Folia Zool. – 57(1–2): 90–99 (2008)

Genetic diversity of *Misgurnus fossilis* populations from the Czech Republic and Slovakia

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Received 2 January 2007; Accepted 21 March 2008

A b s t r a c t . Monitoring of the analysed populations of the endangered fish species *Misgurnus fossilis* was conducted using microsatellite analysis and sequencing of a part of the control region. Absolutely first microsatellite markers for weather loach were found. Six polymorphic microsatellite loci were prepared, five of which were tested. Number of alleles per a locus ranged from 3 to 5. All studied populations are differentiated one from another (F_{ST} = 0.205 - 0.367). All sampled populations contained unique alleles. Sequential analysis of the mitochondrial control region showed great haplotype similarity of the studied populations which come from one widely spread haplotype H_1, and thus suggested possible hypothesis of recent spreading from one source.

Key words: weather loach, endangered species, control region, cross-species amplification, microsatellites

Introduction

Weather loach, *Misgurnus fossilis* (Linnaeus, 1758), is an inconspicuous European species whose distribution area spreads from Spain to the Volga River (H a n e l & L u s k 2005, F r o e s e & P a u l y 2007). In a number of countries it is included in the Red List and is protected by national legislation. According to Council Directive No. 92/43/EEC, the "Sites of Community Importance" (SCI) have been defined for selected populations in EU countries. Occurrence of weather loach is attached to specific biotopes in floodplains of bigger rivers. Reconstruction and regulation of rivers has lead to a significant decrease in these original natural biotopes. Marked increase in intensity of fish production in ponds has also lead to limitation of weather loach occurrence in these alternative biotopes. In the Czech Republic, *M. fossilis* was found in 120 map squares (11.1 x 12.0 km) during the period 1964–2005, while recently it was confirmed only in 37 squares. The effort for recovery of the original species diversity of fish biota is reflected also in the activity to re-stock weather loach into sites from where it disappeared in the past due to various reasons. We also register efforts for establishing of artificial breeding aimed at gaining fish stock for the above mentioned purpose. The necessary conservation of genetic diversity should be based on knowledge of the contemporary status of weather loach populations and on its acceptance during restoration of extinct populations or support for the endangered ones. Therefore the aim of our study was to prepare necessary microsatellite and sequencing markers and thus gain the first pieces of information about genetic diversity of selected *M. fossilis* populations.

Materials and Methods

Sample collection and DNA isolation

We investigated altogether 49 specimens of *M. fossilis* from the drainage areas of the North, Baltic and Black Sea. These specimens were caught using electrofishing at the sites mentioned below during the years 2004–2005.
Czech Republic: North Sea – the Lužnice River (locality Halámky, 10 individuals); Black Sea – confluence of the Dyje and Morava Rivers (loc. Soutok, 10 ind.); the Morava River (loc. Týnec, 5 ind.); Baltic Sea – the Odra River (ponds, 3 ind.); the Opava River (loc. Štěpán, 5 ind.).
Slovak Republic: Black Sea – the Bodrog River (loc. Svätá Mária, 10 ind.); the Ida River (1 ind.); the Belžanský stream (5 ind.). The overall genome DNA was isolated from fins preserved in alcohol using the phenole-chloroform-isoamylalcohol method (Sambrook et al. 1989).

Microsatellite analysis

The very first microsatellite markers in weather loach were prepared using the cross-species amplification method. The microsatellite loci were isolated from the partial genome library of *M. anguillicaudatus*, constructed by Morishima et al. (2001, 2002). Primers were synthesized from the flanking regions of sixteen microsatellites: Mac2, Mac3, Mac4, Mac15, Mac24, Mac32, Mac35, Mac37, Mac40, Mac43, Mac45, Mac47, Mac49, Mac56, Mac57, Mac63. Preliminary testing of microsatellite markers was performed in 15 µl mastermixes (Mac43 and Mac47: 2.0 mM $MgCl_2$) under the following PCR conditions: 94 °C for 1 min, followed by 30 cycles (38 cycles for final PCR) of 94 °C for 30 s, annealing at 50–59 °C for 30 s, and an extension temperature of 72 °C for 40 s, followed by a final extension of 72 °C for 10 min. PCR fragments were identified on 1.7% agarose gel in SB electrophoretic buffer (Sodium boric acid conductive medium, 10mM NaOH and pH adjusted to 8.5 with H_3BO_3) for preliminary determination of suitability of selected primers.

