

Example of the application the microsatellite DNA fragments in the study of farmed European catfish (*Silurus glanis*, L.) broodstock

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Abstract. European catfish, *Silurus glanis* L., is the second largest freshwater fish in Europe. The species is very popular among the farmers, as it is one of the most promising European aquaculture species. Despite the growing importance of European catfish in freshwater aquaculture, the genetic data available on this species are still limited. The main purpose of the present study was to develop a reliable, feasible genetic protocol for future studies on European catfish populations and broodstocks in Poland. The genetic characteristics of the tested fish group were based on genetic parameters such as the polymorphism information content (PIC), the effective population size (N_e), the inbreeding coefficient (F_{is}), and the Garza-Williamson index (M), among others. Additionally, the potential effects of a genetic bottleneck on the genetic variation of the broodstock were examined. The genetic analysis protocol described in this study can be used to establish genetic-based records for European catfish broodstocks, including for sperm cryobanking. This approach will be useful for elaborating the selection procedures that allow for optimal assemblages of spawning pairs in artificial reproduction. The application of the genetic analysis protocol in practice will permit maintaining high quality in European catfish broodstocks.

Keywords: *Silurus glanis*, microsatellite loci, gene diversity, broodstock

Introduction

European catfish, *Silurus glanis* L., is an economically, ecologically valuable representative of the Siluridae fish family. It is widely distributed across Central and Eastern Europe, inhabiting lakes and the slow-flowing rivers that connect them (Kottelat and Freyhof 2007, Alp et al. 2011). European catfish is the second largest freshwater fish species in Europe after the beluga sturgeon, and it usually reaches body lengths from 100 to 200 cm and weights from 15 to 30 kg (Kottelat and Freyhof 2007). European catfish prefers muddy river bottoms and employs a demersal life strategy. It is a nocturnal, piscivorous predator that locates its prey using its senses of smell and hearing and its lateral line (Pohlman et al. 2004).

European catfish is a very popular in freshwater aquaculture, which is why it is one of the most promising European aquaculture species. Factors such as high meat quality, rapid growth, and efficient feed use make rearing this species very attractive. Additionally, the development of modern techniques for breeding, rearing,

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feeding, and genome manipulation facilitate more efficient production of the species in aquaculture (Legendre et al. 1996, Smitherman et al. 1996, Florczyk et al. 2014). For example, European catfish reared in recirculating aquaculture systems (RAS) can reach weights of 1.5 kg in 7 to 8 months. Since 1993, the total production of the species in Europe has been increasing continually (Linhart et al. 2002, FAO 2015). Thanks to a large supply of the stocking material, European catfish is also more frequently released into open waters. This species is mainly stocked as a supplement to local populations with the aim of increasing the angling attractiveness of inland waters. In many European countries, including France, Italy, the Netherlands, Spain, and the UK, European catfish is considered as a major attraction for anglers.

In Poland the species inhabits almost all inland and brackish waters with the exception of cool montane and submontane rivers. In Polish waters the European catfish attains sexual maturity between 3 and 6 years of age. European catfish population sizes have recently declined because of overfishing and water pollution. Currently, the species does not occur in high abundance, and does not play a significant role in commercial catches. Annual fishery catches of European catfish in Poland are just several tons (Draszkiewicz-Mioduszevska et al. 2014). It is estimated that about 600,000 fry and 50 tons of heavy European catfish stocking material are released annually into open waters by the Polish Anglers Association (PZW) and other authorized fisheries enterprises (Mickiewicz et al. 2014, PZW 2015).

Despite the increasing importance of European catfish in freshwater aquaculture, the available genetic data for this species remain limited. To date, only a few preliminary genetic studies focusing on the allozyme and microsatellite and mitochondrial DNA analyses of wild European catfish populations have been performed (Krieg et al. 1999, 2000, Triantafyllidis et al. 1999a, 1999b, 2002). Presently, there are numerous European catfish broodstocks that have been established for aquaculture and stocking material production. Broodstocks are usually established and supplemented by fish originating from the natural environment. It is necessary to sacrifice

males to collect semen for artificial reproduction (Legendre et al. 1996, Brzuska and Adamek 1999). The conditions of aquaculture pose risks of adverse changes in the gene pools of reared fish stocks, resulting in the loss of genetic diversity, decreased heterozygosity, and inbreeding depression. Genetically monitoring reared fish stocks is a very useful tool in modern aquaculture that permits focusing on biodiversity. Permanent genetic monitoring facilitates the more effective management of living resources, and contributes to preventing adverse changes in genetic structure.

