Microsatellite variability in nutria (*Myocastor coypus*) genetic resource in the Czech Republic

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ABSTRACT: Nutria (*Myocastor coypus*) is a large rodent native to South America which was introduced worldwide originally with the intention of fur farming. Three colour forms (Standard, Moravian Silver, and Prestice Multicolour) fall into the Farm Animal Genetic Resources of the Czech Republic protected by the National Programme on Conservation and Utilization of Plant, Animal and Microbial Genetic Resources for Food and Agriculture coordinated by the National Focal Point. The objective of our investigation in the Czech Republic was to establish microsatellite panel for nutria and determine microsatellite variability in the local nutria population, levels of genetic diversity within and among subpopulations with respect to colour form and the levels of inbreeding within subpopulations. The genetic variability was studied in 64 animals by investigating 11 microsatellite markers (*McoD214*, *McoD217*, *McoD59*, *McoD69*, *McoC124*, *McoC203*, *McoD60*, *McoB17*, *McoC118*, *McoA04,* and *McoD228*) analysed by multicoloured capillary electrophoresis. The whole population showed a moderate level of genetic variability in terms of number of alleles (5 alleles per locus) or heterozygosity (50.4%) and the Prestice Multicolour form exhibited the highest level of diversity. Particular attention should be paid to organizing mate to minimise inbreeding, especially in the Standard colour form (F_{IS} = 0.238) which showed the highest level of inbreeding out of the three colour forms. High combined exclusion probability (CEP) values (99.5, 94.5, and 99.9%) implied that the panel of microsatellite markers established in this study was usable for individual identification or routine parentage testing in nutria population in the Czech Republic.

Keywords: nutria; genetic resource; microsatellite marker; diversity; inbreeding

Nutria (*Myocastor coypus,* coypu) is a large rodent native to the southern parts of South America which has been introduced almost worldwide (including Africa, Asia, Europe, and North America) since the early 1900's, in many cases with the original intention of fur farming (Baroch et al., 2002; Carter and Leonard, 2002). In wide open spaces, nutria is semi-aquatic herbivore established in various climate and natural conditions in the surroundings of lakes, ponds, marshes, sluggish rivers or wetlands.

In the Czech Republic, nutrias are bred in a few different colour forms, three of them (Standard Colour – SC; Moravian Silver Colour – MSC; Prestice Multicolour – PM) are endangered and belong to the Farm Animal Genetic Resources which are protected by the National Programme on Conservation and Utilization of Plant, Animal and Microbial Genetic Resources for Food and Agriculture coordinated by the National Focal Point, member of the European Regional Focal Point for Animal Genetic Resources (ERFP). The SC, MSC, and PM colour forms are kept under *in situ* conservation programme with the objective to maintain their genetic variability. Nowadays the nutria population in the Czech Republic counts approximately 2000 animals of all colour forms but only 262 animals (117 SC, 73 MSC, 72 PM) are included in the Farm Animal Genetic Resources so most of nutrias are bred out of the Farm Animal Genetic Resources (Kaplan, 2011).

Microsatellite analysis is a useful tool for species characterization, revealing genetic diversity, inbreeding level or monitoring of population changes

in a small population. The objective of our investigation was to determine microsatellite variability in the three colour forms of nutria kept in the Czech Republic and the levels of genetic diversity within and among the colour forms.

MATERIAL AND METHODS

Samples

The experiment involved 64 unrelated animals born within the years 2008 and 2010 representing three colour forms of nutria: Standard Colour (SC), Moravian Silver Colour (MSC), and Prestice Multicolour (PM) which were maintained according to the *in situ* conservation programme. A total of 64 individual samples were collected from 11 different herds involved in the study: SC (n = 23 from 6 herds), MSC (n = 14 from 6 herds), PM (n = 27 from 4 herds). Total genomic DNA was extracted from hairs or blood. The samples were collected and stored at 8°C (blood with sodium citrate as anticoagulant, hairs in paper bags) until DNA extraction according to standard protocols of Jetquick® tissue DNA spin kit and Jetquick® blood and cell DNA spin kit (Genomed GmbH, Löhne, Germany). Extracted DNA was stored at –20°C until microsatellite analysis.

