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Application of chicken microsatellite markers to molecular monitoring of the experimental population of Japanese quail (*Coturnix japonica*)*

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Chromosome homology is highly conserved between chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) and a few chromosome rearrangements occurred in the evolution of these two species. Microsatellite markers are invaluable tools in molecular biology for genome analysis, mapping, search for quantitative trait loci, and phylogenetic studies. In the current study, 25 microsatellite sequences for molecular analysis of 98 Japanese quail (49 of each sex) were used including 11 chicken microsatellite markers which had been analysed by other authors in chicken genome research. Eighty-two percent (9/11) of chicken primer pairs amplified individual loci in Japanese quail and together with the remaining primers were polymorphic. The H_E and PIC values indicated that the most (14/23) of the selected microsatellite sequences are useful as genetic markers. The study contributed to a better understanding of the Japanese quail genome, and the successful cross-species amplification led to identification of polymorphic markers that will be indispensable for identifying homologous regions on chromosomes.

KEY WORDS: genetic diversity / Japanese quail / microsatellite markers

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Japanese quail (*Coturnix japonica*) is a species of considerable economic importance in a number of countries [Minvielle *et al*. 2004]. Moreover it is frequently used as a model laboratory animal [Kayang *et al*. 2004]. Generaly, birds have a constant number of chromosomes and show relatively low susceptibility to chromosome rearrangements [Kayang *et al*. 2006]. Japanese quail and chicken are closely related. Both species have a karyotype of 2n=78 chromosomes and a similar genome length of 1.2×10^9 bp [Kayang *et al*. 2004]. It was shown that except for chromosomes 1, 2, 4 and 8, the location and the order of genes in the Japanese quail genome was similar to those in chicken genome [Shibusawa *et al*. 2001].

Microsatellite sequences, also known as Short Tandem Repeats (STR), are widely used as valuable genetic markers due to their dense distribution in the genome, wide variability, co-dominant mode of inheritance and easy genotyping. Kayang *et al*. [2000, 2004] identified over 100 microsatellite sequences in the Japanese quail and used them to build the first microsatellite linkage map, which spans 576cM and contains 58 *loci* assigned to 12 linkage groups and one specific group for Z chromosome [Kayang *et al*. 2004]. The same authors found that the use of STR is problematic when applied to mapping of microchromosomes because they contain a small number of STR. Highly conservative sequences were found in homologous chromosomes of both species [Kayang *et al*. 2003, Shibusawa *et al.* 2001]. Kayang *et al.* [2006] showed that the chicken genome contains microsatellite sequences characteristic for the Japanese quail (88.4%) and that they are highly similar in both species (63.6-100%). Roussot *et al.* [2003] and Kayang *et al*. [2006] reported that the identified genetic markers in Japanese quail are, on average, twice as polymorphic as those in the domestic chicken. Research to sequence the Japanese quail genome is relatively recent [Pang *et al*. 1999, Inoue-Murayama *et al.* 2001]. The use of the similar microsatellite sequences in closely related species, whose genome is partly mapped, proved to be successful. Such approach offers opportunity for a more rapid sequencing of the Japanese quail genome [Inoue-Murayama *et al*. 2001, Kayang *et al*. 2000, 2002, 2003, 2006, Kikuchi *et al.* 2005].

Material and methods

Animals

The birds were kept in the experimental facility at the Department of Genetics and Animal Breeding Faculty of Animal Sciences at Warsaw University of Life Sciences. They originated from the heterozygous base population obtained by two-stage reciprocal crossing of three lines of Japanese quail maintained over 25 generation in the Department. The crossbred population, after 6 generation of random mating of the monozygous pairs become the base population of this experiment. The birds were mated in order to minimize the inbreeding rate. Blood for molecular analysis was collected, following decapitation, from 98 birds of the base population (49 of each sex). Blood samples were stored at -20°C.

