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Low Genetic Diversity of the Turopolje Pig Breed

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Summary

We have performed a genetic diversity study of the Turopolje pig breed. Microsatellite genotyping on ten loci recommended by International Society for Animal Genetics (ISAG) and Food and Agriculture Organization (FAO) as well as mitochondrial D-loop sequencing were chosen as methods. Allele numbers, effective allele numbers (Ne), polymorphism information content (PIC), observed and expected heterozygosities were calculated. Mitochondrial sequences were compared to the sequences in the National Center for Biotechnology Information (NCBI) database. We have found relatively low genetic diversity at ten microsatellite loci and no differences in the partial D-loop sequence. Reasons for and consequences of low genetic diversity are discussed.

Key words: autochthonous pig, genetic diversity, microsatellites, mitochondrial D-loop

Introduction

Turopolje pig is one of the two existing critically endangered autochthonous Croatian pig breeds (http://dad.fao.org/). Its origin is still unclear. The most widely accepted hypothesis is that Turopolje pig originates through local domestication with introgression from other European pig breeds. Berkshire, Cornwall and Large white were considered as the possible contributors (*1*). Until the 1950s this breed was numerous and widespread in the continental Croatia, because it is well adapted to the local environment, mostly marshy woodlands and meadows. For example, in 1933 Turopolje pig accounted for 20 % of all pigs reared in the country (*2*). The production system was based on the low food input and utilization of local natural food sources, such as acorn, grass, and roots. Consequently, Turopolje pig breed represented a major food source at the time. However, due to the changes in animal production system and the loss of interest for »lard type« pigs, the population was dramatically reduced and currently consists of only approximately 250 individuals. The role of Turopolje pig in the meat production at present is small, except locally in the region of Turopolje. Even so, it remains important because of the cultural, historical and ecological reasons. Today, there is growing awareness of the need to study and maintain genetic diversity of domestic animals, including pigs. The recognition of that fact resulted, among other things, in the publication of the World watch list for domestic animal diversity (*3*), which assigned the Turopolje pig to the critically endangered category. A project aiming to preserve and reestablish the Turopolje pig breed started in 1996 (*4*). Although the Turopolje pig is morphologically well defined and unique breed, so far no molecular genetic studies have been performed. Research was mostly focused on establishing the meat and slaughter quality (*5,6*), litter size (*2*) and the productivity under outdoor system (*5*). The studies showed a potential and specificity of this breed which could be used for making high quality meat products and for improving commercial pig breeds. As a part of a larger effort to preserve this breed and to maintain genetic biodiversity in domestic animals in Croatia, a study of ge-

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netic diversity in the Turopolje pig breed was performed. We were especially interested in the consequences of the recent population bottleneck, which Turopolje pig population experienced in the middle of the 20th century.

Materials and Methods

Blood samples were taken from 35 purebred Turopolje pigs. All samples were collected at a location in Turopoljski lug, where the majority of the remaining population of the Turopolje pig breed is kept. The total DNA was isolated from 0.5 mL of whole blood using modified phenol chloroform procedure (*7*) and stored at 4 °C. Ten microsatellite loci were selected for analysis according to the recommendations by ISAG-FAO (*8*) and amplified (see Table 1). Latest ISAG-FAO guidelines recommend the use of seven out of ten microsatellite loci (*9*). All chosen loci were dimeric CA repeats except CGA locus, which has two tetrameric CTTT and one dimeric CT repeat region. Oligonucleotides for amplification of the selected loci were synthesized at MWG – the genomics company, Ebersberg, Germany. One primer in each pair contained fluorescent marker Cy5. Microsatellites were amplified in a total volume of 25 μL for 30 cycles. The mixtures contained approximately 50 ng of genomic DNA, 1x PCR buffer (Amersham Pharmacia Biotech), 1 pmol of each primer, 0.5 nmol of each dNTP and 1 U of Taq polymerase (Amersham Pharmacia Biotech). PCR reactions were performed according to the previously published procedure (*10,11*) or with minor modifications in Biometra® T personal thermocycler. Primer sequences and annealing temperatures for ten microsatellite loci are given in Table 1. PCR products were checked by electrophoresis on 1.5 % agarose gels. The size of the PCR products (microsatellite alleles) was determined on ALFexpress™ DNA sequencer using AlleleLocator™ 1.03 software (Amersham Pharmacia Biotech). ALFexpress™ Sizer™ 50–500 was used as a size standard. Electrophoresis was performed on 8 % denaturing polyacrylamide gels (ReproGel™ High Resolution, Amersham Pharmacia Biotech).

