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POPULATION GENETIC STUDY OF THE LIVE COMMON CARP GENEBANK OF HAKI (RESEARCH INSTITUTE FOR FISHERIES AND IRRIGATION, SZARVAS, HUNGARY) USING MICROSATELLITE DNA MARKERS AND PCR-RFLP ANALYSIS

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1. Preliminaries and objectives of the study

Common carp (*Cyprinus Carpio* L.) is ecomomically the most important fish species in Hungary. The local specialists have great breeding knowledge and the country has sufficient natural conditions for this species. Based on this facts the better understanding of the genetic background of the different carp starins and the description of their gene reserve is crucial for the following selection work. Hungary possesses impressive genetic resources of common carp confirmed by the good attributes/qualities of the different local strains and the very good performance of the different hybrid carp lines. The conservation of the local strains is essential and ensure a good basis for the future selection. Common carp (*Cyprinus carpio* L.) strains from the most significant Hungarian fish farms were collected with the leading of János Bakos in the Research Institute for Fisheries and Irrigation (HAKI, Szarvas, Hungary) from 1962. Later the genebank was completed by other native and foreign strains and landraces. The original objective of maintaining the *ex situ* live gene bank was the genetic improvement of common carp through developing highly productive hybrids for production purposes. Today, 18 Hungarian strains (landraces) and 13 “foreign” strains (collected from Central and Eastern Europe, as well as from Asia) are maintained at the institute. Recently, beside the “production-supporting” objectives, the maintenance of the genetic diversity of these starins/genotypes is becoming an increasingly important goal of the genebank.

Today the high standards of molecular genetic methods allows us to describe and analyze this unique gene reserve and based on the results new conservation strategies can be made. New hybrid carp strains can be

selected with higher performace. The broad spectrum of origin of the strains assure a good basis for phylogenetic studies of this species. This unique genetic background also makes possible the selection of disease resistant lines. The DNA level studies can be extremely useful in gene reservation and conservation work.

The main objectives of the study:

- Description of the genetic variability of 15 common carp strains originate from the live gene bank of the Research Institute for Fisheries and Irrigation (HAKI, Szarvas, Hungary) using microsatellite DNA markers.

- Description of allle numbers and allele frequencies on each loci, searching for individual alleles.

- Description of the genetic distance between the different strains.

- Searching for results of inbreeding.

- Study of the danubian and tisza wild carp groups and the description of the variability of their mitochondrial DNA.

2. Material and methods

2.1. The studied strains

In this study the genetic variability of 15 common carp strains (danubian wild, tisza wild, amur wild, szeged mirror, felsősomogy mirror, nagyatád mirror, sumony mirror, mórichely mirror, szarvas 15 mirror, szarvas 22 mirror, tata scaly, fresinet scaly, ukrainien scaly, vietnam scaly and koi) maintained at the genebank were analysed using seven microsatellite DNA markers. The PCR-RFLP analysis of the mitochondrial NADH-3,4 dehydrogenase (ND-3/4) and NADH-5,6 dehydrogenase (ND-5/6) genes of the danubian wild carp and the tisza wild carp was also carried out. All together 565 fin samples were collected from the 15 strains and the fin clips were preserved in 96% ethanol.

2.2. DNA extraction and quality/quantity control

The whole genomic DNA was extracted from the samples after the digesting of the samples' protein content with *Proteinase-K* with high salt concantration and different washing steps (**Miller et al., 1988**). Before the analysis the concentration and quality of the DNA samples were controlled using agarose gelelectrophoresis and spectrophotometry (Smart™Spec Plus, Bio-Rad). The optimal 80-150 ng/µl DNA content was achieved by the dilution or the repeated extraction of the samples.

2.3. Analysis of he mikrosatellite DNA markers

During the study 7 mikrosatellite DNA markers (tandemly repeated DNA fragments, units between 2-4 bp) were used (**Crooijmans és mtsai., 1997**). The primers were fluorescent (Cy5) end labeled.