DNA isolation

Genomic DNA was extracted from the whole blood using a modified version of the method described by Hillel *et al*. [1989]. Fifty µl of whole blood was dissolved in 500 µl TNE buffer (1.0M Tris-HCl, pH 7.5; 5M NaCl; 0.5M EDTA; 10% SDS) with 4 µl proteinase K (SIGMA) at a working concentration of 20 µg/µl. After mixing, all samples were transferred to a water bath and incubated at 55°C for at least 30 min. Proteins were removed using a 550 µl mixture of phenol/chloroform/isoamyl alcohol, followed by 550 µl chloroform extractions. Genomic DNA was precipitated with 96% ethanol, dried, and dissolved in MilliQ water.

Characteristics of microsatellite sequences

Twenty-five microsatellite sequences (Tab. 1) representing twelve chicken chromosomes were studied in the experiment. The choice of microsatellite sequences was guided by the results of other studies, in which the high expected heterozygosity (H_E) and Polymorphism Information Content (PIC) of the selected microsatellite sequences in chickens were reported [Zhou and Lamount 1999, Wimmers *et al*. 2000, Wardęcka *et al*. 2002, Kerje *et al.* 2003, Jacobsson *et al.* 2004, Lujiang *et al*. 2006, Tadano *et al.* 2007a,b]. The microsatellite sequences selected for this study were characterised by high similarity of to 5' and 3' flanking sequences, both in the chicken and in the Japanese quail [Pang *et al*. 1999, Inoue-Murayama *et al*. 2001].

PCR amplification

The microsatellite loci were amplified by PCR using selected STR primers, with forward primers labeled with Cy5. Primer sequences were taken from the ArkDB database of the Roslin Institute [http://www.thearkdb.org] and from the NCBI website [http://www.ncbi.nlm.nih.gov]. Out of the 25 primer pairs selected for the analysis, 11 were used so far only for amplification of STR sequences in the chicken (ADL150, ADL0225, ADL0272, ADL0306, LEI0071, MCW0088, MCW0102, MCW0145, MCW0154, MCW0184, and MCW0210) but not in Japanese quail. Each PCR amplification was conducted in a 25 µL reaction mixture, which included 20 pmol of each primer (TIBMOLBIOL), 100 µM deoxynucleoside triphosphate (POLGEN), 5 µL of 10x reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 50 mM KCl, 0.01%) (SIGMA), 1.0 U of Taq DNA Polymerase (SIGMA) and approximately 20 ng of genomic DNA as a template. The PCR procedure, using TrioBlock (BIOMETRA, Germany), was as follows: first denaturation at 94°C for 5 min, then 30 cycles of denaturation 95°C for 30 s, annealing at primer specific temperature for 45 s, extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min (Tab. 2). After obtaining the amplification products of the examined loci, the alleles were sized with an automated sequencer (ALF Express II, PHARMACIA ELKABE) using Alfwin Fragment Analyser 1.03 software.

Table 1. Characteristics of 25 microsatellite sequences used in current study

Chicken					Japanese quail			
locus name[a)]	chromo-some No	GenBank accession number	repeat	reference[b)]	*locus* name	GenBank accession number	repeat	reference[b)]
ADL0023	5	L23905	(ca)5(cg)4(ca)9	3			no repeats	11
ADL0024	3	L23906	A10N66(ca)6	3			(ct)3N77(ct)3tt(at)3N43(ct)4	11
ADL0037	1	L23912	(cag)3N9(ca)3(ca)8	3	UBC0001	AF121113	(cag)3N9(ca)3ta(ca)5	9, 11
ADL0038	10	L23916	(gt)7(at)9	3				
ADL0106	10	G01550	(tg)9	5	GUQ0004	AB038399	(tg)3	9
ADL0134	1	G01754	(ca)17	2, 5	GUQ0002	AB038397	(ca)7	9
ADL0142	6	G01567	(ac)11ag(ac)7	5	UBC 0002	AF121114	(at)3t(ct)11a(ac)7	9
ADL0143	4	G01568	(tg)10(ta)5	5	UBC0003	AF121115	(at)9N18(ag)3	11
ADL0150	1	G01575	(gt)13	5				
ADL0206	15	G01626	(gt)23	5	UBC0006	AF123886	(ac)3(tg)7	11
ADL0225	13	G01645	(ac)9	5				
ADL0255	4	G01675	(tg)11	5	GUQ0007	AB038402	(tg)9	9
ADL0257	2	G01677	(ca)14(caa)10	5			No repeats	11
ADL0272	10	G01692	(tg)11	5				
ADL0306	3	G01721	(tg)11	5				
ADL0315	7	G16117	(ct)8(ca)9	1	UBC0004	AF121116	(ct)6(ca)5	11
ADL0366	1	G29072	(ac)14	4			(ag)3	11
HUJ0006	3	L10294	(ac)N	10	UBC0005	AF121117	(ac)9	11
LEI0071	1	X82814	(ac)N	8				
MCW0088	2	L40056.1	(tg)N	6				
MCW0102	1	L40073	(ca)13	6				
MCW0145	1	L43656.1	(tg)20	6				
MCW0154	Z	L43659	(ca)20	6				
MCW0184	2	G31975	(tg)N(ta)	7				
MCW0210	5	G31987	(tg)32	7				