Identification of a new microsatellite was done using the tailed primer method. This method utilizes a two-part primer, which consists of a standard primer sequence and a „tail", which is added to 5´-end of the primer sequence (5´-CACGACGTTGTAAAACGAC-3´). The tail sequence corresponds to a standard primer such as M13 universal primer. Allelic polymorphism was detected on automated DNA sequencer Beckman Coulter CEQ™ 8800.

Data analysis

Genetic polymorphism was estimated for each population as the number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E). The significance of the spatial variation in gene diversity among populations was estimated by performing a hierarchical analysis of the molecular variance model (AMOVA), as described in Michalakis & Excoffier (1996). Allelic distribution, test for locus conformity with HWE, Wright's F-statistics, Nei's genetic distance and principal coordinates analysis (PCA), as well as the statistic data mentioned above were computed using GenAlEx 6 package (Peakall & Smouse 2006).

PCR amplification of mitochondrial DNA and sequencing

The control region, specifically its whole central domain and a part of ETAS domain (extended termination-associated sequences), was selected as a mitochondrial marker. For the control region amplification, PCR primers designed from common carp sequences were used (Thai et al. 2004): Carp-Pro (5´-AACTCTCACCCCTGGCTACCAAAG-3´) and Carp-Phe (5´-CTAGGACTCATCTTAGCATCTTCAGTG-3´). The PCR reaction was performed in TGRADIENT Thermocycler (Whatman Biometra) under the following conditions: 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, and an extension temperature of 72 °C for 1 min, followed by a final extension of 72 °C for 5 min. The PCR products were purified by means of a precipitation PEG/Mg/NaAc (26% Polyethylene glycol, 6.5 mM $MgCl_2$. $6H_2O$, 0.6 M NaAc.$3H_2O$). Purified PCR were sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The analysed sequence was repeatedly sequenced from both sides to check the quality. The pertinence of the sequence analysed was verified by comparing it with the NCBI database.

Phylogenetic analysis

A part of the control region was analysed in six populations, see Table 1. The haplotype (Hd) and nucleotide diversity (π) was estimated using DnaSP 4.0 (Nei 1987, Rozas et al. 2003).

For the reason of mutual comparison, the sequences from GenBank database of the *Paramisgurnus dabryanus* (DQ105316) species: two sequences of *M. anguillicaudatus* from the sites Guangxi (AY600879) and Xianning Hubei (DQ105312), as well as two sequences of *M. bipartitus* (valid name is *M. mohoity*) from the sites Yanji Jilin (DQ105311) and Haerbin Heilongji (DQ105309) were included in the cumulative dataset. As an outgroup a sequence of *Gobio gobio* (AJ388392) was used. Control region DNA sequences were aligned using the Clustal W algorithm and adapted using the Lasergene 6 software (DNASTAR, Inc.).

The web-based ModelTest 3.8 (Posada 2006) was used as a tool for selection of the best-fit model of nucleotide substitution. The HKY+I □model was determined for the tested dataset according to Akaike information criterion (AIC). Estimation of the phylogenetic relationships of the haplotypes was performed using neighbour joining (NJ) algorithm and according to the optimality criteria: maximum parsimony (MP) and maximum-likelihood (ML) and also using Bayesian inference (BI). Sequences were imported into PAUP* 4.0B.10 (Swofford 2002) and MrBayes 3.1.2 (Ronquist & Huelsenbeck 2005) for phylogenetic analysis. For NJ analysis, HKY+I distances were calculated. Non-parametric bootstrap analyses with 1000 pseudo-replicates were performed to obtain estimates of support for each node of the NJ trees. For MP tree construction, the unweighted parsimony analysis was employed using the branch-and-bound search. The confidence levels in the resulting relationship were also assessed using the bootstrap procedure with 1000 replications. ML search was performed under the HKY+I+Γ model (the same value –lnL as for HKY+I model) with the branch-and-bound algorithm on 100 bootstrap replicates. Likelihood settings of the best-fit model (HKY+I+Γ) based on the hierarchical likelihood ratio tests (hLRTs) were as follows: base frequencies (A = 0.3241, C = 0.1887, G = 0.1517 and T = 0.3355); ti/tv ratio = 1.2141; proportion of invariant sites 0.4167; and the shape parameter of the gamma distribution 0.7510.

Table 1. Control region sequence haplotypes and variable sites in the 474 bp sequenced region of *M. fossilis* (dots indicate equality with haplotype H_1). Haplotype frequencies, haplotype and nucleotide diversity within the six populations (Bel, Belžan stream; Ida R.; Luž, Lužnice R.; Mor, Morava R.; Odr, Odra R.; Opa, Opava R.).