The PCR (GeneAmp 2700 PCR system, Applied Biosystems) protocol consisted of a 2 minutes long denaturation step on 94°C, followed by 30 cycles of 40 seconds on 94°C (denaturation), 50 seconds on 55°C (primer-annealing) and 90 seconds on 72°C (elongation), followed by the terminal cycle at 72°C for ten minutes to assure the expression of the terminal tranferase activity of the *Taq* polimerase enzyme (Sigma-Aldrich) In order to separate and observe the different alleles in a given locus the PCR products were loaded on a 7% denaturing poly-acryl-amide gel (32% formamide, 5% urea) using the ALF Express II (Amersham Biosciences) fragment analyzer machine. To determine the size of the different alleles size standards (50, 150 and 300 bp long size standards, Amersham-Biosciences) and standard samples (the lenght of these samples is alredy known) were loaded next to the amplified fragments. Fragment Analyser 1.03 (Amersham-Biosciences) software was used to analyze the gels.

2.4. The PCR-RFLP study of the mitochondial ND-3/4 and ND-5/6 genes

25 Tisza wild carp and 35 Danubian wild carp were studied with the PCR-RFLP method. The essentials of the method are the following: The 2 PCR amplified gene segments are digested with restriction endonuclease enzymes and than the products are loaded on agarose gel. If there is a difference in the mtDNA (mitochondrial DNA) sequence of the terminal regions of the

genes, the enzyme cuts the fragment at a different position, so the cuted fragments will be of a different lenght. The lenght polimorphism can be easily detected on agarose gel. **Gross et al., (2002**) found 2 restriction endonuclease enzymes at NADH-3,4 dehidrogenase (ND-3/4) gene and 4 restriction endonuclease enzymes at NADH-5,6 dehidrogenase (ND-5/6) gene wich are applicable to differentiate between the European and Asian maternal lines.

The mtDNA was extracted with the above mentioned method. 2 PCR reactions were applied to amlify the terminal regions of the ND-3/4 and ND-5/6 genes in order to detect the restriction fragment lenght polimorphism.

The primers needed for the amplification were designed on the basis of the whole sequence of the mtDNA of common carp (**Chang et al., 1994**). The PCR reactions were carried out in 50 μl-es reaction volume int he presence of the following components:: 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 0.1 mM dNTP's, 0.2 μM of each primer and 0.5 unit *Taq* DNA polymerase (MBI-Fermentas). The PCR (GeneAmp 2700 PCR system, Applied Biosystems) protocol consisted of a 3 minutes long denaturation step at 95°C, followed by 35 cycles of 30 seconds at 94°C (denaturation), 40 seconds on 55°C (primer-annealing) and 90 seconds at 72°C (elongation), followed by the terminal cycle at 72°C for ten minutes. The approximate length of the two products were 2400 bp (ND-3/4) and 2600 bp (ND-5/6).

The PCR products of ND-3/4 gene were digested using *Hinf*I, *Hpa*II, *Alu*I and *Taq*I restriction endonucleases while the PCR products of the ND-5/6 gene were digested using *BsuR*I and *Eco47*I restriction endonucleases. Afterwards the digested products were loaded on a 2% agarose gel in order to separete the different fragments The lenght of the fragments were compared to a 100-bp size ladder (200bp-3000bp, MBI-Fermentas).

2.5. Data analysis

Allele frequencies, mean number of alleles, observed (H_o) and expected (H_e) heterozygosity values were calculated by the GENEPOP software in case of all strains (**Raymond and Rousset, 1995).** The probability values of deviation from the Hardy-Weinberg equilibrium (t-test) and the heterozygote deficiency probability values were also calculated by the GENEPOP software.

The individual alleles were detected using the Convert 1.31-es software (**Glaubitz, 2004**).

To calculate and compare the pairwise fixation indexes (F_{st}), to calculate allelic richness (A_r) and to detect the allele numbers at each loci the F-Stat software was used (**Goudet, 1995**).

