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Assessing Paternity in Japanese Quails (*Coturnix Japonica*) Using Microsatellite Markers - Inferences for Its Mating System and Reproductive Success

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■Keywords

Coturnix japonica, parentage testing, polyan-
dry, quail, SSRs.

ABSTRACT

Microsatellite markers were analyzed in Japanese quails, *Coturnix japonica*, using different methodologies (PAGE and automated genotyping), in order to evaluate their use in paternity testing. Ten animal triplets composed by a female and two males were used to mate and generate an offspring. Paternity was determined in five-day-old embryos, and the data generated by fluorescent labeled and tailored primers in PCR and further automated genotyping were robust. Three microsatellite markers were polymorphic ($N_a = 5-8$, $H_e = 0.75$) and no loci were found to deviate significantly from Hardy-Weinberg equilibrium or showed any evidence of linkage disequilibrium ($p > 0.05$). A slight heterozygote deficiency and some incompatibilities between the female known parent and its offspring that involved homozygous genotypes were observed at *GUJ0001* locus and may indicate the presence of null alleles. Although a reduced set of microsatellite primers were applied, it was possible to determine the paternity of 96.87% of the embryos, using combined data of three loci. The approach was useful for parentage inferring in a captive population of *C. japonica* and the results evidenced a potential polyandric mating system in the species, in which no advantage mechanism of last-male sperm precedence seems to occur.

INTRODUCTION

The development of DNA-based markers has had a revolutionary impact on gene mapping and, more generally, on several other animal and plant genetics research data, such as measurement of gene flow and migration, assignment of individuals to their population of origin, measurement of effective population size, and detection of past demographic bottlenecks (Jehle & Arntzen, 2002; Gholizadeh & Mianji, 2007). Besides, the implement of the polymerase chain reaction (PCR) allowed the development and application of several DNA markers (Marle-Koster & Nel, 2003). Microsatellites or SSRs (Simple Sequence Repeats) are now one of the most widely used molecular markers in several eukaryotic species, specially due to their relative ease of scoring and ability to exhibit high levels of polymorphism (Wolfgang *et al.* 2011). Several studies have revealed that microsatellites are not just useful in population analyses, but also suitable for the measurement of genetic parameters such as number of effective alleles (N_e) and rare alleles (Bartfai *et al.* 2003), and also for the characterization/identification of genetic alterations (Powierska-Czamy, 2003), and individual identification (Shurtliff, 2005). Today, microsatellites represent the most useful DNA markers in genotyping and paternity analyses (Webster & Reichart, 2005; Shurtliff, 2005).

In birds, analyses using molecular markers specially modified the understanding of social and reproductive behavior, leading to the



detection of extra-pair copulation in several species (Griffith *et al.*, 2002; Masters *et al.*, 2003; Fossoy *et al.*, 2006). As extra-pair fertilizations are common in birds, reliable assessment of paternity is essential. The excluding paternity tests in birds began through allozyme analyses and, later, minisatellite repeats were used for this purpose. Currently, microsatellites are the most widely used molecular markers to elucidate genetic relationships in different bird species (e.g. Alcaide *et al.*, 2005). However, one of the main limitations of using these markers in birds refers to the reduced frequency of microsatellite repeats in this animal group, mainly at the sex chromosomes and microchromosomes (Primmer *et al.*, 1997).

Microsatellite markers were recently isolated from the Japanese quail *Coturnix japonica* as an effort to contribute to the development of reference maps for this avian species, which were mainly obtained using microsatellite repeats of chicken (Pang *et al.*, 1999; Kayang *et al.*, 2000; Kayang *et al.*, 2004; Kikuchi *et al.*, 2005). The Japanese quail was first domesticated in Japan around the 15th century as a pet song bird (Kovach, 1974). In addition of being reared for egg/meat production, it is becoming increasingly used as an experimental research animal, due to several characteristics, such as its small body size, short generation interval, and high egg production (Pisenti *et al.*, 1999). However, in contrast with chickens (*Gallus gallus*), little is known about the Japanese-quail genome. As paternity analyses were only performed in Japanese quail using multilocus DNA fingerprinting (Matthews *et al.*, 2007), the goal of the present study was to implement a paternity test analysis for this species by performing a multiple mating strategy and genotyping of adult males and females and their offspring using microsatellite markers. The generated data were used to support behavioral analyses of this species.

