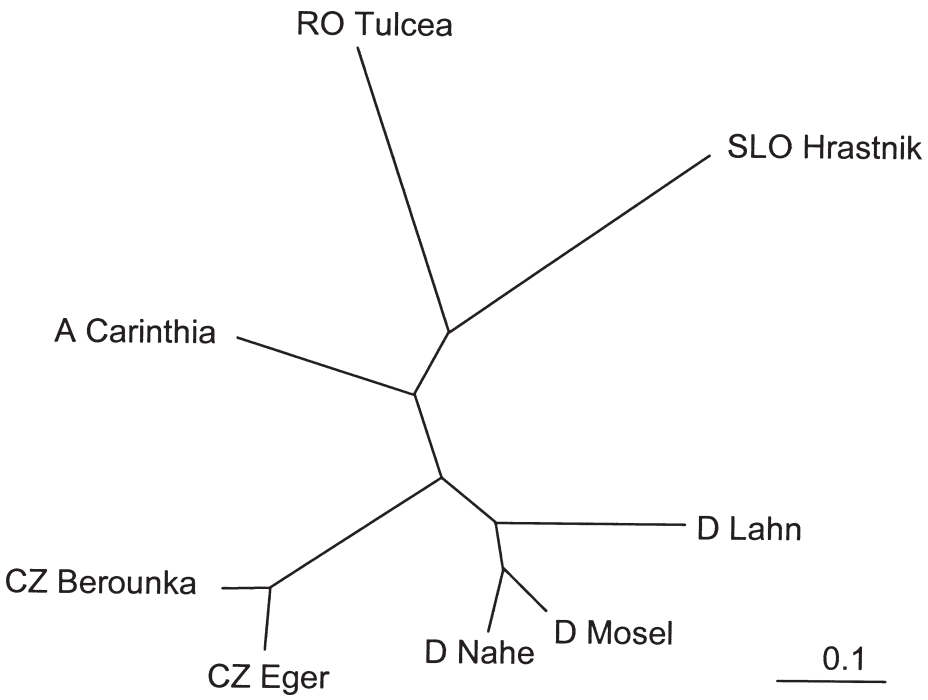

Fig. 3. Unrooted neighbour joining phylogram, based on data from six microsatellite loci and calculated with CAVALLI-SFORZA and EDWARDS' chord distance.

Ungewurzeltes Neighbour Joining Phylogramm, basierend auf den Daten von sechs Mikrosatelliten-Loci und berechnet mit dem Distanzmaß von CAVALLI-SFORZA und EDWARDS.

3.3 Genetic variation within populations

To infer information on the within-population variation, genetic diversity indices were compared between the isolated populations and the populations of the continuous distribution range.

According to cytochrome *b* sequences, genetic diversity was clearly reduced in the northern populations. In four of the five isolated populations only one haplotype was

detected (Tab. 1). Only in the Nahe population, two haplotypes differing by a single nucleotide substitution were present. Also, the Carinthian population was homogeneous for a single cytochrome *b* haplotype, whereas in the Slovenian and Romanian populations two and three haplotypes occurred (Tab. 1).

Genetic diversity indices inferred from microsatellite data are presented as means ± standard deviations of all six loci in Table 3. Indices may be underestimated at least for the Czech population from river Eger because of the potential occurrence of null alleles and non-detectable point mutations at locus μNt2, as discussed above.

Genetic diversity was reduced for all parameters in the isolated populations in Germany and the Czech Republic when compared to more southern populations. In the Nahe population all six loci were monomorphic, reflecting lowest possible genetic variation of this population according to microsatellite data. In accordance with cytochrome *b* data of the three southern populations the one from the edge of the continuous distribution range in Carinthia (Austria) was less diverse than the more central populations from Slovenia and Romania.

FRIEDMAN ANOVA determined highly significant differences ($p < 0.001$) between populations for all indices given in Table 3. WILCOXON matched pairs tests yielded no significant differences of genetic diversity between the five northern populations. Also, no significant differences were detected between indices of the Lahn and Mosel populations in comparison to the Carinthian population, and most indices were not significantly different between the populations of Tulcea (Romania) and Hrastnik (Slovenia).