Nei's genetic distances ($D_{a)}$ between strains and the the dendrogram (Neighbour Joining method- NJ) based on them were generated by the Populations software (**Langella, 1999**). To visualize the dendrogram the Treeview software was used (**Page, 1996**).

The assignment test (self-classification, Bayesian method) was carried out by the GeneClass software (**Piry et al., 1999**).

3. Results

3.1.1. Genetic variability within the strains

During the analysis of seven microsatellite loci altogether 218 alleles were detected. The mean allele number per locus ranged between 18 (MFW28) and 55 (MFW7) at the different loci. The mean allele number per locus at the different strains did not show the same high level of variance. The lowest value was found at the amur wild carp (10 alleles/locus) while the highest value was found at the szeged mirror carp (15 alleles/locus). The observed heterozygosity (H_o) values at the different loci were extremely diverse. In case of koi on a given locus (MFW1) the H_o was equal to zero which means all individuals were homozygotes on this locus, while on an other locus the koi, the vietnam scaly carp, the ukrainien scaly carp and the felsősomogy mirror carp shoved extremly high H_o (H_o=1), which means all individuals were heterozygotes at this locus.

The mean observed heterozygosity (mean H_o) values were not so diverse. They ranged between 0.44 (koi) and 0.77 (danubian wild carp) while the mean expected heterozygosity (mean H_e) values were found between 0.75 (ukrainien scaly carp) and 0.89 (danubian wild carp, mórichely mirror carp). **Kohlmann et al., (2005)** in a very similar study found the mean allele numbers per locus between 2.50 és 14.25 while the mean H_o ranged from 0.630 to 0.829. In his study the mean H_e values were between 0.709 and 0.775. The danubian wild carp, the tisza wild carp, the amur wild carp and the koi were analysed in both studies. In case of all the four strains higher mean number of alleles per locus were found in my

study. 14.71; 13.42; 10.00 and 8.14 in my study and 4.75; 5.25; 2.50 and 3 in the German study respectively. The mean H_o values were very similar in both studies (0.74; 0.77 ; 0.69 and 0.44 in the present study and 0.79; 0.72; 0.63 and 0.49 in Kohlmann's study).

Altogether 45 individual alleles were found during the study. The highest number of individual alleles was found at the ukrainien scaly carp (7). The tisza wild carp and the tata mirror carp aslo had six individual alleles. The lowest number of individual alleles was found at the nagyatád mirror carp (0), while the szarvas 22 mirror carp, the sumony mirror carp and the felsősomogy mirror carp showed 1 individual allele. It is typical of the different strains variability that some of the cultured strains have 6 individual alleles while an other cultured strain shows no individual allele. There is a huge difference between the two Hungarian wild carps as well. The tisza wild carp has 6 individual alleles while the danubian wild carp shows only 2.

The mean H_e exceeded the mean H_o in case of all strains which is an important sign of inbreeding.

The lowest mean allelic richness was detected at koi carp (mean A_r=3.81) while the highest value was found at the mórichely mirror carp (mean A_r=4.72). In case of the allelic richness the strains showed a more balanced picture than in case of mean number of alleles per locus. The mean allelic richness (mean A_r =4.61) of the two Hungarian wild carp strains (tisza wild carp and the danubian wild carp) exceeds both the Hungarian cultured strins' (mean A_r =4.35) and the „foreigner" strains' (mean A_r =4.34) mean allelic richness. The felsősomogy mirror carp showed extremly low allelic richness on locus MFW6 (A_r=2.57) and locus MFW16 (A_r=t2.98). These are the lowest A_r data in the study. The highest allelic richness was found on locus MFW7 at the vietnam scaly carp (A_r=5.7).