MATERIAL AND METHODS

Animal samples

The Ethical Committee on Animal Experimentation (CCEA) of the Biosciences Institute of UNESP approved this study with Japanese quails (*Coturnix japonica*; Galliformes, Phasianidae) under protocol number 42/05.

Two-day-old individuals were obtained from a private farm (Granja Suzuki, São Paulo, SP, Brazil) and maintained in cages at maximum density of 141 birds/m², as suggested by SEBRAE (Serviço Brasileiro

de Apoio às Micro e Pequenas Empresas de São Paulo, 2005). During the first month, birds were fed a grower feed with 24% crude protein and 2,900 kcal metabolizable energy (ME)/kg. Feed and water were supplied *ad libitum*. Environmental temperature was maintained at 37-39°C during the first week and it was further reduced to 25°C during the three subsequent weeks. Birds were transferred to individual cages (16 x 24 x 18cm) at approximately 45 days of age, and were fed a commercial feed (Purina) with higher calcium (3.5 to 4.0%) and lower protein (20 to 22%) contents than the previous feed and 2,900 kcal ME/kg. Temperature was maintained at 20-25°C temperature and a photoperiod of 14 hours of light/10 hours of dark was applied.

Bird selection and sexual interaction

Adult females and males (older than 45 days), presenting regular lay and prominent cloacal gland, respectively, and that had previous sexual experience were chosen to compose 10 groups (ten triplets, each composed by a female and two males). In order to maintain the sexual dimorphism of the species, in each group, the female was heavier than the males, and the two males presented a similar weight. In order to correctly identify the males in each group, one bird was marked in the legs.

A behavioral analysis was performed during the period when the birds' sexual behavior frequency is higher (between 14:30 and 17:30 pm; Delville *et al.*, 1986), using a specific cage made of transparent acrylic. The cage, consisting of a central area and two lateral compartments of acrylic walls (adapted from White & Galef, 1999), was used in order to maintain the three animals separated during 1 minute - the female in the central area and each of the two males in one of the lateral compartments. After this short adaptation period, the acrylic walls of the lateral compartments were removed, and the three birds were allowed to interact for 15 minutes. The number of copulations of each male was recorded. After the behavioral analysis, birds were separated and the eggs of each female were collected from the first day until the 12th day of the behavioral experiment and maintained in an electric incubator at 37.5°C and relative humidity of 50-70% during 5 days.

Dna isolation and amplification

Genomic DNA was extracted from 800 µL of blood collected from the brachial vein of the adult animals and of 5-day-old embryos generated by the eggs of



each female, using a TNEs digestion buffer and phenol-chloroform, as detailed in Sambrook & Russel (2001). The extraction procedure was performed twice in order to ensure good-quality DNA. DNA integrity and quantity was checked on 1% agarose gel by direct comparison with a standard marker (Low DNA Mass Ladder - Gibco.Brl) using the software program Stratagene EagleSight v. 3.22, and in a spectrophotometer (Nano Drop ND-1000 Spectrophotometer - Thermo Fisher Scientific). Five primer sets specific to microsatellite loci of different repetitions, originated from *Coturnix japonica* (Kayang *et al.*, 2000) (Table 1), were initially tested using the DNA samples of some adult individuals in order to test the effect of DNA, $MgCl_2$, primers, and *Taq* DNA polymerase concentrations, to determine the optimum annealing temperature, and to optimize the reproducibility of the PCR assays. Further DNA amplifications using two different methodologies were carried out for all adult individuals for each experimental group and in their generated embryos. Subsequently, a PAGE (Polyacrylamide Gel Electrophoresis) and an automated genotyping analysis were performed. All birds were genotyped at least three times by each methodology, using independent PCR amplifications to avoid genotyping errors.

