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Detection of hybridization and species identification in domesticated and wild quails using genetic markers

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A b s t r a c t. Hybridization is particularly widespread in birds and can affect species status and recovery. The common quail *Coturnix coturnix* is a protected game species that has undergone significant population decrease due to habitat changes. The release of Japanese quail *C. japonica* and or hybrids for restocking has been occurring since the 1970's. Both species have not developed reproductive isolating mechanisms and hybridization is occurring. Species distinction based on morphology and male callings is difficult. In this work cytochrome *b* gene and five microsatellite loci were used with the aim of establishing an identification test for quails sampled in Portugal. Cytochrome *b* gene revealed to be of promising use to identify the quail maternal lineage. Success in species assignment with the studied microsatellite loci was moderate to identify samples with suspicion of being hybrids with common quail maternal lineage.

Key words: *Coturnix coturnix, Coturnix japonica,* microsatellites, cytochrome *b*, hybrids

Introduction

Human-mediated hybridization has increased in the last centuries when plant and animal species were introduced inadvertently or voluntarily in new continents or islands (e.g. red deer, A b e r n e t h y et al. 1994; polecat, D a v i d s o n et al. 2000). Hybridization is common in birds and many conservation problems have been reported in this group. Examples are the white-headed duck (M u ñ o z - F u e n t e s et al. 2007) and the black duck (M a n k et al. 2004) which suffered severe population decline due to the introduction of exotic species. Such hybridization events can cause negative effects due to outbreeding depression, potentially leading to the elimination of unique genotypes, an increase of sterility or a lack of adaptability to the environment (e.g. P r i m a c k 2002).

The common quail (*Coturnix coturnix* Linnaeus, 1758) is a partial migrant with a distribution that extends through Eurasia, North Africa and the Indian sub-continent. In Europe, where it is used as a game species, its populations have undergone a serious decrease in the last 20 to 40 years, because of droughts, overhunting and the decrease of traditional farming practices (D e r é g n a u c o u r t et al. 2002, G u y o m a r c h 2003). As a consequence, restocking has been taking place in a number of European countries such as France, Greece, Italy, Portugal and Spain, aiming to maintain hunting stocks. However,

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most releases appear to be performed with the related Japanese quail (*Coturnix japonica* Temminck et Schlegel, 1849) or with hybrids between the two quails. The common quail is now protected under the Bern Convention and defined as a species with unfavorable conservation status under the Bonn convention. It is in the red list of six European countries and its hunting has been banned in eleven (EUNIS 2006).

The Japanese quail is distributed in the North-East of Asia and has hence little overlap with the common quail. The two species are naturally sympatric only in the Baikal region in Russia and in the Khentei region in Mongolia and it is not known whether they hybridize in these areas (G u y o m a r c h 2003). However, laboratory studies have shown that hybridization is possible (D e r é g n a u c o u r t et al. 2002, 2005a) and that the hybrids can cross with both parental species (D e r é g n a u c o u r t et al. 2002). Portuguese authorities have forbidden the release of quails to the wild since 2001 (*Portaria* 464/01 of 8 May) but the fact that the two species and the hybrids have phenotypic similarity makes the detection and quantification of hybridization difficult. The difficulty to control the use of Japanese and hybrid quails for hunting restocking has led the European Species Management plan for *C. coturnix* calls for the use of molecular forensic tools to control the game breeders and to protect common quail natural populations (G u y o m a r c h 2003). The fact that individuals from species (*C. coturnix* and *C. japonica*) and their hybrids have all been observed in common quail breeding areas in France, Italy, Portugal and Spain (G u y o m a r c h 2003), suggests that it is urgent to develop such molecular tools and apply adapted statistical methods to quantify admixture, so as to advise proper conservation strategies. A recent genetic study by B a r i l a n i et al. (2005) has identified hybrids in Spain and Italy. However, for the individuals sampled in Portugal introgression was not detected.

In the present study we use cytochrome *b* (cyt *b*) gene sequences and microsatellite markers to detect and quantify the amount of admixture in common quail samples from Portugal. We then discuss the practical implications of our results to conservation issues such as the control of game farmers and the introduction of Japanese and hybrid quails in the wild.

Material and Methods

B i o l o g i c a l s a m p l e s

We obtained blood, liver and feather samples from *C. coturnix* (n=35)*; C. japonica* (n=39) and putative *C. coturnix* x *C. japonica* hybrids (n=22, see below) in a total of 96 animals. *C. coturnix* individuals were captured in the wild by hunters, with 20 samples originating from the North and 15 from the South (Fig. 1). Areas of species occurrence in Portugal, are shown in Fig. 1 (G u y o m a r c h & F o n t o u r a 1993, F o n t o u r a et al. 2000), even though we could not obtain samples from all the locations.. *C. japonica* individuals were obtained from a farm in the North-West of Portugal. Putative *C. coturnix* x *C. japonica* hybrids were collected in a farm located in the South-West of Portugal. While the owner of this farm claims to the Forestry Services to be producing and selling common quail, he apparently also sells what he calls “special quails” to hunting associations. The Forestry Services suspect him of producing *C. coturnix* x *C. japonica* F1 crosses. These samples were included in this study in order to test the accuracy of the methods used to identify individuals of *Coturnix* species and also to analyze the type of hybridization in captivity occurring in Portugal. For simplicity, these individuals will be referred to as hybrids in this study.