3.1.2. Genetic differences between the strains

Nei's D_a distance was used to measure the genetic distance between the strains (**Nei et al., 1983**) The highest genetic distance was found between the koi and amur wild carp (D_a=0.7466). The koi has a relatively high genetic distance from all other strains as well. The smallest genetic distance between the koi and other strains was 0.5 (ukrainien scaly carp). In case of the hungarian strains the bigest genetic distance was found between the tisza wild carp and the felsősomogyi mirror carp (D_a=0.4436). The smallest genetic distance was found between the szeged mirror carp and the mórichely mirror carp (D_a=0.125). These two stranis are originating from relatively distant parts of the country but during their breeding history both strains were crossed with varászló mirror carp to improve their body shape. The nagyatád mirror carp has also low genetic distance from these strains. The reason of this is the same. The nagyatád mirror carp was selected from the varászló mirror carp (**Bakos és Gorda 2001, Bakos, 2006**). Based on the genetic distance data the danubian wild carp and the tisza wild carp differentiate significantly from each other and also from other strains. The two native strains are situated at the neighbouring branches of the dendrogram based on the D_a genetic distance data. **Kohlmann et al., (2005)** found the same in their study. Based on the distance matrix all the external strains belongs to the same branch of the tree except the fresinet scaly. At the formation of this strain the szeged mirror carp and the tata scaly carp were used as a basis. This can be the explanation of the small genetic distance between these strains. The felsősomogy mirror carp and the sumony mirror carp are also situated at the same branch of the tree. These strains are originated from the same part of the country and sometimes

exchange of brood fish occured (**Bakos, J. 2006**). The szarvas 22 mirror carp also belongs to this branch of the tree because the basis of this strain was a selected group of sumony mirror carp.

The pairwise F_{st} values between the strains showing a similar picture to the genetic distances.

The assignment test (self-classification, Bayesian method) based on the 7 examined microsatellite loci was able to assign 85.8% of the individuals into their original strain. Individuals from 4 (amur wild carp, vietnam scaly carp, koi, tisza wild carp) strains were assigned to their original strain with a 100% accuracy. In case of all the Hungarian cultured strains several individuals can be found wich are misclassified by the software. This is an important sign of the mixed status of the strains and the close relationship between them. In the study of **Kohlmann et al., (2005)** the assignment test had an impressive 90.25% result. This exceeds my 85.8% result. The difference can be explained considering the higher genetic distances between the examined strains. In Kohlmann's study the genetic distances (D_a) ranged between 0.212 and 0.975 while in the present study the distances was found between 0.125 and 0.746.

3.2. Results of the PCR-RFLP study of the ND-3/4 and ND-5/6 mitochondrial genes

PCR-RFLP analysis using the restriction enzymes *Hinf*I, *Alu*I, *Hpa*II and *Taq*I at ND-3/4 and *BsuR*I and *Eco47*I at ND-5/6 mitochondrial genes, that represent about 25% of the total common carp mitochondrial genome by DNA sequence length, did not give evidence for mixing of European and Asian carp in the two strains examined.

All individuals showed the typical European haplotype providing evidence of the purity of these strains from the matrilineal perspective.

This fact can have importance fom the conservation point of view. Several European carp strains (ie.:ropsha) has Asian ancestors.

Some individuals from these strains can escape to the natural water bodies or can accidentaly mixed into any pure European strain in the live genebank. With this kind of studies the individuals with Asian ancestors can be excluded from breeding and establishment to natural waters.

4. Conclusions

Common carp strains maintained at the Research Institute for Fisheries and Irrigation (HAKI, Szarvas, Hungary) were found to be genetically heterogenic so that the genebank posseses impressive genetic resources. This is prominently true in the case of our two native wild carp strains (danubian and tisza wild carp). However several strains showing the signs of inbreeding.

Most of the strains maintained at the live genebank are genetically close to each other (in some cases the genetic distances are extremely low). The genetic distances are relatively small even between populations that are originated geographically far from each other. However the strains can be distingusihed well by the seven examined microsatellite DNA markers. The native wild carp strains are distinct both from each other and from all other strains. Variability within the strains exeeds the variation between strains in several cases.

The whole stock of the live genebank is deviate significantly from the Hardy-Weinberg equilibrium that support the theory of inbreeding and possible bottleneck phenomenon in some strains.