		Variable sites							Populations/specimens					
		0	1	2	3	4	4	4						
	Haplotype	9	9	9	0	2	2	3						
		5	9	8	0	2	9	3	Bel	Ida	Luž	Mor	Odr	Opa
	H_1	A	T	C	T	A	C	C	2	1		1	2	2
	H_2	·	A	·	·	·	·	·	1					
Haplotype	H_3	·	·	·	C	·	·	·	2			3		2
frequencies	H_4	G	·	·	C	·	·	·				1		
	H_5	·	·	·	·	T	·	·					1	
	H_6	·	·	T	·	·	·	·						1
	H_7	·	·	·	C	·	A	A			2			
Haplotype diversity									0.800 ± 0.164	1.000 ± 0.000	0.000 ± 0.000	0.700 ± 0.218	0.667 ± 0.314	0.800 ± 0.027
Nucleotide diversity									0.00211 ± 0.00059	0.00000 ± 0.00000	0.00000 ± 0.00000	0.00169 ± 0.00064	0.00141 ± 0.00066	0.00211 ± 0.00058

Bayesian analysis was performed using MrBayes 3.1.2 program. Starting from a random tree, four Markov chains were run for 1 x 10^6 generations with sampling frequency of 100. The HKY+ Į model was specified. The application Tracer 1.2 (Rambaut & Drummond 2003) was used to view the output of the *sump* file generated by MrBayes. Trees generated prior to reaching stationarity were discarded as burn-in. We then took the resulting 50% majority rule consensus tree.

A haplotype network was constructed to estimate the genealogical intraspecific relationships employing the statistical parsimony (Templeton et al. 1992) implemented in the TCS 1.21 program (Clement et al. 2000).

Results and Discussion

The first insight into weather loach population structure-microsatellite analysis

For preparation of microsatellite markers in weather loach 16 pairs of primers have been tested for the time being (the analyses continue). Of the 16 loci tested, 5 were polymorphic, 1 was slightly polymorphic, 5 were monomorphic, and 5 pairs of primers did not amplify any PCR product (Table 2).

For the time being, the population statistics of *M. fossilis* has been based on testing of three populations, 10 individuals each, and therefore represents only a preliminary view of population structure of the analysed populations. Six polymorphic loci with 2 – 5

Table 2. Microsatellite locus designation, repeat motif, PCR results of the test of 16 loci (Y = PCR product was detected, N = no PCR product), annealing temperature (T_a), allele size range in base pair (bp), number of private alleles, mean values expected (H_E) and observed heterozygosity (H_O); x = not specified.

Locus	Repeat motif	PCR product	T_a (°C)	No. of alleles	Allele size range (bp)	Private alleles	Mean H_O	Mean H_E
Mac2	(CA)14	Y	59	4 (7)	75-83	1	0.269	0.346
Mac3	(CA)17	Y	50	5 (6)	80-86	2	0.403	0.418
Mac4	(CA)39	N	x	x (4)	x	x	x	x
Mac15	(CA)7	N	x	x (6)	x	x	x	x
Mac24	(CA)11	Y	59	4 (3)	89-100	3	0.276	0.237
Mac32	(TG)19	N	x	x (3)	x	x	x	x
Mac35	(CA)11	N	x	x (4)	x	x	x	x
Mac37	(CA)15	Y	59	5 (5)	77-87	3	0.633	0.517
Mac40	(CA)6 -AA -(CA)9	N	x	x (5)	x	x	x	x
Mac43	(CA)8-TG -(CA)8 -TG-(CA)3	Y	59	3 (3)	69-73	1	0.462	0.382
Mac45	(CA)10	Y	54	1 (5)	72	0	x	x
Mac47	(CA)16	Y	59	1 (4)	104	0	x	x
Mac49	(CA)21	Y	54	1 (6)	73	0	x	x
Mac56	(TG)21	Y	59	1 (3)	74	0	x	x
Mac57	(CA)23	Y	59	2 (3)	74-76	0	x	x
Mac63	(CA)16	Y	59	1 (4)	88, 92	0	x	x