Number of alleles was determined by direct counting. Effective number of alleles (*12*), observed (Hobs) and expected (Hexp) heterozygosities were calculated using POPGENE (*13*) program. Polymorphism information content (PIC) was calculated with CERVUS (*14*). Program Bottleneck (*15*) was used to test the microsatellite data for recent effective population size reductions – bottlenecks.

Primers »MitF« (GTA TGC AAA CCA AAA CGC CAA GTA) and »MitR« (CCA GCT ACA ATT GAT TTG ACT GTG) were designed to amplify a 557 bp long fragment (excluding the primers) of the porcine mitochondrial D-loop between sites 15458 and 16064 of the complete pig mtDNA sequence used as a reference (GenBank AJ002189) (*16*). Mitochondrial PCR products were directly sequenced on ALFexpress in both directions using ThermoSequenase Cy5 Terminator Kit (Amersham Pharmacia Biotech). DNA sequences were analyzed and stored using Lasergene sequence analysis software (DNAStar, Madison, WI).

Results

All microsatellite loci were successfully amplified and PCR products were separated and sized. Allele numbers and frequencies for all 10 loci are shown in Table 2. Number of alleles and effective number of alleles, polymorphism information content, observed and expected heterozygosity of each marker in both populations are given in Table 3.

Locus S0227 was monomorphic in all 35 analyzed Turopolje pigs. Even some of the polymorphic loci (CGA, SW742, S0005 and S0068) exhibited the predominance of just one allele with frequencies ranging from 0.914 to 0.986. The highest number of alleles was only 4 (Ne= 2.06), found on locus SW936. The mean number of alleles was 2.4 (Ne=1.47), the mean observed heterozygosity was 0.306 and PIC was 0.222. In general, observed heterozygosities were close to the expected values with the exception of loci S0355, S0090 and SWR1941, where the excess of heterozygotes was found. However, no statistically significant deviations from HW equilibrium were observed in Turopolje pig population.

We did not get statistically significant results after testing our data for recent genetic bottleneck with methods proposed by Cornuet and Luikart (*15*).

Table 1. Chromosomal locations, sequences of oligonucleotide primer pairs and annealing temperatures for 10 microsatellite loci

Locus	Chromosomal location	Forward primer	Reverse primer	Annealing temperature/°C
CGA	1 p 2.1 –	ATAGACATTATGTAAGTTGCTGAT	GAACTTTCACATCCCTAAGGTCGT	55
S0355	15 q1.2 – q1.4	TCTGGCTCCTACACTCCTTCTTGATG	TTGGGTGGGTGCTGAAAAATAGGA	55
S0090	12	CCAAGACTGCCTTGTAGGTGAATA	GCTATCAAGTATTGTACCATTAGG	55
SW742	16	AATTCTACTTCTGGGGAGAGGG	CTTTTGGGAACATTTCTGCC	60
SW936	15 q2.5	TCTGGAGCTAGCATAAGTGCC	GTGCAAGTACACATGCAGGG	58
S0227	4 p1.4 – p1.5	GATCCATTTATAATTTTAGCACAAAGT	GCATGGTGTGATGCTATGTCAAGC	55
S0005	5 q2.1 – q2.4	TCTTCCCTCCTGGTAACTA	GCACTTCCTGATTCTGGGTA	59
S0068	13	AGTGGTCTCTCTCCCTCTTGCT	CCTTCAACCTTTGAGCAAGAAC	62
SWR1941	13	AGAAAGCAATTTGATTTGCATAATC	ACAAGGACCTACTGTATAGCACAGG	62
SW830	10 p	AAGTACCATGGAGAGGGAAATG	ACATGGTTCCAAAGACCTGTG	62

Table 2. Allele sizes, numbers and frequencies on 10 microsatellite loci in Turopolje pig