[a)]Marker names come from the name of the laboratory: ADL - USDA ARS Avian Disease and Oncology Lab, HUJ - Hebrew University of Jerusalem, MCW - Microsatellite Chicken Wageningen, GUQ - Gifu University Quail, UBC - University of British Columbia.
[b)]Reference papers: [1][Cheng 1996]; [2][Cheng 1999]; [3][Cheng and Crittenden 1994]; [4][Cheng and Ponce de Leon 1996]; [5][Cheng *et al*. 1995]; [6][Crooijmans *et al*.1996]; [7] [Crooijmans *et al*. 1997]; [8][Gibbs *et al*. 1997]; [9][Inoue-Murayama *et al*. 2001]; [10][Khatib and Soller 1993]; [11][Pang *et al*. 1999].

Statistical

The frequency of identified alleles in the microsatellite sequences, the observed heterozygosity (H_O), the expected heterozygosity (H_E), the Polymorphism Information Content (PIC), the compliance between the observed and expected genotype frequencies based on Hardy-Weinberg exact test using Cervus 3.0.3. software [Kalinowski *et al*. 2007], and the frequency of genotypes using ALFREQ software [Tereba and Tereba, 2005] were calculated.

Results and discussion

Out of the 25 microsatellite sequences selected for molecular monitoring of the analysed Japanese quail population, PCR product was obtained for 23 STR. The PCR reaction for 11 chicken microsatellite sequences in Japanese quail yielded a PCR product for 9 STR sequences, no amplification product was obtained only for the ADL0225 and MCW0102 sequences (Tab. 2). In earlier studies, Pang *et al*. [1999], who used chicken specific primers, amplified only 22.9% of microsatellite sequences in Japanese quail (11 STR sequences out of 48 analysed), while Innoue-

Table 2. Description of 23 microsatellite markers used in current study

Locus	Annealing temperature (°C)	H_O	H_E	PIC	No of genotype	H-W exact test[a)]
ADL0023	56	1.000	0.615	0.536	2	***
ADL0024	46	0.620	0.698	0.652	15	NS
ADL0037	47	0.531	0.517	0.402	4	NS
ADL0038	48	0.235	0.393	0.355	7	*
ADL0106	47	0.622	0.807	0.785	25	*
ADL0134	48	0.602	0.729	0.680	13	NS
ADL0142	55	0.822	0.677	0.613	13	NS
ADL0143	47	0.898	0.689	0.636	11	***
ADL0150	51	0.469	0.381	0.338	4	NS
ADL0206	61	0.429	0.589	0.497	6	***
ADL0255	52	0.786	0.767	0.723	13	***
ADL0257	46	0.122	0.182	0.176	8	ND
ADL0272	55	0.959	0.868	0.850	29	*
ADL0306	47	0.914	0.639	0.567	7	***
ADL0315	55	0.010	0.611	0.527	4	***
ADL0366	60	0.990	0.574	0.479	3	***
HUJ0006	61	0.388	0.449	0.347	3	NS
LEI0071	46	0.439	0.466	0.437	9	NS
MCW0088	56	0.989	0.735	0.685	7	***
MCW0145	51	0.959	0.636	0.561	6	***
MCW0154	48	0.957	0.754	0.703	7	***
MCW0184	56	0.463	0.670	0.618	9	NS
MCW0210	51	0.235	0.295	0.250	3	ND
Mean	-	0.628	0.597	0.540	6.826	-