Microsatellite loci amplification with common primers and page genotyping

Each PCR reaction mixture consisted of 1x reaction buffer (10mM Tris-HCl pH 8.0, 50mM KCl, 1.5mM $MgCl_2$ - USB), 1.25mM of each dNTP (Invitrogen), 5 μ M of forward and 5 μ M of reverse primer (Table 1), 50ng of genomic target DNA, and 1.25U of *Taq* DNA polymerase (USB), in a total volume of 25 μ L. Amplifications were carried out in a Mastercycler Personal thermocycler (Eppendorf) with the following

cycle program: a first denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55-65°C for 1 min (Table 1), and 72°C for 1 min, and a final extension step at 72°C for 5 min. A negative control, consisting of all the reaction components, except for the template DNA, was also included to monitor any possible contamination. Reactions products (6 μ L) were subjected to 6% and 8% non-denaturing polyacrylamide gel electrophoresis for approximately 6 hours at 120V. DNA bands were visualized after Ag-nitrate staining (Sambrook and Russel 2001) and molecular weights were estimated using standard DNA markers (10 bp DNA and 1Kb DNA Ladders - Invitrogen). The photo-documentation was performed under white light (Hoefer UV-25) using the computer program Stratagene EagleSight v. 3.22 (Stratagene).

Microsatellite loci amplification using labeled primers and automated genotyping

The same five sets of primers (Table 1) were also applied in the DNA samples using the protocol described by Schuelke (2000). A tail (5'-TGT AAA ACG ACG GCC AGT-3' (M13(-21)) was added to each forward primer and universal primers M13(-21) were labeled with the fluorescent compounds FAM (6-carboxy-fluorescein), NED (naptiletilenediamine dihydrochloride) or HEX (hexaclaro-6-carboxy fluorescein). Each PCR reaction mixture consisted of 1x reaction buffer (10mM Tris-HCl pH 8.0, 50mM KCl, 1.5mM $MgCl_2$ - USB), 0.4 μ L of dNTPs (2.5 mM), 0.5 μ L of reverse primer (8pmol/ml), 0.5 μ L of M13(-21) primer (8pmol/ml) labeled with one of the fluorescent compounds, 0.5 μ L of forward primer with the tail (2pmol/ml), 0.2 μ L of $MgCl_2$ (50 Mm), 50ng of genomic target DNA, and 1.25U *Taq* DNA polymerase (USB), in a total volume of 10 μ L. Amplifications were carried out in a gradient

Table 1 – Microsatellite loci characterization of *Coturnix japonica*.

Locus	Repeat motif	Primer sequence (5'-3')	N	Na	Ta (°C)	allele size (bp)	H _o	H _e	Reference
GJ0001	(CA) ₇ TG(CA) ₁₃	F: GAAGCGAAAGCCGAGCCA R: CAGCACTTCGGAGCACAGGA	90	5	65	247-255	0.61	0.73	present data
GJ0006	(CA) ₁₄	F: TGGGATGATAATGAGGTACGG R: AGGATAGCATTTCAGTCACGG	88	8	55	127-139	0.82	0.81	present data
GJ0025	(CA) ₉	F: CCTGAGCGAATACACAAGT R: AGTGTTAGGTGAGGACTGCT	-	-	60	245	-	-	Kayang <i>et al.</i> (2000)
GJ0030	(CA) ₃₁	F: TGCACCAATCCCAGCTGTTT R: AACGCACAATGGAAAGTGGG	-	-	64	181	-	-	Kayang <i>et al.</i> (2000)
GJ0037	(CA) ₁₀ C(CA) ₂	F: CCATTCCTCCATCGTTCTGA R: GGGAAGGAGTGTAGGAAAGA	92	8	55	190-206	0.63	0.71	present data

N, number of genotyped individuals; Na, number of alleles; Ta, PCR annealing temperature; H_o, observed heterozygosity; H_e, expected heterozygosity.



Mastercycler Personal thermocycler (Eppendorf) with the following cycle program: a first denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55-65°C for 45 s, and 72°C for 45 s, plus 8 more cycles of 94°C for 30 s, 53°C for 45 s, and a 72°C for 45 s, and a final extension step at 72°C for 10 min. A negative control, consisting of all the reaction components except template DNA, was also included to monitor any possible contamination. Reaction products (3µL) were first subjected to 1% agarose gel electrophoresis for 45 minutes (100V/150A) in order to verify the amplification efficiency. Four micro liters of the PCR products were further diluted in 26 µL of water. After dilution of the PCR products, 2 µL were mixed with 7.75 µL of 0.1% Tween 20 and 0.25 µL of ET-ROX. Samples were denatured at 95°C for 3 minutes and submitted to electrophoresis for 51 minutes at 10 kV using a 96 plate of the capillarity nucleotide sequencer MegaBACE1000 (GE Healthcare Life Sciences). Allele sizes were estimated using a DNA marker (ET-550R - GE Healthcare Life Sciences), and the genotyping data, referring to the definition of the allele's peaks, were obtained by the computer program MegaBACE Fragment Profile v 1.0 (GE Healthcare Life Sciences).