The PCR-RFLP studies show that the danubian wild carp and tisza wild carp strains maintained at the live genebank of Research Institute for Fisheries and Irrigation (HAKI, Szarvas, Hungary) are representing the typical European haplotype.

5. New Scientific Results

1. The examined 7 microsatellite DNA markers proved to be applicable to distinguish between the strains (85% of the individuals was successfully assigned to its original strain). The 7 loci showed high genetic variance in common carp strains originated from the live genebank of HAKI. Altogether 218 alleles were detected. The alle number per loci ranged between 18 and 55. The mean allele number of the different strains ranged between 8.14 and 15.

2. Common carp strains kept at the live genebank are showing the signs of inbreeding. At most of the loci they deviate from the Hardy-Weinberg equilibrium and the mean observed heterozygosity is lower than the mean expected heterozygosity in all cases

3 The Hungarian cultured strains of the gene bank were found to be genetically close to each other and showing low differentiation (D_a: 0,125-0,4436, F_{st}:0,004 -0,16).

4. The danubian wild carp and the tisza wild carp are genetically different from each other and from all the other strains as well.

5. PCR-RFLP analysis did not give evidence for mixing European and Asian carps in the cases of the danubian and tisza wild carp. All individuals showed the typical European haplotype providing evidence for the matrilineal purity of these strains.

6. Recommendations

In order to decrease inreeding and maintaine the live genebank new individuals from the same strains should be added to the broodstock. The new individuals should be tested with molecular genetic tools before entering the stock. The specialists of the institute should try to increase the number of propagated individuals while (in order to get closer to panmictic reproduction) producing the new generations of the maintained strains.

Further examinations are recommended in order to compare the strains maintained in the genbank with the wild populations and with the cultured strains maintained at carp breeders.

The PCR-RFLP studies show that the danubian wild carp and tisza wild carp strains maintained at the live genebank of Research Institute for Fisheries and Irrigation (HAKI, Szarvas, Hungary) are representing the typical European haplotype. Based on this phenomenon and the microsatellite results from the nuclear genome it can be stated that the two native strains are representing a highly valuable genetic resource. The institute should increse the number of broodfish by introducing captured individuals from the original habitats (preliminariy phenotypical and molecular genetic studies arre strongly suggested to ensure the origin of the new individuals).

Further studies, applying diagnostic markers from the nuclear genome will be necessary to exclude the possibility of paternal introgression of foreign strains.

7. Publications in the field of the dissertation

Publications in English:

E., Froufe, I., Magyary, **I., Lehoczky**, S., Weiss. (2002) mtDNA sequence data supports an Asian ancestry and single introduction of the common carp into the Danube Basin. Journal of Fish Biology 61: 301-304

I., Lehoczky, I., Magyary, C., Hancz, S., Weiss, (2005) Preliminary studies on the genetic variability of six Hungarian common carp strains using microsatellite DNA markers. Hydrobiologia 533(1): 223-228.

Lehoczky, I., Jeney, Z., Magyary, I., Hancz, C. and Kohlmann, K. (2005) Preliminary data on genetic variability and purity of common carp (*Cyprinus carpio* L.) strains kept at the live gene bank at Research Institute for Fisheries, Aquaculture and Irrigation (HAKI) Szarvas, Hungary. Aquaculture. Special Issue: Genetics in Aquaculture VIII. 247: 45-49.

Publications in Hungarian:

Lehoczky, I., Magyary, I., Hancz, Cs. (2002) Hat hazai pontyfajta genetikai változatossága mikroszatellit DNS markerekkel vizsgálva, Állattenyésztés és Takarmányozás. 51.1: 9-18

Presentations:

Lehoczky, I., Magyary I., Schlötterer, C., Weiss, S. és Hancz, Cs., (2000) Néhány hazai pontyfajta DNS szintű vizsgálata. XXIV. Halászati Tudományos Tanácskozás, HAKI. Szarvas, május 24-25. Összefoglalók. 23. pp.