	N	rel. location	P	A (x ± SD)	R (x ± SD)	H_o (x ± SD)	H_e (x ± SD)	D (x ± SD)
Germany Lahn	11	isolated	3	1.67 ± 0.82	1.59 ± 0.68	0.485 ± 0.229	0.474 ± 0.016	0.24 ± 0.26
Germany Mosel	18	isolated	3	1.5 ± 0.55	1.38 ± 0.44	0.111 ± 0.096	0.234 ± 0.177	0.12 ± 0.17
Germany Nahe	7	isolated	0	1 ± 0	1 ± 0	–	–	0
Czech Rep. Eger	28	isolated	3	1.67 ± 0.82	1.46 ± 0.60	0.191 ± 0.237	0.332 ± 0.238	0.17 ± 0.24
Czech Rep. Berounka	8	isolated	3	1.67 ± 0.82	1.58 ± 0.70	0.250 ± 0.216	0.322 ± 0.188	0.16 ± 0.22
Austria Carinthia	16	edge	5	2.83 ± 1.47	2.26 ± 0.89	0.325 ± 0.184	0.435 ± 0.167	0.37 ± 0.23
Slovenia Hrastnik	6	central	6	3.83 ± 1.33	3.83 ± 1.33	0.278 ± 0.228	0.667 ± 0.183	0.71 ± 0.19
Romania Tulcea	7	central	6	5.17 ± 1.72	4.91 ± 1.55	0.587 ± 0.302	0.726 ± 0.121	0.74 ± 0.12

Tab. 3. Genetic diversity of eight populations of dice snakes based on microsatellite data. Presented are sample sizes (N), relative location of the population in the distribution range of the dice snake, number of polymorphic loci (P) and means ± standard deviations for the average number of alleles per locus (A), allelic richness (R), observed (H_o) and expected (H_e) heterozygosities and genetic diversity according to NEI (D).

Genetische Diversität von acht Würfelnatter-Populationen basierend auf Mikrosatelliten-Daten. Angegeben sind die Stichprobengrößen (N), die relative Lage der Population im Verbreitungsgebiet der Würfelnatter, die Anzahl polymorpher Loci (P) sowie jeweils Mittelwert ± Standardabweichung für die mittlere Anzahl Allele pro Locus (A), den Allelreichtum („allelic richness", R), die beobachtete (H_o) und erwartete (H_e) Heterozygotie und die genetische Diversität nach NEI (D).

4 Discussion

4.1 Population differentiation

The main goal of this study was to carry out a first assessment of the genetic structure of the central European dice snake populations. Clear evidence for differentiation of the isolated populations in Germany and the Czech Republic from those of the

continuous distribution range was found. Furthermore, the data provided evidence for genetic divergence of the three German populations, with the Lahn population being significantly different from the Mosel and Nahe populations.

In snakes, strong population subdivision is often found even on a small spatial scale (O'BRIEN 1994, GIBBS et al. 1997, PRIOR et al. 1997, PROSSER et al. 1999). Narrow habitat requirements and limited dispersal abilities of these animals contribute to the development of highly structured populations with little or no gene flow between them.

There is good evidence that the central European dice snakes descend from animals that survived the last Pleistocene glacial period in refugia on the Balkans. In post-glacial times, the ancestral populations started expanding northwards following the main river systems of the Danube, the Elbe, and presumably the Rhine (GRUSCHWITZ et al. 1999, GUICKING et al. 2002). Successive bottlenecks during northward range expansion, reduced gene flow through isolation, and small population sizes promote genetic drift and result in reduced genetic variation and increased differentiation (LESCIA & ALLENDORF 1995, TABERLET et al. 1998). Also, different environmental conditions at the edge of a species' distribution range may lead to different regimes of natural selection and further increase divergence of isolated populations (LESCIA & ALLENDORF 1995). The colonization history and the fact that no private microsatellite alleles were found in the isolated populations indicate that genetic drift rather than mutations have caused the observed differentiation.

There was no genetic evidence of differentiation between the two Czech populations which is in concordance with field observations of a continous distribution of the dice snake in the Czech Republic (M. GRUSCHWITZ, S. LENZ, pers. comm.). This demonstrates the genetic homogeneity of the animals that were reintroduced to the river Elbe in eastern Germany.