STATISTICAL ANALYSES

Allele frequency, number of alleles per locus, observed (H_o) and expected (H_e) heterozygosity, deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium (Fisher test) were evaluated using Excel Microsatellite Toolkit and the computer program GenePop version 4.0.6 (Rousset, 2008).

RESULTS AND DISCUSSION

Five microsatellite primer sets were selected as they revealed polymorphic loci in a previous analysis using DNA samples of one male and one female of *C. japonica* that were randomly selected from a domesticated population from Japan (Kayang *et al.*, 2000). In the present study, an initial test was performed using DNA samples of one bird triplet, composed by a single female and two males (F15, M29 and M30), and of its generated embryos on a PCR approach using non-labeled primers and further genotyping through PAGE. Although the PCR conditions initially followed the protocol described by Kayang *et al.* (2000), several changes were made in order to achieve better amplification results (see present methods description).

In every case, allele sizes were similar to those described by Kayang *et al.* (2000) in their original report. The primers sets for the *GUJ0006* and *GUJ0037* loci generated alleles with very diverse sizes in the female and two males analyzed, which easily allowed the correct identification of each animal genotype (Figure 1). It was also possible to determine all offspring genotypes by the use of the *GUJ0006* marker as well as their paternity. The determined genotypes of the analyzed individuals and the inferred paternity of the embryos using data for the *GUJ0006* and *GUJ0037* loci are presented in Table 2. The primers for the *GUJ0001* and *GUJ0030* loci also allowed the amplification of microsatellite repetitions in the analyzed individuals. However, it was not possible to correctly determine the entire genotype of the birds due to the presence of some diffuse bands. The other selected microsatellite marker (*GUJ0025*) failed to generate amplification products.

Table 2 – Genotypes of the analyzed adult individuals from one triplet of birds, composed by a female and two males, and of their generated embryos, and the inferred offspring paternity. Data were obtained using two sets of non-labeled primers in PCR (for the *GUJ0006* and *GUJ0037* loci) and polyacrylamide electrophoresis genotyping.

	Loci				
	<i>GUJ0006</i>			<i>GUJ0037</i>	
Birds	Allele size		IP	Allele size	
F15	113	119		170	176
M29	111	113		176	176
M30	119	121		176	178
E52	111	113	M29	170	176
E53	113	113	M29	176	176
E54	111	119	M29	176	176
E55	119	119	M30	170	176
E56	119	119	M30	170	178
E98	113	113	M29	-	-
E102	111	119	M29	170	176
E108	113	119	M30	176	176

F, female; M, male; E, generated embryo; IP, inferred paternity.

The genotyping failure may be due to primer binding site mutation ("null alleles"), which leads to allele non-amplification, or miscoring of allele banding patterns (Callen *et al.*, 1993; Dakin & Avise, 2004). Out of these, the most important source of error is probably the incorrect calling of alleles. In particular, the presence of "stutter bands", generated by