The main purpose of the present study was to develop a reliable, feasible genetic protocol for future studies on European catfish broodstocks in Poland.

Materials and methods

Sample collection and DNA extraction

Pectoral fin clips from a total of 20 European catfish specimens were sampled non-invasively from a broodstock at Wasosze Fish Farm near Konin, Poland. The fin clips were fixed by drying, and then kept in envelopes at room temperature until DNA extraction. Genomic DNA was extracted from the collected fin tissues using the Genoplast Tissue Genomic DNA Extraction Mini Kit (Genoplast, Poland) following the manufacturer's protocol.

PCR amplification

Five microsatellite loci, *S3-10*, *S5-F*, *S3-25*, *S1-40* and *S7-159* (Krieg et al. 1999), were amplified using the polymerase chain reaction (PCR) technique. Reaction mixtures were prepared in a total volume of 25 μ l with a 0.7 μ l DNA template ($4.6 \pm 0.5 \mu\text{g ml}^{-1}$), 6.0 μ l of 10x PCR reaction buffer (100mM KCl, 100mM $(\text{NH}_4)_2\text{SO}_4$, 200mM Tris-HCl pH 8.5, 20mM MgSO_4 , 1% Triton X-100), 1.0 μ l of each primer, 0.5 μ l (500 μ M) of each deoxynucleotide triphosphate (dNTP) and 0.3 unit TaqDNA polymerase RUN (A&A Biotechnology, Poland). Re-distilled water was used to bring the

reaction mixture to the desired final volume. Amplification was performed with a Mastercycler gradient thermocycler (Eppendorf, Germany) under the following conditions: initial denaturation at 94°C for 3 min, followed by 34 cycles at 94°C for 30 s, annealing at 58–60°C (Table 1) for 30 s, elongation at 72°C for 45 s and a final elongation step at 72°C for 7 min. The quality of obtained PCR products were assessed by electrophoresis of samples in 1.5% agarose gel with ethidium bromide DNA bands visualization.

Genotyping

Genotyping of microsatellite DNA fragments was conducted using an Applied Biosystems 3130 Genetic Analyzer. In order to enable genotyping of the PCR products, forward primers were fluorescently labeled. The GeneScan 600 LIZ size standard was utilized as a reference for determining the length of the examined DNA fragments. The results were visualized using computer software provided by the manufacturer Genemapper v4.1 and Data Collection Software v3.0 (Applied Biosystems, California, USA). Genetic profiles containing the list of alleles detected within the studied loci were prepared for each fish.

Data analysis

Microsatellite allele frequencies, the number of allele per locus, allelic range, allelic richness (A_r), and fixation index (F_{is}) were computed using Fstat computer software (version 2.9.3.2) (Goudet 2002). The observed (H_o) and expected heterozygosity (H_e), Garza-Williamson index (M), and Linkage Disequilibrium (LD) were calculated using Arlequin software (version 3.5) (Excoffier and Lischer 2010). The Exact Hardy-Weinberg equilibrium (H-WE) test for each locus was performed in GenPop software (version 4.2.1) (Rousset 2008). Moreover, global Hardy-Weinberg equilibrium probability tests were conducted with Fisher's method, and Smouse's multilocus analysis implemented in GenPop and PopGene software (version 1.3.2) (Yeh and Boylet

1997), respectively. Shannon's index (I) and the polymorphism information content (PIC value) for each loci within the investigated broodstock of European catfish were also calculated using PopGene and PowerMarker (version 3.25) (Liu and Muse 2005) software. The effective population size (N_e) was assessed for the examined European catfish specimens with the NeEstimator computer program (version 2.01) (Do et al. 2013). The linkage disequilibrium method was used for computing N_e , where the lowest allele frequency used was 0.02. Calculated N_e values were subsequently corrected for underestimation from sampling errors with jackknifing 95% confidence intervals (CIs). A test for bottleneck assessment was conducted using Bottleneck computer software (version 1.9) (Piry et al. 1999) via the two methods implemented. The first method tested for departure from the mutation drift equilibrium based on heterozygosity excess or deficiency. Recent broodstock bottlenecks were tested in the examined European catfish broodstock with the Stepwise Mutation Model (SMM) and Infinite Allele Model (IAM). This method is based on the assumption that in non-bottlenecked broodstock (close to mutation drift equilibrium) the value of expected heterozygosity (H_e) is equal to Heq (heterozygosity expected in a mutation-drift equilibrium). The excess of H_e over Heq is evidence of a severe reduction in broodstock effective size that can occur because of a bottleneck. Statistical tests were performed using the one-tailed Wilcoxon signed rank test. The second method was to test allele frequency distribution. A population that does not suffer any bottleneck is expected to show a normal L-shaped distribution. In contrast, a bottlenecked population exhibits mode shifts (Luikart and Cornuet 1998).