Subdivision of the three German populations was also suggested due to differences in pholidosis and colouration (LENZ et al. 2000). However, because of several records of dice snakes in the Rhine valley during the late 19th and early 20th century (GRUSCHWITZ et al. 1993), it was generally assumed that the three German populations had been in contact until very recently. Only during the last decades, isolation of the three populations from each other became evident. The Lahn population was the first that was separated from the river Rhine due to extensive alteration of riverside habitats during the 19th and 20th century. However, this seems not sufficient to explain the observed strong genetic divergence from the other two German populations. More likely, genetic differentiation of the three populations dates back much further in time. This raises the possibility that even under natural conditions genetic exchange between populations was much more restricted than generally assumed. Because of the location of the river Lahn east of the Rhine and of the rivers Mosel and Nahe west of the Rhine, the observed separation of the Lahn population may reflect a barrier function of the Rhine, suggesting that dispersal followed mainly along the shoreline. Moreover, anthropogenic influences may have led to considerable fragmentation of the German populations already long before the 20th century. However, most interesting in this context probably is the possibility that the observed differentiation may reflect different origins of the populations, suggesting that the Lahn and the Mosel and Nahe rivers may have been founded during independent colonization events. As only very scarce fossil records are available to infer information on colonization routes of the dice snake in central Europe [e. g. MARKERT (1976) reports fossil bones of *N. tessellata* from the upper Danube valley from the Holocene], the only possibility to

gain further insight into this topic may be the performance of a comprehensive genetic study of all possible ancestral populations on the Balkans.

4.2 Within-population diversity

Genetic investigations revealed very low within-population diversities for the isolated dice snake populations in Germany and the Czech Republic. This is consistent with general assumptions on the genetic status of small isolated populations that are prone to increased genetic drift and have suffered from founder effects during colonization (LESCIA & ALLENDORF 1995, TABERLET et al. 1998). However, extremely low genetic variation may also reflect anthropogenically driven population bottlenecks during the last century. For both Mosel and Lahn populations severe breakdowns as a consequence of habitat destruction and anthropogenic disturbance have been documented (GRUSCHWITZ et al. 1999). At the Mosel, the dice snake was thought extinct before a small relict population was rediscovered in 1975 (GRUSCHWITZ 1978). The relictual Lahn population had constant sizes of 100-150 animals during the last decades. However, a detrimental ageing of the entire population in the early 1980s indicated low reproductive success and very low effective population sizes (LENZ & GRUSCHWITZ 1993). Surprisingly, according to microsatellite data, the Nahe population is genetically least diverse. This population was generally assumed to be genetically most variable, as it was the largest population in Germany both in the past and at present. In spite of population declines in the 20th century, numbers never fell below 250 individuals (GRUSCHWITZ et al. 1993). The observed low genetic variation in the Nahe population is unlikely to have resulted by chance alone, as the study specimens were collected at different localities. More likely, the monomorphic status of all six analyzed microsatellite loci may reflect a former genetic bottleneck that has not been detected by demographic surveys.

4.3 Implications for conservation

Our data extend the current knowledge on the status of German dice snakes and have several implications for population management.

Genetic distinctiveness emphasises the independent evolution and confirms the conservation value of the central European dice snakes. In Germany, the Lahn population and at least one of the other populations should be given highest priority for conservation to preserve a maximum amount of genetic diversity.

Within-population variations are extremely low and are even below those reported for introduced populations in Switzerland that trace back to no more than 20-25 individuals (MEBERT 1993, GAUTSCHI et al. 2002). This emphasizes the devastating impact that population declines had on the genetic status of the central European populations.

Low genetic diversity is assumed to have detrimental effects on the long-term viability of populations through inbreeding depression and reduced adaptability (FRANKHAM 1995, FRANKHAM 1998, CRNOKRAK & ROOF 1999, KELLER & WALLER 2002). Especially in peripheral populations that often live in suboptimal environmental conditions, genetic variation is considered very important to maintain adaptive diversity (LESCIA & ALLENDORF 1995). Although the quantitative importance of loss of genetic variation is difficult to predict for any given population (BOOY et al. 2000), there is strong evidence for reduced fitness in inbred snake populations (MADSEN et al. 1996, ÚJVÁRI et al. 2002).