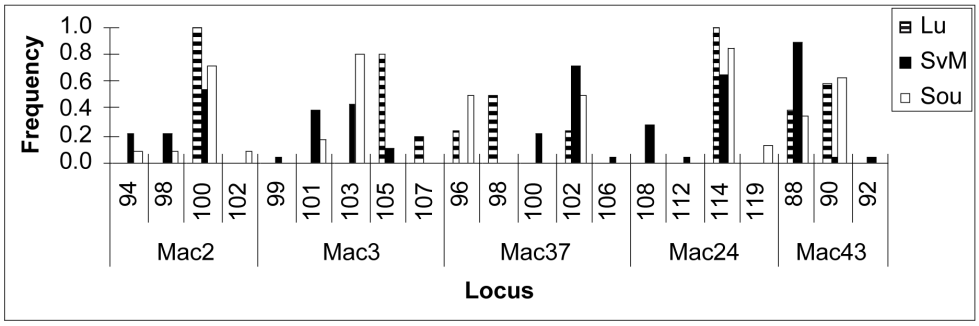

Fig. 1. Allele frequencies of five loci in three populations of *Misgurnus fossilis*. Lu, Lužnice River; Sou, Soutok; SvM, Svätá Mária.

alleles per locus were identified. Among others, Table 2 also compares polymorphism of alleles of the pertinent locus in *M. anguillicaudatus* and *M. fossilis* (figures in parentheses specify the number of alleles per locus; tested in 6 individuals of *M. anguillicaudatus*; Morishima, personal communication). Lower polymorphism of alleles of the tested *M. fossilis* populations in comparison with *M. anguillicaudatus* was evident.

In five polymorphic microsatellite loci tested, 16, 12 and 9 alleles were identified in populations from Svätá Mária, Soutok and the Lužnice River (Fig.1). In case of the population from the Lužnice River the microsatellite loci Mac2 and Mac24 were monomorphic. Expected heterozygosity (He) per locus ranged from 0.204 to 0.636. The highest value of He was found in the population from Svätá Mária, then from Soutok and the Lužnice River. The total number of private alleles in the population from the site Svätá Mária was six, from Soutok and the Lužnice River two.

The result of Chi-square test (χ^2-test) for HWE was not statistically significant (P>0.05) for the populations from the sites Svätá Mária and Soutok. In case of the individuals from the Lužnice River the result of χ^2-test is not given, due to bias resulting from monomorphy of the above mentioned Mac2 and Mac24 loci.

The statistically significant F_{ST} value lies outside the F_{ST} distribution generated by random permutation (9999 permutations) for confirmation of the null hypothesis (graph not provided). So all the three populations are differentiated one from another, the individual

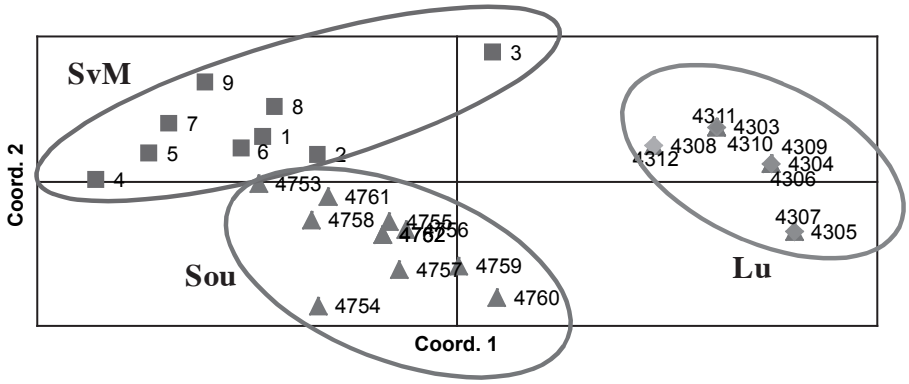

Fig. 2. Multivariate technique – Principal coordinate analysis (PCA) of three populations. Lu, individuals from Lužnice River; Sou, individuals from Soutok; SvM, individuals from Svätá Mária.

F_{ST} values ranged from 0.205 to 0.367. Very high F_{ST} values suggest decrease in the flow of genes between the studied populations and the influence of genetic drift upon fixation of different alleles.

The Analysis of Molecular Variance (AMOVA) showed that the overall variance can be explained as follows: 67% by variance within populations, 20% by variance among populations and 13% by variance between the drainage areas of the North and Black Seas. The multivariate technique – Principal Coordinate Analysis (PCA, Fig. 2) – documents mutual differentiation of the populations graphically and at the same time highlights closer proximity of the populations from the sites Svätá Mária and Soutok which belong to the same Black Sea drainage area. The Nei genetic distance values of the tested populations ranged from 0.273 to 0.496 and the highest values were identified in the specimens from the Lužnice River of the North Sea drainage area (Lužnice vs. Soutok 0.334 and Lužnice vs. Svätá Mária 0.496).