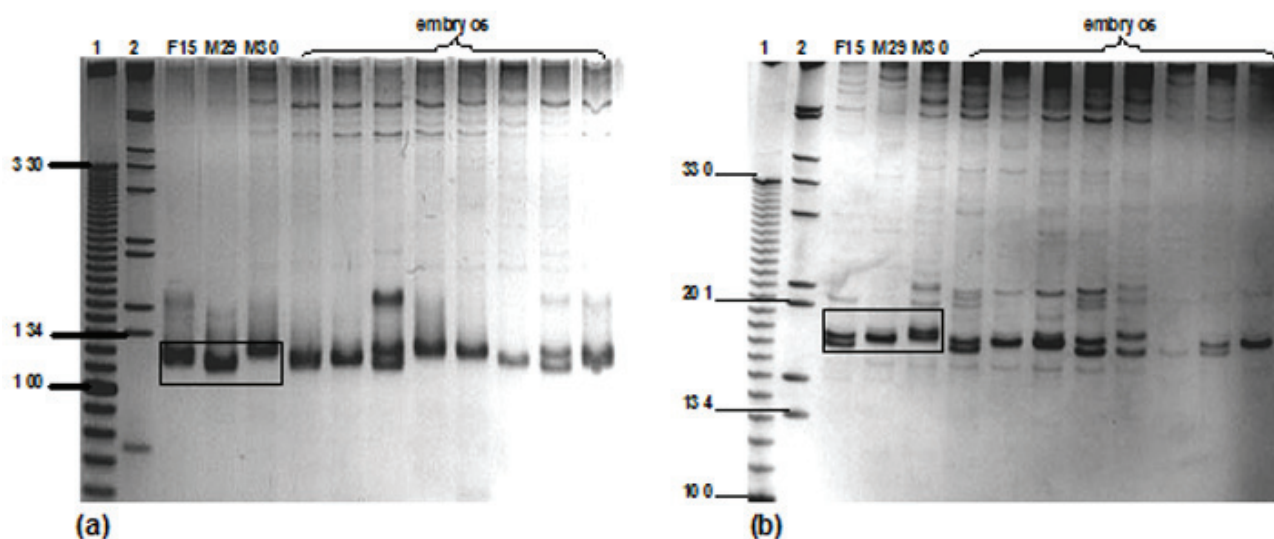


Figure 1 – Polyacrylamide gel electrophoresis (PAGE) results obtained with the non-labeled primers for the (a) *GUJ0006* and (b) *GUJ0037* microsatellite loci of *Coturnix japonica*. (1) 10 bp DNA molecular marker, (2) 1Kb DNA molecular marker, (F) female, (M) males. The box evidences distinct alleles present in the adult female and males that belong to one of the analyzed triplets of birds. The genotypes of the eight generated embryos are also evidenced.

slippage of DNA polymerase during PCR, and also due to polyacrylamide gel analysis, can make it difficult to score alleles reliably, especially when there are large signal intensity differences between alleles and/or the lengths of two alleles in a heterozygous individual differ by only a few nucleotides (Fernando *et al.*, 2001; Johansson *et al.*, 2003). Due to the limited rate of genotyping success in *C. japonica* achieved by the use of common primers in PCR and subsequent PAGE analysis, another microsatellite genotyping test through the use of fluorescent labeled and tailed primers and further automated genotyping was performed.

An initial fluorescent PCR test was performed using the five selected microsatellite primers sets and the DNA samples of one bird triplet (F15, M29 and M30) and its embryos. Amplification products were firstly confirmed by agarose gel electrophoresis and were further submitted to automated analysis. Consistent amplification products were obtained with the use of labeled primers for the *GUJ0001*, *GUJ0006* and *GUJ0037* loci. As also observed in PAGE analyses, no amplification products were detected for the *GUJ0025* locus and there were no consistent results for the *GUJ00030* locus, which led us to eliminate these markers from further analyses. Although the primers sets for the *GUJ00025* and *GUJ00030* loci were previously successfully amplified in *C. japonica* (Kayang *et al.*, 2000), these results were obtained in the analysis of only one male and one female randomly sampled from a hatchery colony originated from a wild population in 1970 (Kayang *et al.*, 2000). Even though we cannot discard the possibility of technical amplification problems,

the birds analyzed in the present study probably have a different origin and the failure in obtaining PCR results may be due to the high microsatellite mutation rate, which is estimated to occur between 10^{-3} and 10^{-5} times per locus per generation (e.g. Hancock, 2000). Moreover, recent data have evidenced that the Japanese quail presents a high mutation rate in its overall genome, since several mutations were found to have occurred in different genes in this species during the last 50 years (Tsudzuki, 2008).

A comparison between the results of the PCR with non-labeled primers/PAGE and the PCR with labeled primers/automated genotyping allowed to verify that the allele determination was very similar using the two approaches, considering the presence of 18 additional nucleotides in the alleles obtained by the use of the second methodology due to the forward primer tail (Tables 2 and 3). Minimal differences in the size of the alleles observed when comparing the two employed techniques could be related to the accuracy and/or details of each technique. Despite both methodologies lead to the assumption of homozygous or heterozygous individuals and did not influence the parentage analysis, the use of labeled primers/automated genotyping lead to more robust results.