GAUTSCHI et al. (2002) report an association of the occurrence of scale anomalies with the degree of bottlenecking and individual heterozygosity, suggesting that scale anomalies may indicate genetic stress. Also, environmental stress is discussed as a cause for increased occurrence of scale anomalies (GAUTSCHI et al. 2002). In the German dice snakes, scale anomalies occur in more than 80 % of the animals (LENZ et al. 2000), giving further evidence that these populations live under some form of genetic or environmental stress.

Conservation action in the framework of the "Erprobungs- und Entwicklungs-Vorhaben" for the conservation of the dice snake in Germany has greatly improved the situation of the German dice snakes during the last years. Still, genetic data demonstrate a severe loss of variation, predicting that the German dice snakes will need a long time to genetically recover from earlier population breakdowns. Therefore, further fragmentation and reductions of population sizes must be prevented under all circumstances. Continuation of the demographic population monitoring is highly recommended and should be supplemented by a genetic monitoring that may be more sensitive (LUIKART et al. 1998, GAUTSCHI et al. 2002).

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Portrait of an adult dice snake from the river Nahe.

Porträt einer adulten Würfelnatter von der Nahe.

Populationsgenetik der Würfelnatter (*Natrix tessellata*) in Deutschland: Konsequenzen für den Artenschutz

Einleitung

Die Würfelnatter *Natrix tessellata* erreicht in Deutschland ihre nordwestliche Verbreitungsgrenze. Sie ist heute auf drei isolierte Vorkommen an den Flüssen Mosel, Nahe und Lahn begrenzt. Eine im 20. Jhd. erloschene vierte Population an der Elbe wurde kürzlich mit Tieren aus der Tschechischen Republik wiederangesiedelt (SCHMIDT & LENZ 2001). Aufgrund geringer Populationsgrößen und starker Bestandseinbußen, insbesondere durch anthropogen bedingte Habitatveränderungen, ist die Würfelnatter in Deutschland vom Aussterben bedroht (GRUSCHWITZ et al. 1999). Ein umfangreiches Artenschutzprojekt hat in den letzten Jahren wesentlich zur Erholung der Bestände beigetragen (SCHMIDT & LENZ 2001, HERZBERG & SCHMIDT 2001, LENZ & SCHMIDT 2002). Bislang lagen jedoch keine Daten über die genetische Konstitution der deutschen Würfelnattern vor. In der vorliegenden Arbeit untersuchen wir die Populationsgenetik der isolierten deutschen und tschechischen Würfelnatterbestände basierend auf mitochondrialen Cytochrom *b* Gensequenzen und nukleären Mikrosatelliten-Analysen. Aus diesen Daten lassen sich Informationen über die Populationsdifferenzierung sowie zur genetischen Variabilität der einzelnen Populationen abschätzen, die mit traditionellen Methoden nicht zu erhalten wären.

Material und Methoden

Es wurden insgesamt 36 Proben aus den drei deutschen Populationen an Lahn, Mosel und Nahe und 36 Proben aus zwei tschechischen Populationen an den Flüssen Eger und Berounka untersucht (Fig. 1). Zu Vergleichszwecken wurden zusätzliche Proben von Populationen aus dem zusammenhängenden Verbreitungsgebiet berücksichtigt. Diese stammten aus Populationen in Kärnten, Slowenien und Rumänien (Fig. 1). Die in Puffer oder Ethanol konservierten Blut- und Gewebeproben wurden für die Isolierung von DNA genutzt. Mit Hilfe der PCR wurde das gesamte Cytochrom *b* Gen amplifiziert und anschließend sequenziert (GUICKING et al. 2002). Zusätzlich wurde eine Mikrosatelliten-Analyse anhand von sechs ausgewählten Loci durchgeführt (GAUTSCHI et al. 2000). Die Daten wurden mit gängiger populationsgenetischer Software ausgewertet.