Amplification and microsatellite markers genotyping

The panel of 11 microsatellites (*McoD214, McoD217, McoD59, McoD69, McoC124, McoC203, McoD60, McoB17, McoC118, McoA04, McoD228*) was established from 27 microsatellites characterized by Callahan et al. (2005) with regard to possible multiplex polymerase chain reaction (PCR) amplifications. The multiplex PCR was carried out on a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster City, USA) in a total volume of 6.25 µl containing the following reagents: 50 ng genomic DNA as a template, 0.24µM fluorescently labelled and unlabelled reverse primer, 1.2 × PCR buffer (containing 15mM $MgCl_2$, 100mM Tris-HCl, pH 8.3, 500mM KCl, 0.01% gelatin), 336µM of each dNTP and 0.8 U of AmpliTaq Gold® polymerase (Applied Biosystems, Foster City, USA). Labelling of microsatellite markers was following: PET® (*McoD217, McoD214, McoD59*), 6-FAM™ (*McoD69, McoC124*), NED™ (*McoC203, McoD60, McoB17*), VIC® (*McoC118, McoA04, McoD228*) (all dyes Applied Biosystems, Foster City, USA). PCR conditions consisted of one initial denaturation step at 95°C for 15 min, followed by 35 cycles including denaturation at 95°C for 30 s, annealing at 55°C for 90 s and an extension step at 72°C for 90 s, and a final extension step at 72°C for 60 min. PCR product (0.5 µl) and 0.5 µl of a GeneScan™ 500 LIZ® Size Standard were loaded in 11.5 µl of deionized formamide, then denaturated at 95°C for 5 min and inserted into ice.

The polymorphisms of microsatellite markers were determined by multicoloured capillary electrophoresis based on laser scanning of fluorescence-marked fragments on ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, USA) by fluorescent fragment analysis and evaluated by GeneMapper® Software v3.5 (Applied Biosystems, Foster City, USA).

Statistical analysis

Allele frequency, the number of alleles, and polymorphism information content (PIC) (Botstein et al., 1980) were calculated across different loci, population, and subpopulations using Microsatellite Toolkit v.3.1.1 (Park, 2001). Tests for expected and observed heterozygosity (Hendrick, 1983; Nei, 1987) and departure from Hardy-Weinberg equilibrium (HWE) were tested using Arlequin v.3.5 (Excoffier and Lischer, 2010). The exact test with default settings of the Markov Chain Monte Carlo methodology for estimation of deviations from HWE (Guo and Thompson, 1992) and Bonferroni correction for multiple simultaneous tests (Rice, 1989) was applied. FSTAT v.2.9.3 software (Goudet, 2001) was employed in calculations of allelic richness (an estimation of the mean number of alleles per locus corrected by a sample size).

Genetic structure of the nutria populations in the Czech Republic was analysed by Wright's F-statistics (Wright, 1951). Fixation coefficients (F_{IS}, F_{ST}, F_{IT}) were calculated according to Weir and Cockerham (1984), linkage equilibrium expectations used the randomisation method and gene diversity (heterozygosity expected from Hardy-Weinberg equilibrium (HWE) assumptions) (Nei, 1987) were determined by GENEPOP 4.0.10 (Raymond and Rousset, 1995). The number of effective migrants per generation (Nm) was based

on F_{ST} estimates (Weir and Cockerham, 1984) and was calculated also by GENEPOP 4.0.10 (Raymond and Rousset, 1995).

Exclusion probabilities (EP) and combined exclusion probalities (CEP) were calculated according to Jamieson and Taylor (1997).

The genetic distances among colour forms were estimated, a neighbour-joining consensus tree was reconstructed and the tree robustness was evaluated by bootstrapping over loci (1000) using POPGENE v.1.31 (Yeh et al., 1999); CONVERT 1.3.1 (Glaubitz, 2004) was used for the data set conversion to POPGENE formats. Phylogenetic dendrograms were constructed based on Nei's (1987) genetic distance applying the unweighted pair group method with arithmetic mean algorithm (UPGMA, Sneath and Sokal, 1973). The dendrogram was drawn with the software package TreeView v.1.6.6 (Page, 1996).

RESULTS AND DISCUSSION

Genetic variability at microsatellite loci and colour form variability

A total of 55 alleles were identified for the 11 microsatellite loci in nutrias in the Czech Republic. All the microsatellites were polymorphic in the whole population. All markers were polymorphic in the whole population, however the microsatellite *McoD228* was monomorphic in the PM population. A total of 5 exclusive alleles in the SC, 5 exclusive alleles in the PM and only 1 exclusive allele in the MSC forms were observed for 5 loci (*McoD69, McoC203, McoD60, McoC118, and McoD228*) confirming its uniqueness and isolation.

In the whole population, the number of alleles for microsatellite ranged from 2 (*McoB17*) to 11 (*McoD69*). Total mean number of alleles per locus was 5; allelic richness, an estimate of the number of alleles per locus corrected for variation in a sample size, was 5.090. Observed heterozygosity ranged from 0.063 for marker *McoB17* to 0.781 for marker *McoD69*.