Results

In the present study all five examined microsatellite loci were polymorphic within the investigated European catfish specimens. The size of the alleles at individual locus varied between 118 and 254 base pairs

Table 1

Characterizations of the five microsatellite loci used in the study of the European catfish (*S. glanis*) broodstock at Wąsosze Fish Farm in Poland: locus designation, primer sequences, optimal annealing temperature (T_a), allele size range and source reference

Locus	Primer sequence (5'→3')	T_a (°C)	Allele size range (bp)	Reference
S3-10	F: CTACTTGCGACCTACTTGAAC	58	128-138	Krieg et al. 1999
	R: GTAACCCACACAACCTCAGG			
S5-F	F: CCAATTTACCTCAGACTACTTCTG	58	132-141	Krieg et al. 1999
	R: GCACGTGCAAAGTCTCTG			
S3-25	F: CTGGCCGGAGAATAGCCTG	58	210-235	Krieg et al. 1999
	R: GGGCTCATAACATACTCATTTCAC			
S1-40	F: CCCTCCTGCTGGCATCC	58	118-126	Krieg et al. 1999
	R: GCAAAACCTAGAGGTGCTTTAC			
S7-159	F: CTGCTCAATCAAAGTTGGTTC	60	236-254	Krieg et al. 1999
	R: CAAACTAAGTTCAGCCAGGC			

Table 2

Genetic diversity parameters of the European catfish broodstock (*S. glanis*). A_r : allelic richness, A_o : observed alleles, A_e : expected alleles, I : Shannon's index, PIC : polymorphism information content, Fis : fixation index. Fis values statistically significant at: * $P < 0.05$, ** $P < 0.001$

Locus	A_r	A_o	A_e	I	PIC	Fis
S3-10	4.000	4	2.556	1.067	0.529	-0.208
S5-F	3.000	3	2.100	0.792	0.410	-0.905**
S3-25	4.000	4	2.694	1.136	0.565	-0.329*
S1-40	3.000	3	2.000	0.693	0.410	-0.805**
S7-159	3.000	3	2.508	0.983	0.495	-0.608**
Mean	3.400	3.4	2.372	0.934	0.482	-0.571**

(bp). A total of 17 alleles were found in the group of fish studied. The number of alleles per locus ranged from 3 (*S5-F*, *S1-40*, *S7-159*) to 4 (*S3-10*, *S3-25*), at an average of 3.4 alleles per locus (Tables 1 and 2).

The genetic diversity parameters (H_o , H_e , A_r , A_o , A_e , I and PIC) of the European catfish studied are presented in Tables 2 and 3. The average values of the polymorphism information content (PIC) and Shannon's index (I) were 0.482 and 0.934, respectively. The observed allelic richness (A_r) varied from 3.000 to 4.000 in fish studied. The estimated effective population size (N_e) value for the fish group investigated was 26.1 (95% CI = 3.1-∞). The values of the fixation index (Fis) within each loci were between -0.208 and -0.905 with an average of -0.571. All Fis values obtained were statistically significant except for the *S3-10* locus. Moreover, the average value of this indicator was also highly significant (Table 2).

The observed heterozygosity (H_o) within the examined loci ranged from 0.750 to 1.000. In turn, the expected heterozygosity varied from 0.537 to 0.645. Three of the five examined loci (*S5-F*, *S1-40*, *S7-159*) deviated from H-WE, exhibiting heterozygosity excess. Both of the H-WE probability tests applied detected highly significant ($P < 0.001$) global deviation of the loci examined from H-WE expectations. Furthermore, significant ($\alpha = 0.01$) linkage disequilibrium (LD) was found among five of the ten locus pairs in the fish tested. Table 3 also shows the expected heterozygosity in two models of mutation-drift equilibrium (Heq). Heterozygosity excess was detected in all of the analyzed loci in the group of fish tested under the Infinite Allele Model (*IAM*) and Stepwise Mutation Model (*SMM*). Nevertheless, the observed $He > Heq$ differences within each loci were not statistically significant. On the other hand, the