Sequence characteristics

All haplotype sequences of the control region of 474 bp size were deposited in the GenBank (EF090913 - EF090919). The molecular characteristics of the control region: base frequencies A = 0.3241, C = 0.1887, G = 0.1517 and T = 0.3355 and number of parsimony-informative characters = 74. Nucleotide base composition showed high level of A and T (32.4% and 33.6%, respectively), it is similarly specified in insects, where the control region is called the AT-rich region (Hillis et al. 1996).

Phylogenetic analysis

Analysis of the mitochondrial marker control region supports the differentiation of *M. fossilis* from the other compared species with strong bootstraping support (100), both in case of the algorithmic method (NJ), and the methods based on optimality criteria (MP and ML). Similarly the Bayesian analysis determined the value of posterior probability to be 1.00. The values of HKY+I distances highlighted marked sequential difference in comparison with Asian species *M. mohoity* or *M. anguillicaudatus* (15.3–18.6%). On the basis of comparison of available sequences from the GenBank database, *M. fossilis* seems to be sequentially closer to *Paramisgurnus dabryanus* (7.7–8.0%) with strong bootstraping support, as well as high posterior probability value (1.00). The maximum parsimony analysis generated one MPT (length, 223; CI (excluding uninformative characters), 0.809; RI, 0.886; RC, 0.799). The 50% majority-rule consensus tree displays two major clades with strong support (87 and 100, the tree is not shown). The first clade includes the representatives of haplotypes of *M. fossilis* from the drainage areas of three seas (North, Baltic and Black Sea) and of *P. dabryanus*. The second clade includes the representatives of two Asian species, *M. anguillicaudatus* and *M. mohoity*.

The topology of Bayesian tree (Fig. 3), in which the bootstrap values of two tree-building methods (NJ and ML) are displayed together with the values of posterior probability, resembles that of MP tree. That means a strong bootstrap support and posterior probability value of 1.00 for the major clades. The only differences were in more precise identification of the individual representatives of *M. fossilis* populations and also in stronger support for identification of the North Sea basin representative (Lužnice R.). Further subdivision of the representatives of *M. fossilis* is lacking a strong support and what is more, the ML analysis

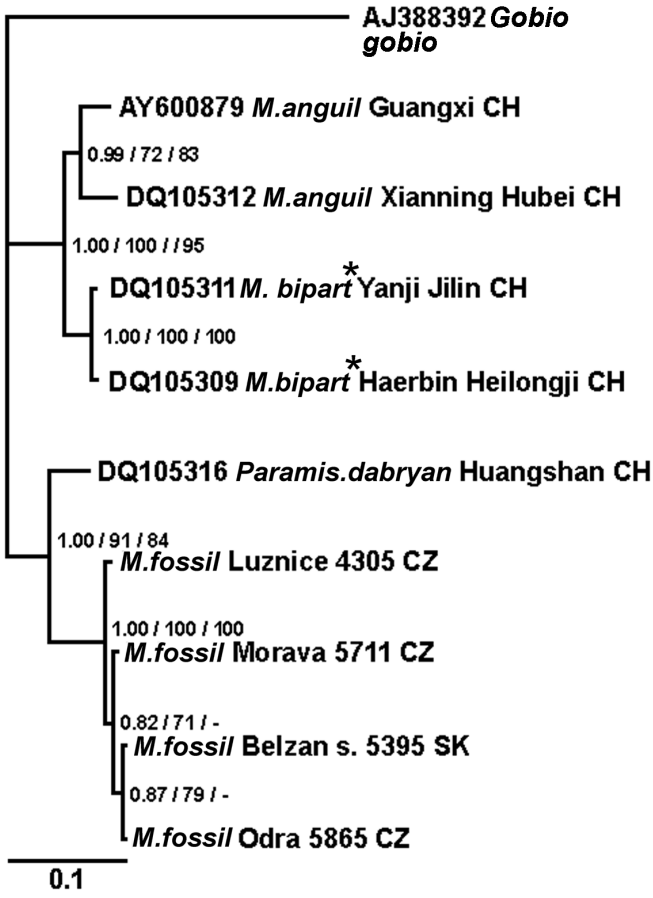

Fig. 3. Bayesian consensus tree resulting from analysis of 474 bp sequence of the control region, with Bayesian posterior probabilities/NJ bootstrap/ML bootstrap listed near the nodes (for details see section Materials and Methods). * valid name is *M. mohoity*. The nominal species name is followed by the name of the locality.