[a)]Exact test of Hardy-Weinberg equilibrium: level of significance: ***P≤0.001, **P≤0.01, *P≤0.05, NS – not significant; ND – not determined owing to a too few number of group.

Murayama *et al.* [2001] only 26.0% (31 out of 120), and Kikuchi *et al*. [2005] only 8.5% (17 out of 200). Despite this relatively low percentage of successful cross-species amplifications, Kikuchi *et al*. [2005] hold the view that when a cross-species amplification is successful and results in polymorphic markers, these markers can be useful for identifying homologous chromosomal regions.

Microsatellite sequences, which were taken from the chicken genome have been successfully used in genome research of other species of birds: turkey [Reed *et al*. 2000, Reed *et al*. 2005], duck [Mukesh *et al*. 2011], ostrich [Huang *et al*. 2008], guinea fowl [Kayang *et al*. 2002], chukar partridge (*Alectoris chukar*) [Tejedor *et al*. 2007] also. It has been shown that the similarity between the chicken and turkey genome is higher than 90% [Reed *et al*. 2007]. Bech *et al.* [2010], taking into consideration that the chicken, turkey and partridge (*Perdix perdix*) belong to the same family, used a conservative 138 STR sequence of chicken and turkey in the study of genetic variation of partridges. In partridge only 55% of the pairs of primers gave the amplification product and 13% of microsatellite sequences were polymorphic. Authors found the mutations in the analyzed sequences, as well as the differences between the species, studied especially as regards the number of repeat microsatellite sequences. Studies of Shibusawa *et al.* [2004] showed that differences between chicken and turkey genome are resulting from chromosomal rearrangements.

Huang *et al.* [2005], using 35 new duck microsatellite markers, were trying to identify homologous *loci* in geese, chickens and peacocks. Duck and goose belong to the order *Anseriformes*, while the chicken and peacock to the order *Galliformes*. The authors obtained the amplification products for 62.86% geese, 14.29% chicken and 11.43% peacock markers. All of them demonstrated simple sequence tandem repeat. Only 5.7% of the primers designed for the ducks could be used in the chicken genome analysis, but none of them could be used for the analysis of the genome of a peacock. In the cited work only 2 chicken *loci* and 14 goose *loci* were found as the true homologues to the tested duck *loci*. In later studies, Huang *et al*. [2006] using 155 microsatellite sequences to create a genetic linkage map for ducks proved that only 49 from them were ortologus for ducks and chickens. The use of chicken microsatellite sequences in the study of genetic variation of the ostrich – species so phylogenetically distant – poses many difficulties. Research conducted by Horbańczuk *et al.* [2007] have failed. The authors have not obtained any amplification product for 29 selected chickens STR sequences applied in the ostrich genome analysis. However, Huang *et al*. [2008] found 13 ortologus microsatellite *loci* in chicken and ostrich. These results indicate the possibility of conducting research using primers derived from a species belonging to the same order, however, the use of primers in the study of genetic variability of the species from different order may be difficult, because of the substantial difference between the homology of respective sequences [Huang *et al*. 2005].

Most of the selected microsatellite sequences were highly polymorphic (Tab. 3 and 4). These findings are consistent with the earlier results reported by Pang *et al.* [1999] and Inoue-Murayama *et al*. [2001], who showed very high level of similarity between

chicken and Japanese quail in the 5' and 3' flanking regions of 14 STR sequences out of the 23 used in our study.