Ergebnisse

Die genetischen Daten belegen eine deutliche Differenzierung der deutschen und tschechischen Würfelnatterpopulationen sowohl voneinander als auch von den Populationen des zusammenhängenden Verbreitungsgebiets (Tab. 1 und 2) und unterstreichen damit die Schutzwürdigkeit der isolierten mitteleuropäischen Bestände. Des Weiteren weisen die genetischen Daten die Lahnpopulation als deutlich differenziert von den Populationen an Mosel und Nahe aus. Diese Differenzierung zeigt sich sowohl durch das Vorhandensein von populationsspezifischen mitochondrialen Haplotypen (Tab. 1, Fig. 2) als auch durch hohe genetische Distanzen, die basierend auf nukleären Mikrosatelliten-Daten berechnet wurden (Tab. 2). Keine beziehungsweise eine nur schwache Differenzierung wurde lediglich zwischen den beiden tschechischen Populationen sowie zwischen den deutschen Populationen an Mosel und Nahe nachgewiesen.

Die genetische Variabilität innerhalb der einzelnen Populationen erwies sich in den isolierten Populationen als deutlich reduziert gegenüber den südlichen Populationen. Dies zeigte sich besonders an den geringeren Werten verschiedener populationsgenetischer Diversitätsindizes für die isolierten mitteleuropäischen Bestände (Tab. 3). Die Nahepopulation erwies sich anhand der Mikrosatellitendaten als die genetisch am wenigsten diverse Population, da in dieser Population alle sechs Loci nur ein einziges Allel aufwiesen. In Bezug auf mitochondriale Daten war die Nahepopulation jedoch die einzige der isolierten nördlichen Populationen, die zwei anstatt eines Haplotypen aufwies (Tab. 1).

Diskussion

Die mitteleuropäischen Würfelnattern stammen mit größter Wahrscheinlichkeit von Tieren ab, die die letzte Glazialzeit in südlichen Refugien auf dem Balkan verbracht und sich erst in postglazialer Zeit von dort nach Norden ausgebreitet haben (GRUSCHWITZ et al. 1999, GUICKING et al. 2002). Wiederholte Gründereffekte während der Ausbreitung, die isolierte Lage sowie geringe Populationsgrößen fördern den Einfluss genetischer Drift und bieten damit eine mögliche Erklärung für die stark reduzierte genetische Variabilität innerhalb der mitteleuropäischen Populationen sowie für die Differenzierung der verschiedenen Populationen untereinander. Schwieriger zu erklären ist die beobachtete Differenzierung der Lahnpopulation von den Populationen an Mosel und Nahe. Bislang ging man davon aus, dass die drei westdeutschen Populationen noch bis vor wenigen Jahrzehnten über den Rhein in engem Austausch miteinander standen. Die deutliche Differenzierung spricht jedoch dafür, dass die Lahnpopulation schon wesentlich früher unabhängig von den Populationen an Mosel und Nahe existierte. Diese Tatsache könnte eine Barrierefunktion des Rheins widerspiegeln, ließe sich aber auch durch die Annahme von mehr als einem postglazialen Besiedlungsereignis in Westdeutschland erklären. Eine endgültige Aussage über die Ursache(n) der Differenzierung zwischen den drei deutschen Populationen lässt sich anhand der vorliegenden Daten leider nicht treffen.

Die geringe genetische Variabilität innerhalb der fünf mitteleuropäischen Populationen ist zum Teil auf den Einfluss natürlicher Faktoren zurückzuführen (z. B. erhöhte genetische Drift in kleinen Populationen bzw. Gründereffekte während der postglazialen Besiedlung), spiegelt zum anderen aber sicher auch anthropogene Einflüsse wider, die besonders in den letzten Jahrzehnten zu starken Bestandseinbußen geführt haben (vgl. GRUSCHWITZ et al. 1999). Die geringe genetische Variabilität innerhalb der Populationen könnte sich langfristig negativ auf die Populationsentwicklung ausüben (sogenannte Inzuchtdepression). Um dies zu verhindern, sind weitere Populationseinbrüche oder Fragmentierungen unter allen Umständen zu verhindern. Es wird außerdem geraten, ein Populations-Monitoring auch in Zukunft durchzuführen und durch regelmäßige genetische Untersuchungen zu ergänzen.