The total number of alleles observed in each loci, the observed and expected heterozygosity and allelic richness are listed in Table 1.

The moderate levels of allelic diversity (5 alleles per locus) and observed heterozygosity (50.4%) detected by these 11 markers across the population are similar to wildlife populations of nutria from Maryland, Louisiana, and Argentina (5 alleles per locus, heterozygosity 46%; Callahan et al., 2005) and these results are also similar to the

Table 1. Summary statistics for 11 microsatellite loci analysed in nutria population in the Czech Republic

Locus	No. of alleles		TNA_O	H_O	H_E	AR	Nm*	F_{IS}	F_{ST}	F_{IT}
	exp.	obs.								
McoD214	6	4	9	0.797	0.837	9	2.478	0.049	0.090	0.080
McoD217	10	8	4	0.641	0.644	4	3.553	0.005	0.080	0.030
McoD59	5	4	4	0.453	0.582	4	1.135	0.223	0.180	0.250
McoD69	11	7	11	0.781	0.846	11	3.004	0.077	0.100	0.100
McoC124	7	4	4	0.578	0.585	4	12.991	0.012	0.010	0.020
McoC203	3	4	5	0.625	0.574	5	4.411	–0.090	0.060	–0.060
McoD60	6	4	5	0.266	0.419	5	1.322	0.367	0.240	0.420
McoB17	2	2	2	0.063	0.393	2	0.881	0.842	0.240	0.850
McoC118	3	4	4	0.609	0.613	4	2.274	0.006	0.100	0.010
McoA04	5	4	4	0.438	0.526	4	12.786	0.170	–0.010	0.160
McoD228	5	3	3	0.094	0.444	3	0.596	0.790	0.450	0.820

exp. = number of alleles expected (according to pooled collections of Callahan et al., 2005), obs. = number of alleles observed in the Czech Republic, TNA_O = total number of alelles observed, H_O = heterozygosity observed, H_E = heterozygosity expected, AR = allelic richness, Nm = number of effective migrants per generation; F-statistics: F_{IS} = intrapopulation fixation index, heterozygote deficiency coefficient, F_{ST} = effect of subpopulations (S) compared to the total population (T), F_{IT} = global deficit of heterozygotes across population

*gene flow estimated from $F_{ST} = 0.25(1 - F_{ST})/F_{ST}$

Table 2. Microsatellite diversity of nutria*s* in the Czech Republic across subpopulations based on 11 loci

Colour form	*N*	TNA_O	*ANA*	AR	H_E ± SD	H_O ± SD	F_{IS}
Standard (SC)	23	43	3.9	3.6	0.533 ± 0.193	0.451 ± 0.239	0.238
Moravian Silver (MSC)	14	37	3.4	3.5	0.557 ± 0.0.145	0.490 ± 0.260	0.126
Prestice Multicolour (PM)	27	44	4.0	3.6	0.578 ± 0.212	0.570 ± 0.289	0.040

N = number of analysed samples per population, TNA_O = total number of alelles observed, *ANA* = average number of alleles per locus, AR = allelic richness, H_O = heterozygosity observed, H_E = heterozygosity expected, SD = standard deviation, F_{IS} = heterozygote deficiency coefficient

levels observed for another rodent, Allegheny woodrat (*Neotoma magister*), where Castlebery et al. (2000) published allelic diversity 5.4 alleles per locus and level of heterozygosity at 54%. Tunez et al. (2009), who analysed 16 microsatellite markers (6 microsatellites were the same as in our study), reported a higher observed heterozygosity (65.8%) in wildlife nutria population from Argentinean Pampas.

The PM form of nutria showed the largest number of alleles per locus (4.0), followed by the SC form (3.9) and the MSC form (3.4). The low level of allelic diversity observed in each subpopulation is consistent with the limited number of individuals of each colour form and certain level of inbreeding. The PM exhibited the highest heterozygosity (0.570), while the heterozygosity in the SC and MSC forms was lower (0.451 and 0.490, respectively). In general, all the three colour forms showed lower heterozygosity estimates than expected. Characteristics of the colour forms are listed in Table 2.

A highly significant ($P < 0.01$) deviation from Hardy-Weinberg equilibrium (HWE) was observed in each colour form, but for different locus: for *McoD59, McoD69,* and *McoD228* in the SC subpopulation, for *McoD60* in the MSC and also in the PM subpopulation. Test for conformance to HWE in nutrias from USA and Argentina revealed that two loci (*McoB17,* which was analysed in our population, and *McoD55*) deviated from expectations after the sequential Bonferroni correction for multiple simultaneous tests (Callahan et al., 2005). In population from Argentinean Pampas Tunez et al. (2009) published that 4 loci significantly ($P < 0.01$) deviated from expectations after Bonferroni correction.