Table 3

Comparison of observed (H_o) and expected (H_e) heterozygosity, expected heterozygosity (Heq) in the Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and the Garza-Williamson index (M) in the European catfish (*S. glanis*) broodstock: P-level of significance. Deviations statistically significant at $P < 0.05$

Locus	H_o	H_e	P	IAM		SMM		M
				Heq	P	Heq	P	
S3-10	0.750	0.624	0.384	0.500	0.229	0.615	0.465	0.364
S5-F	1.000	0.537	0.000	0.388	0.231	0.496	0.444	0.300
S3-25	0.850	0.645	0.263	0.495	0.178	0.615	0.477	0.154
S1-40	0.950	0.537	0.000	0.389	0.220	0.485	0.438	0.333
S7-159	0.950	0.600	0.001	0.384	0.082	0.493	0.206	0.158
Mean	0.900	0.590	-	0.431	-	0.541	-	0.262

Wilcoxon sign test showed significant ($P < 0.05$) overall heterozygosity excess under both the IAM and SMM in the group of European catfish examined. Furthermore, mode-shift analyses indicated a shifted mode of allele distribution. All investigated loci differed in terms of the Garza-Williamson index (M), ranging from 0.154 to 0.364. The average value of M in the investigated fish was 0.262.

Discussion

Microsatellite DNA is commonly used as a universal genetic marker. Molecular screening based on microsatellite DNA markers is essential when determining the degree of relatedness among individuals in fish stocks, identifying species and interspecific hybrids, the genetic monitoring of broodstocks, and verifying genomic manipulation effectiveness, among other things (Frankham et al. 2010, Hellerman et al. 2007, Olsson et al. 2012, Kaczmarczyk and Fopp-Bayat 2013). An important advantage of such analysis is not invasive because only small fragments of tissue (e.g., fins) are required for genetic examinations. This circumstance is particularly important in the case of broodstocks, enabling for assemblages of spawning pairs (Kaczmarczyk and Fopp-Bayat 2013). The present study presents an example of applying microsatellite DNA fragments in the study of

a farmed European catfish broodstock, and this method can be implemented in further work with European catfish stocks.

In Poland European catfish has long been an important aquaculture species since it is a valuable source of high quality, palatable meat. Moreover, in recent years this fish species has also been reared more and more frequently for the production of stocking material released into Polish waters. According to data available from 2013, the total production of this species was about 350 tons, making it the sixth most important species in Polish aquaculture after carp, trout, and sturgeon. In turn, the overall production of European catfish stocking material in Poland for aquaculture and inland water stocking was estimated to be 126 tons. Furthermore, the total share of the species in stocking programs is approximately 5%, and this trend is on the rise (Lirski and Myszowski 2014, Mickiewicz et al. 2014). In the face of the growing importance of European catfish in Polish aquaculture, information regarding the genetic characteristics of existing broodstocks of this species can be very helpful in the management of both aquaculture stocks and stocking programs to supplement wild populations. The genetic analysis protocol described in the present paper can be used to establish the genetic characteristics of European catfish broodstocks, and for sperm cryobanking.

The European catfish individuals examined were characterized by moderate levels of genetic diversity (the mean *PIC* and *I* were close to 0.5 and 1.0, respectively). Similar moderate indices of genetic diversity to those observed in the present study were reported for some wild populations of European catfish and the Aristotle catfish, *Silurus aristotelis* Garman, from southern Europe (Triantafyllidis et al. 1999a, 2002). Lower values of these indices were reported in broodstocks of brook trout, *Salvelinus fontinalis* (Mitchill), from Poland (Fopp-Bayat et al. 2010). On the other hand, higher genetic diversity parameters were observed in the Soldatov catfish, *Silurus soldatovi* Nikolskii & Soin, from northern China and in Siberian sturgeon, *Acipenser baerii* Brandt, broodstock from Poland (Fopp-Bayat 2010, Quan et al. 2007).