Although allele determination problems still remain when genotyping is automated, including the presence of stuttering or shadow bands (a leading ladder of minor products preceding the primary allele peak), which leads to difficulties in allele calling, particularly when analyzing dinucleotide repeats on heterozygous individuals with two alleles of close sizes (Hall *et al.*,



Table 3 – Genotypes of the analyzed adult individuals from ten bird triplets, each composed by a female and two males, and of their respective generated embryos, and the inferred offspring paternity. Data were obtained using three sets of primers, including a fluorescent labeled primer, in PCR (for the GUJ0001, GUJ0006 and GUJ0037 loci) and automated genotyping. Allele incompatibilities between the female known parent and its offspring for the GUJ001 locus, which may reflect the presence of null alleles, appear in bold. Parentage incompatibilities related to the GUJ0037 locus are also evidenced in bold.

Loci										
Animal	GUJ0001			GUJ0006			GUJ0037			Paternity assignment
	IP			IP			IP			
F04	253	253		133	141		190	200		
M07	249	255		133	137		194	206		
M08	249	249		129	137		198	202		
E05	249	249		133	137		198	200	M08	
E07	249	253		133	137		198	200	M08	
E08	249	249		133	141	M07	200	206	M07	
E11	249	253		129	133	M08	190	202	M08	
E17	253	255	M07	-	-		190	194	M07	
E33	249	249		129	133	M08	190	202	M08	
E34	253	255	M07	133	141	M07	194	200	M07	
E121	249	249		137	141		198	200	M08	
E123	249	253		129	141	M08	198	200	M08	
F06	253	253		131	141		204	206		
M11	251	251		129	137		200	200		
M12	251	255		133	139		200	204		
E65	251	253		129	141	M11	200	206	M11	
E66	253	253		133	141	M12	200	206	M12	
E67	251	251		129	131	M11	194	206	M11	
E68	251	253		129	131	M11	194	204	M11	
E69	251	253		137	141	M11	194	204	M11	
E70	251	253		133	141	M12			M12	
E124	251	255	M12	133	141	M12	204	204	M12	
E126	251	251		129	131	M11	200	204	M11	
E127	253	255	M12	139	141	M12	204	206	M12	
F08	251	253		137	141		194	200		
M15	249	251		139	139		200	200		
M16	249	255		133	137		204	204		
E24	251	251	M15	139	141	M15	200	200	M15	
E110							200	204	M16	
E111	251	255	M16	133	137	M16	194	204	M16	
E116	249	253		137	141	M16	200	204	M16	
E119	249	251		133	141	M16			M16	
F09	255	255		127	131		200	204		
M17	249	255		129	129		202	206		
M18				127	127		200	200		
E25	255	255		127	127	M18	200	200	M18	
E26	255	255		127	131	M18	200	200	M18	
E37	253	255		127	127	M18	200	200	M18	
E38	253	255		127	131	M18	200	204	M18	
F12	251	255		131	133		200	200		
M23	253	253		129	141		200	206		
M24	255	255		133	141		204	204		
E41	251	253	M23	131	141		200	200	M23	
E42	253	255	M23	129	131	M23	200	200	M23	
E43	251	255	M24	133	133	M24	200	204	M24	



	Loci									
Animal	GUJ0001			GUJ0006			GUJ0037			Paternity assignment
			IP			IP			IP	
E44	251	253	M23	133	141		200	200	M23	M23
E45							200	204	M24	M24
E46	251	253	M23	129	131	M23	200	200	M23	M23
E63	251	253	M23	131	141		200	206	M23	M23
E64	251	255	M24	133	141		200	204	M24	M24
E109				129	131	M23	200	200	M23	M23
E112	251	255	M24	133	141		200	204	M24	M24
E122	255	255	M24	133	133	M24	200	204	M24	M24
F13	251	253		133	139		190	200		
M25	255	255		131	141		200	204		
M26	251	255		131	137		190	200		
E47	251	255		139	141	M25	190	200		M25
E48	251	255		131	139		190	204	M25	M25
E49	251	255		131	133	M25	190	200		M25
E50	251	255		139	141	M25	190	200		M25
E51	251	255		131	139		200	200		
F14	255	255		141	141		204	204		
M27	249	255		139	139		198	204		
M28	247	249		127	127		194	200		
E77	255	255	M27	139	141	M27	204	204	M27	M27
E78	255	255	M27	139	141	M27	198	202	M27	M27
E79	255	255	M27	139	141	M27	204	204	M27	M27
E80	255	255	M27	139	141	M27	204	204	M27	M27
E81	255	255	M27	139	141	M27	196	204		M27
E82	249	255					204	204	M27	M27
F15	255	255		133	137		194	200		
M29	255	255		129	133		200	200		
M30	251	255		137	141		194	200		
E52	255	255		129	133	M29	194	202		M29
E53	255	255		133	133	M29	194	206		M29
E54	255	255		129	137	M29	200	200		M29
E55	251	255		137	141	M30	200	200		M30
E56	255	255		137	137	M30	200	200		M30
E98	255	255		133	133	M29	194	200		M29
E102	255	255		129	137	M29	194	200		M29
E108	255	255		133	137		200	202		
F16	249	251		131	137		200	202		
M31	251	255					200	200		
M32	249	249		133	141		200	200		
E57	251	251	M31	137	139		200	200		M31
E114	249	251	M31	133	133		194	200		M31
E117	249	249	M32	131	133		200	202		M32
F17	253	255		135	137		200	204		
M33	251	253		137	139		194	206		
M34	253	253		137	141		200	204		
E83	253	255		137	137		200	206	M33	M33
E105	253	253		135	139	M33	202	206	M33	M33
E106	253	255		137	137		200	204	M34	M34
E107	253	255		137	137		204	204	M34	M34