No linkage disequilibria were detected between any pair of loci surveyed in the USA or Argentina by Callahan et al. (2005) and also by Tunez et al. (2009). In our study we observed linkage disequilibria between 6 pairs of loci (between *McoD59* and *McoC203* high significant ($P < 0.01$) linkage disequilibrium was observed) – see Table 3.

Table 3. Linkage disequlibrium between analysed pairs of 11 loci in nutrias in the Czech Republic

	McoD214	*McoD217*	*McoD59*	*McoD69*	*McoC124*	*McoC203*	*McoD60*	*McoB17*	*McoC118*	*McoA04*	*McoD228*
McoD214	–										
McoD217	0.893	–									
McoD59	0.140	0.656	–								
McoD69	0.298	0.599	0.697	–							
McoC124	0.436	0.322	0.972	0.543	–						
McoC203	0.049*	0.921	0.009**	0.030*	0.689	–					
McoD60	0.052	0.916	0.067	0.485	0.906	0.595	–				
McoB17	0.923	0.027*	0.044*	0.096	0.187	0.262	0.568	–			
McoC118	0.147	0.573	0.028*	0.096	0.422	0.926	0.316	0.090	–		
McoA04	0.086	0.775	0.466	0.945	0.143	0.873	0.117	0.766	0.758	–	
McoD228	0.214	0.170	0.392	0.197	0.979	0.944	0.930	0.212	0.717	0.580	–

*$P < 0.05$, **$P < 0.01$

Table 4. Parentage testing characteristics of 11 microsatellite loci analysed in nutrias in the Czech Republic

Locus	TNA_O	PIC	PE1	PE2	PE3
McoD214	9	0.811	0.669	0.499	0.846
McoD217	4	0.588	0.392	0.224	0.571
McoD59	4	0.527	0.336	0.179	0.507
McoD69	11	0.822	0.688	0.521	0.864
McoC124	4	0.511	0.311	0.170	0.462
McoC203	5	0.524	0.337	0.176	0.513
McoD60	5	0.378	0.220	0.089	0.360
McoB17	2	0.314	0.157	0.076	0.244
McoC118	4	0.553	0.355	0.197	0.525
McoA04	4	0.473	0.291	0.143	0.452
McoD228	3	0.350	0.179	0.097	0.275

TNA_O = total number of alelles observed, PIC = polymorphism information content; paternity exclusion probabilities: PE1 = one parentage exclusion, PE2 = one parental genotype unknown, PE3 = parentage exclusion

The polymorphism information content (PIC) values calculated for each loci ranged from 0.314 (*McoB17*) to 0.822 (*McoD69*) in the whole population. In the whole population, only 4 loci (*McoD69, McoB17, McoA04,* and *McoD228*) showed the PIC value below 0.5 and the average PIC in our samples was 0.530 and only 2 loci proved to be highly informative (*McoD214* and *McoD69*). The PIC values in the SC ranged from 0.368 (*McoD217, McoC118*) to 0.764 (*McoD69*), in the MSC from 0.292 (*McoC203*) to 0.758 (*McoD214*), and in the PM from 0 (locus *McoD228* was monomorphic) to 0.721 (*McoD69*). Table 4 shows PIC and other values for parentage testing.

Individual PIC values were highly correlated with the number of alleles detected for each corresponding locus. The same tendency was observed in all probabilities of paternity exclusion – one parentage exclusion (PE1), one parental genotype unavailable (PE2), and parentage exclusion (PE3) – the lowest values were found in the microsatellite marker *McoB17* which showed the lowest variability and the highest values in *McoD69*. The combined exclusion probabilities (CEP1, CEP2, and CEP3) were 99.5, 94.5, and 99.9%, respectively. The high CEP values indicate that the panel of microsatellite markers is appropriate for routine parentage testing. In horse, cattle or pig populations CEP values are above 99% (Putnová et al., 2003, 2011; Kourková et al., 2009). This implies that the panel of microsatellite markers established in this study was usable for individual identification or routine parentage testing in nutria population in the Czech Republic. For CEP values increasing it is possible to replace the microsatellite *McoB17* by a microsatellite with a higher variability.