Schlagwörter: Cytochrom *b* Gen; Mikrosatelliten-Analyse; Naturschutzgenetik; Populationsdifferenzierung; Squamata: Colubridae: *Natrix tessellata*.

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Appendix (next page):

Locus- and population-specific allele frequencies in the eight sample populations of *N. tessellata* in Europe, based on an analysis of six microsatellite loci. For each locus and population, the identified allele lengths are specified and absolute and relative frequencies are indicated.

Locus- und populations-spezifische Allel-Häufigkeiten in den acht untersuchten Populationen von *N. tessellata* in Europa, basierend auf sechs Mikrosatelliten-Loci. Für jeden Locus und jede Population sind die nachgewiesenen Allel-Längen und ihre zugehörigen absoluten und relativen Häufigkeiten angegeben.

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Authors: DANIELA GUICKING, Institut für Pharmazie und Molekulare Biotechnologie, Abteilung Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany; Milchlingstraße 8, 34121 Kassel, E-Mail: daniela.guicking@gmx.de; MICHAEL WINK, Institut für Pharmazie und Molekulare Biotechnologie, Abteilung Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany, E-Mail: wink@uni-hd.de; ANDREA HERZBERG, Heidestraße 25, D-56154 Boppard, Germany, E-Mail: andrea.herzberg@t-online.de.

Population	μNt8			μNt7			μNt6			μNt5			μNt3			μNt2		
	Allele	abs.	rel. %	Allele	abs.	rel. %	Allele	abs.	rel. %	Allele	abs.	rel. %	Allele	abs.	rel. %	Allele	abs.	rel. %
Germany Lahn	151	22	100	180	8	36.4	187	15	68.2	116	22	100	145	15	68.2	206	22	100
				186	14	63.6	189	6	27.3				147	7	31.8			
							191	1	4.5									
Germany Mosel	151	36	100	186	25	69.4	151	3	8.3	116	36	100	145	34	94.4	206	36	100
				188	11	30.6	153	33	91.7				147	2	5.6			
Germany Nahe	151	14	100	186	14	100	153	14	100	116	14	100	145	14	100	206	14	100
Czech Rep. Eger	151	56	100	182	56	100	153	31	55.4	116	54	96.4	145	56	100	196	15	27.8
							189	23	41.0	118	2	3.6				200	39	72.2
							191	2	3.6									
Czech Rep. Berounka	151	16	100	182	15	93.8	153	10	62.5	116	16	100	145	16	100	196	2	12.5
				184	1	6.2	189	6	37.5							200	13	81.3
																202	1	6.2
Austria Carinthia	151	24	75	186	22	68.8	151	1	3.1	116	29	90.6	145	32	100	196	8	25.0
	161	8	25	188	10	31.2	189	1	3.1	122	2	6.3				198	1	3.1
							195	19	59.4	124	1	3.1				202	19	59.4
							199	9	28.1							206	4	12.5
							203	2	6.3									
Slovenia Hrastnik	151	4	33.3	180	2	16.7	151	1	8.3	116	1	8.3	145	10	83.3	196	6	50.0
	161	5	41.7	186	4	33.3	181	1	8.3	122	4	33.3	165	2	16.7	202	2	16.7
	163	1	8.3	188	5	41.7	189	5	41.8	124	5	41.7				204	4	33.3
	167	2	16.7	190	1	8.3	191	1	8.3	126	2	16.7						
							203	3	25.0									
							213	1	8.3									
Romania Tulcea	151	1	7.1	178	1	7.1	153	4	28.7	116	9	64.3	145	6	50	198	8	57.1
	153	1	7.1	180	6	42.9	191	2	14.3	122	3	21.5	147	2	16.7	200	4	28.6
	157	3	21.4	184	1	7.1	195	2	14.3	124	1	7.1	165	1	8.3	206	2	14.3
	161	7	50.0	186	2	14.3	197	1	7.1	126	1	7.1	171	2	16.7			
	163	2	14.3	188	2	14.3	199	2	14.3				173	1	8.3			
				190	2	14.3	203	1	7.1									
							205	1	7.1									
							211	1	7.1									