has placed the remaining representatives into a common group (bootstrap support <50%). Therefore, the NJ, MP, ML, as well as Bayesian analyses of the control region produce congruent topologies which in all cases support the monophyly of the species *M. fossilis* (100 % of bootstrap support and posterior probability 1.00) and the monophyly of the Asian species *M. anguillicaudatus* and *M. mohoity* (95–100% bootstrap and posterior probability 1.00). In case of the specimens of *M. anguillicaudatus*, moderate bootstrap support (72–83%) is apparent, which could be linked to the belief concerning hidden species within this species (A r a i 2003).

H a p l o t y p e r i c h n e s s a n d h a p l o t y p e n e t w o r k

The analyzed distribution area is regarded, according to B o h l e n et al. (2007), as the area with the highest genetic diversity of this species. Sequencing analysis of a part of the control region has discovered seven different haplotypes in *M. fossilis* defined by seven various sites and differing by one to three substitutions. Low to high levels of haplotypic diversity

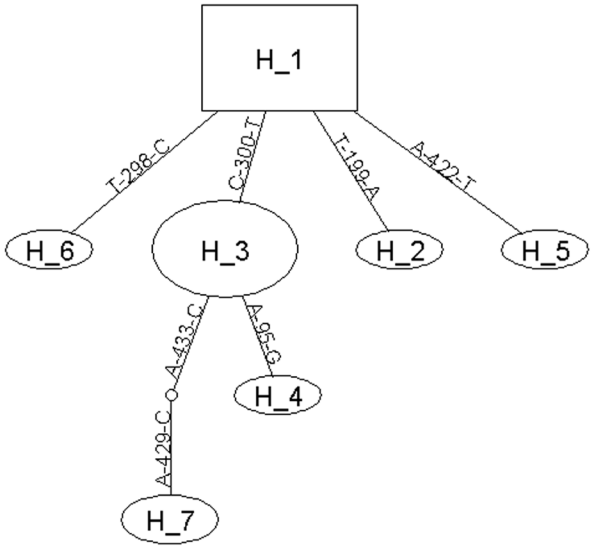

Fig. 4. Unrooted haplotype network based on the control region of the *M. fossilis*. Nodes are labelled with the haplotype; node sizes are proportional to the haplotype frequency. Mutating sites are noted along the branches.

(0.000–1.000) and low nucleotide diversity (0.00000 – 0.00211) were observed over all populations (Table 1).

Haplotype 1 (H_1) represents the main haplotype line, which was found at five of the six investigated sites. The second most frequently occurring haplotype 3 (H_3) was found at three sites. Haplotype 7 (H_7) was typical only for the population from the Lužnice River. The haplotype distribution is given in Table 1.

The haplotype network shows genealogical relationships among seven detected haplotypes of *M. fossilis* (Fig. 4). The numbers of the individual haplotypes in the specimens tested are depicted proportionally in the graph. Haplotype 1, which occurs at almost all studied sites, is at the same time the basic source haplotype from which the other haplotypes were derived. The most derived is haplotype 7 which occurs only in the population from the Lužnice River belonging to the North Sea drainage area. The analysed populations proved to be very similar which is documented by low number of variable positions, as Table 1 shows. The values of inter-population HKY+I distance ranged from 0.2% to 0.4% in the population from the Black and Baltic Sea drainage areas, in case of comparison with the population from the North Sea drainage area (Lužnice River) it was up to 0.8%.

In conclusion, the analysis of the control region showed great haplotype similarity of the studied populations. The analysis of microsatellites seems to be a more useful molecular technique for assessment of the *M. fossilis* population structure as it unambiguously distinguishes the individual populations and also provides the necessary diagnostic parameters for their conservation and management.

A c k n o w l e d g e m e n t s

This research was carried out in the Institute of Aquaculture in Stirling and supported by the European Community – Access to Research Infrastructures (ARI) Action of the Improving Human Potential (IHP) Programme (contract no. HPRI-CT-2001-00180). The research is part of project no. VaV-SM/6/3/05 supported by a grant from the

Ministry of Environment of the Czech Republic. We are grateful to B.J. McAndrew, H. Whitaker, J. Taggart for advice and technical assistance and our Japanese colleagues K. Morishima and K. Arai for provided *M. anguillicaudatus* markers. We also thank Prof. R. Mann for linguistic revision and two anonymous reviewers for their helpful comments.

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