Genetic structure, gene flow and genetic relationship among nutria subpopulations

The negative intrapopulation fixation index (F_{IS}) value (–0.090) was observed only for the marker *McoC203*; this negative value indicates higher observed heterozygosity value than expected and the excess of heterozygotes – see Table 1. On the other hand, F_{IS} values of the markers *McoB17* and *McoD228,* in which the lowest heterozygosity and the lowest allelic richness were determined, were rather high (0.842 and 0.790, respectively, 10 markers in total showed values higher than 0), suggest heterozygotes deficit and high level of inbreeding. Similarly, for microsatellites *McoD228* and *McoB17,* the F_{IS} estimates were considerably higher than the F_{ST} (the effect of subpopulations (S) compared to the total population (T)) estimates, suggesting that the infrastructure of the populations is influenced by nonrandom mating. Total F_{IS} index was 0.089, which indicates heterozygotes deficiency in all population analysed in our study.

In the three colour forms of nutria occurring in the Czech Republic, the F_{IS} values ranged from 0.040 in the PM to 0.238 in the SC and indicated strong heterozygote deficiency in the SC (0.238) and MSC (0.126) and suggest matings between closely related individuals. Interestingly, the SC

Table 5. F_{ST} coefficients (below diagonal; heterozygote deficiency coefficient) and Nei's genetic distances (above diagonal) between subpopulations of nutria in the Czech Republic

	Standard (SC)	Moravian Silver (MSC)	Prestice Multicolour (PM)
Standard (SC)	+	0.254	0.202
Moravian Silver (MSC)	0.142*	+	0.217
Prestice Multicolour (PM)	0.128*	0.126*	+

*$P < 0.05$

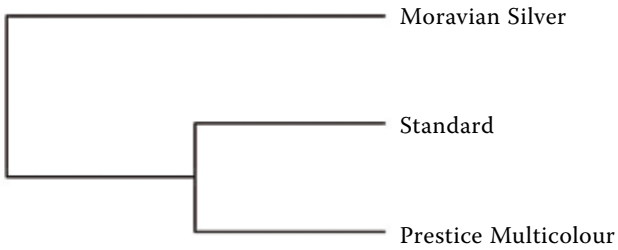

Figure 1. Dendrogram constructed from Nei's genetic distances by unweighted pair group method with arithmetic mean algorithm (UPGMA) shows the genetic relationships among three colour forms of nutrias included in the Farm Animal Genetic Resources of the Czech Republic

is the most numerous colour form in the Czech Republic. Although *in situ* conservation preservation programmes for nutrias in the Czech Republic aim to avoid inbreeding, the total F_{IT} index (the global deficit of heterozygotes across population), which is a good indicator of inbreeding, amounted to 0.207 presenting an excess of homozygotes of 20.7% in our population.

The population differentiation of three subpopulations of different nutria colour form is presented by pairwise F_{ST} coefficients. The pairwise F_{ST} values between subpopulations, which were significantly higher than zero ($P < 0.05$) in all cases, are shown in Table 5. The F_{ST} coefficients varied from 0.126 between the PM and the MSC forms to 0.142 between the SC and the MSC forms. These results suggest that 12.6 or 14.2% of the genetic variability was explained by differences among subpopulations while the remaining variability is explained by the variation within subpopulations.

Gene flow among the subpopulations is shown by the estimates of the number of migrants per generation *Nm* (where *N* is a total effective number of nutrias and *m* is the migration rate), see Table 1. The highest *Nm* was estimated for the marker *McoC124* (12.991) and the lowest for the marker *McoD228* (0.596) indicating high gene flow among subpopulations of nutria for the marker *McoC124*.

Nei's genetic distances, calculated pairwise between the subpopulations were, as expected, less than 1 and varied from 0.202 to 0.254 – see Table 5. The UPGMA (unweighted pair group method with arithmetic mean algorithm) dendrogram clarifies the genetic relationship among subpopulations of nutria in the Czech Republic according to the colour form – see Figure 1.

CONCLUSION

Results of the study revealed that colour forms of nutria (SC, MSC, PM), which fall into the Farm Animal Genetic Resources protected by the National Programme on Conservation and Utilization of Plant, Animal and Microbial Genetic Resources for Food and Agriculture, are an important reservoir of genetic variation and of genetic uniqueness and removal of any of the three colour forms would result in a loss of genetic diversity. In our study the PM form showed the highest level of genetic diversity while the SC has already been suffering from the lack of heterozygosity and showed the highest level of inbreeding out of the three studied colour forms. In order to preserve these valuable genetic resources, effective measures have to be taken to increase effective population sizes and thus prevent increase of inbreeding. High CEP values implied that the panel of microsatellite markers established in this study was usable for individual identification or routine parentage testing in nutria population in the Czech Republic.

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