The molecular analyses of the Japanese quail population showed that the number of alleles varied from 2 for the HUJ0006 and MCW02010 sequence up to 10 for the ADL0106 and ADL0272 sequences (Tab. 3 and 4). The average number of alleles (5.0) was calculated as the average number of distinct alleles found at each *locus*

Table 3. Frequency of alleles for 14 microsatellite sequences (high similarity of 5' and 3' flanking sequences in chicken and Japanese quail) in the analysed population of Japanese quail

Locus	Current study		Other authors		*Locus*	Current study		Other authors	
	allele size (bp)	freuquency	allele size (bp)	ref. [b)]		allele size (bp)	freuquency	allele size (bp)	ref. [b)]
ADL0023	156	0.168	132	5	ADL0142	220	0.033	204-214	5
	158	0.500				222	0.422		
	162	0.332				224	0.339	210	1
	n[a)]	98				226	0.178	214	1
ADL0024	276	0.005	206	5		228	0.028		
	278	0.011				n	90		
	280	0.022			ADL0143	152	0.005	138	5
	282	0.044				154	0.117		
	284	0.027				156	0.454		
	286	0.261				158	0.026		
	288	0.462				160	0.097		
	290	0.141				162	0.291		
	294	0.027				164	0.010		
	n	92				n	98		
ADL0037	182	0.541	174	1	ADL0206	103	0.454	95-101	1
	186	0.439	176	1;5		105	0.102		
	198	0.020	180	1;5		107	0.444		
	n	98				n	98		
ADL0038	156	0.169	130	5	ADL0255	122	0.005	98	1
	180	0.051	140	5		124	0.286	108	1
	198	0.005				126	0.280	111	4
	202	0.760				128	0.036		
	204	0.015				130	0.005		
	n	98				132	0.194		
ADL0106	180	0.107	154	1		136	0.194		
	182	0.082				n	98		
	184	0.092			ADL0257	132	0.015	118	5
	186	0.061				134	0.046		
	188	0.036	164	1		136	0.903		
	190	0.015	165	3		138	0.031		
	192	0.02				140	0.005		
	194	0.112				n	98		
	196	0.378			ADL0315	244	0.383	236-250	5
	198	0.097				246	0.143		
	n	98				248	0.474		
ADL0134	114	0.199	106	1		n	98		
	128	0.066	108	1	ADL0366	230	0.408	226-232	5
	130	0.265	110	2		232	0.505		
	132	0.077	112	1		234	0.087		
	134	0.393				n	98		
	n	98			HUJ0006	103	0.337	99-107	5
						105	0.663		
						n	98		

[a)] n – number of birds.
[b)]Reference papers: [1][Inoue-Murayama *et al*. 2001]; [2][Inoue-Murayama *et al*. 2008a]; [3][Inoue-Murayama *et al*. 2008b]; [4][Inoue-Murayama *et al*. 2008c]; [5][Pang *et al*. 1999].

Table 4. Frequency of alleles for 9 microsatellite sequences (previously used for the chicken genome research only) in the analysed population of Japanese quail

Locus	Allele size (bp)	Frequency
ADL0150	334	0.015
	338	0.036
	344	0.184
	348	0.765
	n[a)]	98
ADL0272	166	0.102
	168	0.041
	170	0.102
	172	0.046
	174	0.260
	176	0.061
	178	0.056
	180	0.128
	184	0.092
	188	0.112
	n	98
ADL0306	126	0.086
	128	0.457
	130	0.376
	132	0.081
	n	93
LEI0071	154	0.005
	156	0.102
	158	0.102
	160	0.714
	162	0.072
	164	0.005
	n	98
MCW0088	252	0.375
	254	0.119
	260	0.006
	262	0.028
	264	0.273
	266	0.199
	n	88
MCW0145	246	0.459
	248	0.041
	264	0.372
	266	0.128
	n	98
MCW0154	162	0.245
	164	0.261
	168	0.255
	170	0.239
	n	92
MCW0184	243	0.105
	245	0.200
	247	0.495
	249	0.005
	251	0.195
	n	95
MCW0210	202	0.179
	204	0.821
	n	98

[a)]n – number of birds.