According to the current results, the studied group of European catfish was not in H-W equilibrium. Most (60%) of the studied loci deviated from the H-W equilibrium, suggesting that both the genotype and gene frequencies fluctuated continuously as a response to the various stocking conditions. The average *F_{is}* value was negative in the specimens examined, and exhibited significant excess of heterozygotes against Hardy-Weinberg expectations. This significant excess is most probably a consequence of non-random selection of spawners under hatchery conditions (Luikart and Cornuet 1999). Additionally, the global deviation observed of the studied fish group from H-WE seems to support this hypothesis. Similar observations are frequently reported for other fish stocks kept under controlled conditions, e.g., for Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum); Arctic charr, *Salvelinus alpinus* (L.); brook trout, *S. fontinalis*; paddlefish, *Polyodon spathula* (Walbaum); European huchen, *Hucho hucho* (L.) (Kim et al. 2004, Ditlecadet et al. 2006, Fopp-Bayat et al. 2010, Kaczmarczyk et al. 2012, Kucinski et al. 2015).

In most studies, the characterization of linkage relationships between microsatellite loci used in genetic analyses is usually disregarded. Significant, stable LD typically means that the microsatellite loci used are physically linked; however, LD can also be

caused by small population size, non-random mating, sampling bias, recent admixture, or genetic drift (Frankham et al. 2010). In the current study, only microsatellite locus *S5-F* displayed evident LD, where it was observed with relation to all the other microsatellite loci used. All LDs occurred in proportion (five pairs of loci of ten possible combinations), which might not be expected by chance alone. The results obtained on LD imply that the studied group of European catfish specimens could be affected by genetic drift effect as is hypothesized by Hill and Robertson (1968) with populations of small size.

Despite the bottleneck test applied in the present study which did not indicate any statistically significant differences in *He* > *Heq* within each loci, the Wilcoxon sign test and mode-shift analyses showed evidence of a bottleneck under *IAM* and *SMM*. Similarly, the average value of the Garza-Williamson index ($M=0.262$) suggested that the tested group of European catfish could have suffered a bottleneck or a founder effect in the past. Based on numerous reports in the literature, values of the Garza-Williamson index of 0.8 or higher are specific to populations that were not subjected to size reduction in the past (Lampila et al. 2009, Li et al. 2010). Lower values of the index (about 0.7 or less) might indicate that a population has undergone a recent reduction in size (Garza and Williamson 2001). Moreover, low values of *M* (below 0.4) are specific for remnant populations and might indicate an essential reduction in population size in the past (Tzika et al. 2008, Kaczmarczyk and Żuchowska 2011).

The influence of captive conditions on the genetic structure of fish stocks is very complex and difficult to predict (Duchesne and Bernatchez 2002). Widespread practices such as mixed-milt fertilization, systematic spawner selection based on specified phenotypes, or feeding under controlled conditions can lead to the depletion of fish stock genetic diversity (Waples 1999, Glover et al. 2001, Wedekind et al. 2007). Using modern spawner selection methods is essential to preserving a high level of genetic diversity, and helps to prevent the inbreeding effect (Fopp-Bayat 2010). However, it should be empathized that within small, isolated fish stocks the risk

of significant changes in their genetic structure is much greater from genetic drift than from inbreeding (Hellerman et al. 2007, Frankham et al. 2010). Analysis of effective population size (N_e) parameters for farmed fish stocks is a very effective tool for determining their vulnerability to genetic drift effect (Tringali and Bert 1998, Hoarou et al. 2005). To avoid short-term inbreeding and genetic drift events in farmed fish stocks, the values of N_e should be at least 50 (Frankham et al. 2010). The estimated N_e value for the European catfish examined in the present study was lower than the threshold value of 50, which indicates they are very susceptible to genetic drift. The current results suggest that future management decisions should take this into account and attempts should be made to improve the efficiency of artificial spawning and to increase breeder census.

The genetic registry of fish stocks is a very important tool in sustainable aquaculture management since it permits elaborating the optimal spawner selection protocol during artificial reproduction, while also guaranteeing appropriate fish stock mixing (Huff et al. 2011, Kaczmarczyk and Fopp-Bayat 2013). A properly developed fish stock genetic registry should include basic information such as the origin of fishes used when establishing and supplementing broodstocks, the spawner domestication level, their numbers, sex, age, fecundity, and phenotypic features. In addition, genetic data determined with microsatellite DNA analyses that includes genotype profiles for each specimen is an essential part of any fish stock registry, and it can also be used to cryobank the sperm of valuable, rare fish species (Fopp-Bayat and Ciereszko 2012).

Using the proposed genetic analysis protocol will permit maintaining high quality European catfish broodstocks. Moreover, it will also help to prevent cross-breeding among individuals with similar genetic profiles, which would decrease the quality of the fish material.

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