Lehoczky, I., Magyary, I., Schlötterer, C., Weiss, S. és Hancz, Cs. (2001) Hazai ponty fajták genetikai vizsgálata mikroszatellit DNS markerekkel. XXV. Halászati Tudományos Tanácskozás, HAKI. Szarvas, május 16-17. Összefoglalók. 50. pp.

Lehoczky, I., Magyary, I., Hancz, C., (2002) Preliminary studies on the genetic variability of six Hungarian common carp using microsatellite DNA markers. XXth Genetic Days. Brno, Czech Republic, September 12-13, Book of abstracts. 238. pp.

Lehoczky, I., Jeney, Zs., Gorda, S., Magyary, I., Hancz, Cs., (2003) Előzetes eredmények a ponty (Cyprinus carpio L.) élő génbankjában tartott pontyfajták genetikai változatosságáról és tisztaságáról. XXVII. Halászati Tudományos Tanácskozás, HAKI. Szarvas, május 7-8. Összefoglalók. 12. pp.

Lehoczky, I., Jeney, Z. , Magyary, I. , Hancz, C. and Kohlmann, K. (2003) Preliminary data on genetic variability and purity of common carp (Cyprinus carpio L.) strains kept at the live gene bank at Research Institute for Fisheries, Aquaculture and Irrigation (HAKI) Szarvas, Hungary. VIII-th International Symposium on Genetics in Aquaculture (ISGA). Puerto Varas, Chile, November 11-15, Book of abstracts. 27. pp.

Lehoczky I., Bakos, J., Gorda, S., Magyary, I., Hancz, Cs., Jeney, Zs. (2004) Előzetes eredmények az őshonos ponty fajták genetikai állapotáról *ex-situ* élő fajtabankban. XXVIII. Halászati Tudományos Tanácskozás, HAKI. Szarvas, május 12-13. Összefoglalók. 45-46. p.

Horti, Cs., Révay, T., **Lehoczky, I.**, Jeney, Zs. és Hidas, A. (2004) Génbanki pontyfajták összehasonlító vizsgálata random DNS polimorfizmus alapján. XXVIII. Halászati Tudományos Tanácskozás, HAKI. Szarvas, május 12-13. Összefoglalók. 47. pp.

Lehoczky, I., Bakos, J., Gorda, S., Hancz, C. and Jeney, Z. (2004) Genetic status of indigenous carp (*Cyprinus carpio* L.) strains in ex-situ live gene bank. Biotechnologies for quality. Aquaculture Europe 2004. Barcelona, Spain, October 20-23. European Aquaculture Society Special Publication No.34: 477-478. p.

Lehoczky, I., Wang, S., Zhou, S., Li, S. and Jeney, Z. (2005) Genetic variability of Hungarian and Chinese Common carp (*Cyprinus carpio* L.) strains, characterised by microsatellite and RAPD markers. XL. Croatian

Symposium on Agriculture. Opatija, Croatia, February 15-18. Book of abstracts: 527-528. p.

Lehoczky, I., Bakos, J., Gorda, S., Hancz, Cs., Magyary, I. és Jeney, Zs. (2005) Őshonos pontyfajták (*Cyprinus carpio* L.) genetikai státusza ex-situ élő génbankban. VI. Magyar Genetikai Kongresszus. Eger, április 10-12. (poszter) Összefoglalók. 169-170. p.

Lehoczky I., Magyary I., Hancz Cs., Urbányi B., Horváth Á., Bakos J. és Jeney Zs. (2005) Genommanipulált pontyivadék vizsgálata mikroszatellit DNS markerekkel. XXIX. Halászati Tudományos Tanácskozás, HAKI. Szarvas, május 4-5. Összefoglalók. 23-24. p.

Lehoczky, I., Nagy Z.T., Magyary I., Hancz Cs., Bakos J. és Jeney Zs. (2006) A ponty (*Cyprinus carpio*) magyarországi tenyésztett és természetesvízi populációinak genetikai jellemzése. XXX. Halászati Tudományos Tanácskozás, HAKI, Szarvas, május 24-25. Összefoglalók. 54. pp.