across all *loci*. This result is considerably different from the results reported by Pang *et al*. [1999], Inoue-Murayama *et al.* [2001], Inoue-Murayama *et al*. [2008a,b,c], who noted only several alleles (from 1 to 4) in some of the analysed *loci* in Japanese quail (Tab. 3 and 4). Most repeat motifs in Japanese quail proved shorted than in chicken (Tab. 1). Because of the small number of birds studied by the authors, as well as the type of repeat motif used (Tab. 1), Pang *et al*. [1999] and Inoue-Murayama *et al.* [2001] suggested that more molecular research have to be conducted with a higher number of animals included to be able to confirm or correct the results obtained.

Tables 3 and 4 present allele frequencies for 23 microsatellite sequences both in the whole population. Alleles with a very high frequency were found for each sequence analysed. In chicken of different breeds, lines and utilization types, most of these sequences which were used in our study, are highly polymorphic [Cheng and Crittenden 1994, Gibbs *et al*. 1997, Khatib and Soller 1993, Wardęcka *et al*. 2002 and many others]. The ADL0023, ADL0142, ADL0143, ADL0206, ADL0257, HUJ0006,

LEI0071 and MCW0088 sequences were longer in chicken than in Japanese quail (Pang *et al.* [1999], Innoue-Murrayama *et al*. [2001, 2008a,b,c]) and was confirmed in our study (Tab. 3 and 4). The ADL0024, ADL0038, ADL0106 ADL0150, ADL0255, ADL0306, MCW0145 and MCW0210 sequences were longer in quails than in chickens (Tab. 3 and 4). When comparing the number of alleles found in chickens and in our study (Tab. 3 and 4) higher polymorphism was found in quails than in chickens in the case of 9 STR sequences (ADL0024, ADL0038, ADL0106, ADL0134, ADL0142, ADL0143, ADL0206, ADL0366, ADL0272). In turkeys sequences: ADL0106, ADL0143, ADL0150, ADL0306 had a similar length as in chickens, and sequences: ADL0134, ADL0142, ADL0272, ADL0366 were longer in turkeys than in chickens [Reed *et al*. 2000]. However, sequences: ADL0257 [Reed *et al*. 2000] and ADL0023 [Burt *et al.* 2003] in turkeys and ADL0315 in partridges [Zhou *et al*. 2009] were shorter than in chickens. ADL0134, ADL0150, ADL0257 and ADL0366 sequences were monomorphic in turkey [Reed *et al.* 2000]. Similar results were obtained by Korom *et al*. [2004]. Out of 58 chicken pair of primers, 81% amplified specific microsatellite fragments from turkey genomes, but only 36% were polymorph. ADL0142 in partridge was high polymorphic (H_E>0.7) [Tejedor *et al*. 2007] and there has been no MCW0145 sequence similarity between chicken and ostrich [Horbańczuk *et al*. 2007].

Table 2 presents estimated H_O, H_E and PIC values as well as the number of genotypes for the microsatellite sequences. 100% heterozygotes were found for the ADL0023 microsatellite sequence, and H_O>91% for the other six sequences. The coefficients of H_E ranged from 0.182 (ADL0257) to 0.868 (ADL0272), and PIC values were slightly lower. All of the microsatellite sequences with H_E>0.6 [Ott 1992] can be successfully used in further research on genetic variation in Japanese quail.

The largest number of genotypes was found for the ADL0106 and ADL0272 sequences (25 and 29 genotypes, respectively) (Tab. 2) while the average was 9,044. Differences in the distribution between the observed and expected genotype frequencies (based on Hardy-Weinberg law) were extremely significant (P≤0.001) for 10 microsatellite sequences and significant (P≤0.05) for 3 sequences (Tab. 2) and non significant for remaining ones.

Finally, it may be concluded that the 9 new microsatellite sequences, which had previously been used in chicken studies, can be used in the Japanese quail research. Most of the 23 STR sequences analysed were highly polymorphic, and the H_E and PIC values obtained show that these sequences are suitable as genetic markers, and provide an useful tool for analysis of the Japanese quail population genetic diversity.

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