F, female; M, male; E, generated embryo; IP, inferred paternity.



1996), it is an accurate technique, specially due to the minor handling of samples and to computer software efficiency. Moreover, the strong detection sensitivity of the fluorescent labeled primers produces good results even with minute amounts or even old/degraded template DNA (Schuelke, 2000).

Therefore, we performed additional analyses for the *GUJ0001*, *GUJ0006* and *GUJ0037* loci using the automated genotyping approach. Ten bird triplets (each composed by a female and two males) and their respective embryos were successfully analyzed and polymorphism level was assessed by typing 94 individuals of *C. japonica* (30 adults and 64 embryos) (Table 3). In general, the automated genotyping results were consistent and the alleles were easily determined (Figure 2). All the three analyzed loci were polymorphic ($N_a = 5-8$, mean $H_E = 0.75$) and no locus was found to deviate significantly from the Hardy-Weinberg equilibrium or showed any evidence of linkage disequilibrium ($p > 0.05$) (Table 1). However, the *GUJ0001* locus showed evidence of a slight heterozygote deficiency and some incompatibilities were observed between the female known parent and its offspring that invariably involved homozygous genotypes (Table 3). These results may indicate the presence of null alleles, generally a typical

result from polymorphism due to point mutations or insertions/deletions in the flanking sequence of the locus, such that some alleles lack a functional PCR priming site (Callen *et al.*, 1993; Ishibashi *et al.*, 1996; Jones & Ardren, 2003). Parentage incompatibility was also observed for some embryos using the *GUJ0037* marker (Table 3), a finding that seems to be related to errors on the genotyping analysis and not to "allelic dropout". Nevertheless, it was possible to infer the male's paternity for 96.87% of the generated embryos using combined data of the three analyzed microsatellite loci (Table 3).

The genetic parentage inferences also allowed to evidence no significant difference in the contributions of the two potential male parents of *C. japonica* to the offspring ($p=0.755$). Although these data were observed in an experimental design, they lead to the assumption of a potential occurrence of a polyandric mating system in this species, as already evidenced for the European quail (*C. coturnix*) through a multilocus DNA fingerprinting analysis (Rodriguez-Teijeiro *et al.*, 2003).

The genetic paternity results in *C. japonica* were correlated to sperm and behavioral analyses performed in the same ten bird triplets. Combined data evidenced