Locus	Allele sizes	Allele numbers	Allele frequencies
CGA	277	3	4.29
	285	1	1.43
	289	66	94.29
S0355	251	24	34.29
	266	46	65.71
S0090	243	23	32.86
	254	47	67.14
SW742	205	1	1.43
	217	64	91.43
	221	5	7.14
SW936	92	46	65.71
	94	1	1.43
	100	12	17.14
	104	11	15.71
S0227	233	70	100.00
S0005	228	69	98.57
	236	1	1.43
S0068	225	2	2.86
	241	67	95.71
	251	1	1.43
SWR1941	204	47	67.14
	212	23	32.86
SW830	178	46	65.71
	184	24	34.29

All 35 sampled Turopolje pigs had the identical partial mitochondrial D-loop sequence. The sequence is deposited under Acc. no. AY684145.

Mitochondrial haplotype determined in all Turopolje pigs was previously found at different frequencies in Hungarian Mangalitza (AY232892), Basque pig (AY232891), Pietrain (AY232886), Landrace (AY232884), Duroc (AY232877) and Iberian pig (AY232867) by Alves *et al.* (*17*), and in Large white pig (AB041494; *18*). A recent comprehensive study (*19*) identified this haplotype in numerous European pig breeds and in European wild boar populations. Fig. 1 shows the alignment of partial mitochondrial D-loop sequence from the Turopolje pig breed with different haplotypes representing European and Asian clades.

Discussion

When compared with the results obtained in earlier studies on other European, Chinese and Indian pig breeds, our data indicate a significantly lower genetic diversity in Turopolje pig breed. Previous analysis showed microsatellite allele numbers in the range from 3.22 to 5.84 (Ne 1.51–2.91) in European breeds (*20,21*), 4.351 to 6.108 (Ne 3.399–4.982) in Chinese breeds (*22,23*) and 7 to 7.74 (Ne 5–5.3) in Indian breeds (*24*). Observed and expected heterozygosities and polymorphism information content values were accordingly higher for those breeds. Studies using the same loci (at least six out of ten) were chosen for comparison with our results.

We attribute our findings to the severe demographic bottleneck that Turopolje pig experienced in the middle of the 20th century. As previously mentioned, the method for detecting genetic bottlenecks proposed by Cornuet and Luikart (*15*), based on allele deficiency and heterozygosity excess, could not prove our assumption. Although heterozygosity excess was present on some loci, it was not statistically significant. A possible explanation for this could lie in a very specific recent history of the Turopolje pig breed. As reported before (*4*), the reestablishment of this breed began less than 10 years ago with only 12 sows and 3 boars. Nothing is known about the genetic background of these individuals, *i.e.* breeding and selection practices before the beginning of the conservation project.

Table 3. Observed and effective number of alleles, polymorphism information content, observed and expected heterozygosity over 10 microsatellite loci in Turopolje pig

Locus	No. of alleles	Ne	PIC	Hobs	Hexp
CGA	3	1.1223	0.105	0.114	0.111
S0355	2	1.8202	0.349	0.571	0.457
S0090	2	1.7896	0.344	0.543	0.448
SW742	3	1.1887	0.150	0.171	0.161
SW936	4	2.0571	0.466	0.543	0.521
S0227	1	1.0000	0	0	0
S0005	2	1.0290	0.028	0.029	0.029
S0068	3	1.0903	0.081	0.086	0.084
SWR1941	2	1.7896	0.344	0.543	0.448
SW830	2	1.8202	0.349	0.457	0.457
Mean [standard deviation]	2.4 [0.8433]	1.4707 [0.4155]	0.222	0.306 [0.025]	0.272 [0.067]

Ne = effective number of alleles
PIC = polymorphism information content
Hobs = observed heterozygosity
Hexp = expected heterozygosity