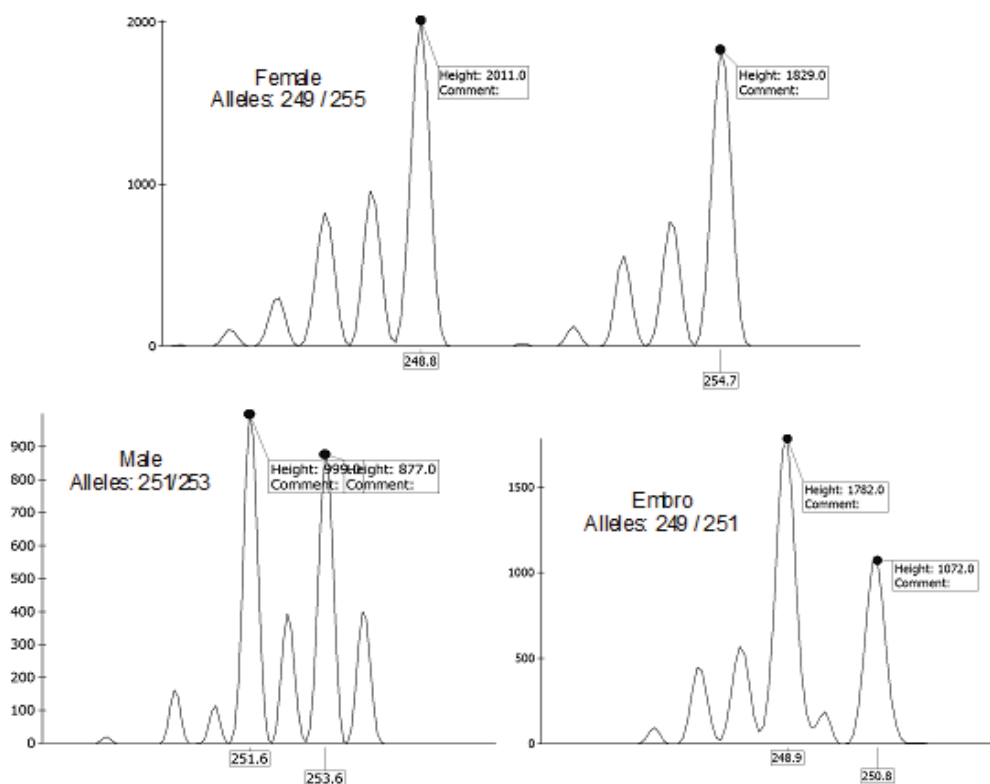


Figure 2 – Examples of electropherograms generated by automated genotyping. Fragment size in base pairs is shown on the horizontal axis and fluorescence is evidenced in the vertical axis.



no sperm selection by females merely based on sperm quality (concentration, vitality, or motility) and that the offspring paternity was also not influenced by the number of copulas by each male or by the male that performed the first or the last copulation (Gomes 2009). This shows that, apparently, a last-male mating advantage, in which spermatozoa from the last male to mate within a brief period results in the fertilization of most eggs, a mechanisms that has been described in several bird species, specially those with SSTs (Sperm Storage Tubules) (Birkhead & Moller, 1998), does not occur in *C. japonica*.

The occurrence of a potential polyandric mating system in Japanese quails was reinforced by several features that may predispose these birds to mating changes, such as lack of parental care by males and a sex ratio strongly biased in favor of males throughout the breeding season (Rodrigues-Teijeiro *et al.*, 2003), which is also reflected in genetic polygamy. Moreover, as mating in Japanese quail frequently fails to result in eggs fertilization (Adkins-Regan, 1995) and female quails cannot use the physical act of copulation to determine that their eggs have been fertilized, copulations from additional males could be a sexual strategy to obtain some advantages. These not only include a higher probability of egg fertilization, but also comprise genetic benefits, such as the acquisition of "good genes" and an increase in genetic diversity of clutches (e.g. Evans & Simmons, 2008).

In conclusion, despite the use of a reduced set of microsatellite primers in the present study, it was possible to determine the paternity of most of the analyzed embryos of *Coturnix japonica* when using combined data of three polymorphic loci, leading to the assumption of a potential polyandric mating system in this species, in which no advantage mechanism of last-male sperm precedence seems to occur. More consistent data could be achieved with the redesign of the PCR primers suspected of producing null alleles in an attempt to recover normal inheritance at the *GUJ0001* locus. A recent model for detecting multiple mating in vertebrates evidenced that, if two males (or females) contribute with equal fertilization success, then only ten offspring and one microsatellite locus with seven alleles seem to be required to ensure that 80% of multiple mated broods are detected. Ninety-five per cent of multiple mated broods can be detected through the analysis of ten offspring and five loci with four alleles (Neff & Pitcher, 2002). The parentage test reported here seems to fit this proposed model and could be a valuable tool for documenting paternity patterns in *C. japonica*. These data can be useful to support behavioral analyses

related to fertility success and also to support selective breeding programs of the species.

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