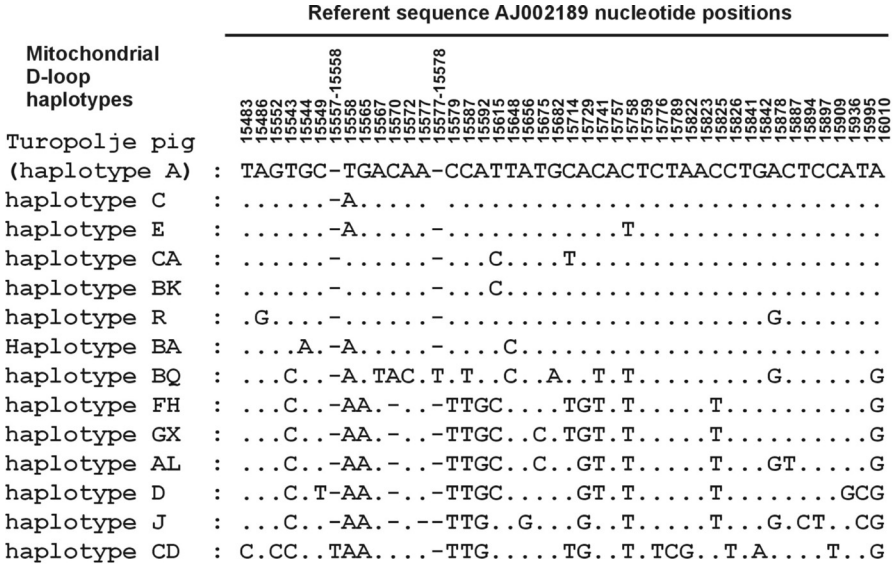

Fig 1. Alignment of mitochondrial D-loop sequences. Haplotypes are named according to Larson *et al.* (*19*). Only variable positions are shown. Nucleotide positions are numbered according to the referent sequence AJ00219. Haplotypes A (Turopolje pig) – AY684145, C – AY884770, E – AY884749, CA – AY232872, BK – AY232858, R – AY884769 and BA – AF535163 belong to the European clade; haplotypes BQ – AY884727, FH – AY884811, GX – AY884784, AL – AY884646, D – AY884708, J – AY884801 and CD – AY884644 belong to the Asian clade

Haplotype A found in the Turopolje pig breed is only present in Europe or European derived populations; it is the most frequent haplotype in the European pig breeds and forms a basis for one of the two core European lineages (lineage A). As it is also present in the European wild boar populations (*19*), it is possible that the Turopolje pig was domesticated locally. There is no evidence of introgressions from other mitochondrial haplotypes in the present population of the Turopolje pig breed. However, the hypothesis of introgression from other pig breeds cannot be completely discarded for two reasons; (*i*) haplotype A is also present in most other European breeds including Berkshire, Cornwall and Large white, (*ii*) strong bottleneck might have eliminated less frequent haplotypes.

Conclusion

Low genetic diversity of Turopolje pig breed presents an additional danger for extinction and emphasizes the need to apply strictest possible conservation measures and prevent further loss of intrabreed diversity.

The prevailing motive for current conservation efforts is cultural and historic importance of Turopolje pig breed. However, the example of Iberian pig (*25*,*26*) shows that it is possible to use autochthonous pig breeds for the production of specialized high quality meat products. Đikić *et al.* (*5*) have already shown that there are similarities in fatty acid composition between the two breeds. Commercial use of Turopolje pig would certainly contribute to the conservation efforts.

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Genetička raznolikost pasmine Turopoljska svinja

Sažetak

U ovom je radu provedeno istraživanje genetičke raznolikosti Turopoljske svinje. Genotipizacija je provedena analizom mikrosatelita i sekvencioniranjem mitohondrijske D--petlje. Odabrano je deset mikrosatelitskih lokusa prema preporuci *International Society for Animal Genetics* (ISAG) i *The Food and Agriculture Organization* (FAO). Izračunat je broj alela, efektivni broj alela (Ne), informacijski sadržaj polimorfizma, te očekivana i opažena heterozigotnost. Mitohondrijske sekvencije uspoređene su sa sekvencijama pohranjenim u bazi *National Center for Biotechnology Information* (NCBI). Na osnovi analize deset mikrosatelitskih lokusa ustanovljena je razmjerno niska razina genetičke raznolikosti. Također je utvrđeno da sve jedinke u uzorku imaju identičnu sekvenciju mitohondrijske D-petlje. U radu su komentirani mogući uzroci i posljedice niske razine genetičke raznolikosti ove pasmine.