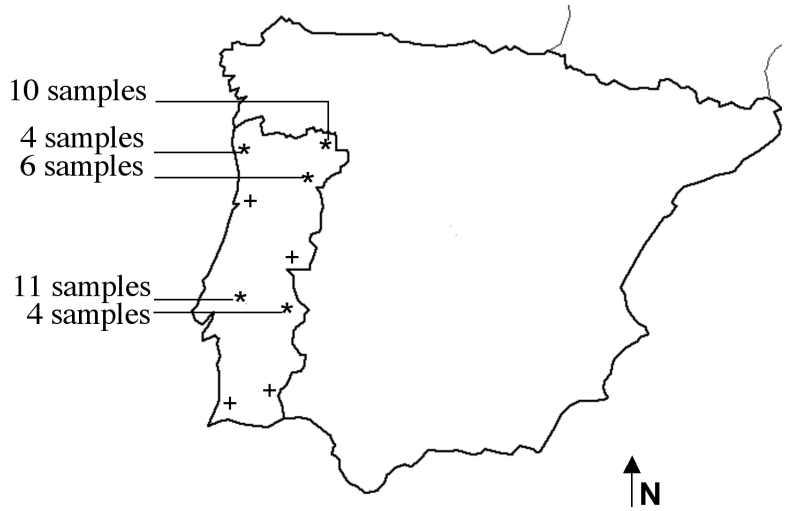

Fig. 1. *C. coturnix* occurrence sites in Portugal. Sites sampled (*) and not sampled (+). The sample sizes are indicated for each location.

Total genomic DNA from blood, liver and feather samples was extracted using commercial kits for Blood and Tissue DNA Purification (Jetquick- Genomed, Löhne, Germany) following manufacturer's instructions. Briefly, DNA extraction was performed by adding the Jetquick-Extraction Solution to each type of sample (100µl of whole blood, 10 mg of liver or 3 feather calamus) and the mixture was incubated for 5 minutes at room temperature. Extraction mixture was transferred to a Jetquick-Column and a series of centrifugations and column washing with Jetquick-Washing Solution was performed to bind the DNA. Jetquick-Elution Buffer was used to recover the DNA that was stored at -20ºC.

Mitochondrial DNA genotyping

A 611 bp fragment of cytochrome *b* (cyt *b*) mitochondrial gene was amplified for all samples using internal primers L14841 (Kocher et al. 1989) and H15646 (Sorenson et al. 1999). Each PCR mix reaction contained 200ng of DNA, 1X buffer (10X buffer, with KCl (-$MgCl_2$), 3.5 mM $MgCl_2$, 0.2 μM of each dNTP, 0.2 ρmol of each primer, 0.8 µg of BSA and 1U Taq (FERMENTAS, St Leon Rot, Germany) in a final reaction volume of 25 µl. The PCR amplification was performed on a Minicycler™ (MJ Research, Watertown, MA, USA) with the following cycling programme: 34 cycles of 94ºC for 30s, 55ºC for 30s and 72ºC for 1min, preceded by 3min at 94ºC and followed by 5min at 72ºC. Amplification success was checked through electrophoresis in a 1% agarose gel. Electrophoresis was performed on a Submarine Gel Electrophoresis System (EC350 Midicell, EC Apparatus Corporation, St. Petersburg, Florida, USA), at 100V, 80 mA for 45 min. Gel image was visualized and photographed with KODAK EDAS 290 system (Eastman Kodak, Rochester, NY, USA). PCR products were cleaned using the DNAce Quick Clean kit (Bioline, Boston, MA, USA) and directly sequenced in both directions using the same amplification primers and the ABI Dye Terminator Sequencing Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). Sequencing was performed on an ABI 310 automated sequencer (Applied Biosystems, Foster, CA, USA).

Microsatellite genotyping

Extracted DNA (50ng) of all samples was used for PCR amplification of eight microsatellite, namely MCW118, MCW135, MCW225, MCW252, MCW280, MCW276, UBC0004 and UBC0005 (Table 1). The MCW markers are *Gallus gallus* derived microsatellite and were developed by Wageningen University (http://137.224.73.223/abgorg/hs/research/molecular/body_mcwtable.html). The UBC markers were described by Pang et al. (1999) for *C. japonica*. PCR reactions were performed according to conditions available in table 1 in a total volume of 10 µl and using 1X Buffer, 0.2 µM dNTP's, 0.8 µg BSA and 0.75 Unit Taq-polymerase (FERMENTAS, St Leon Rot, Germany).

PCR products were separated by electrophoresis in 6 % Long Ranger denaturing gels (0.25 mm thick) for 1–2 h using an automated fluorescence 4200S LI-COR sequencer (Li-cor, Lincoln, NE, USA). Gel image results were analyzed with RFLPScan 3.1 (Scanalytics, CPS Inc., Rockville, M.D., USA). The DNA fragments were sized by reference to the Li-Cor STR marker. Allele identification was based on their base-pair size. Data was stored in RFLPDataBase 3.1 (Scanalytics, CPS Inc., Rockville, M.D. USA) and then transferred to an internal database.

Microsatellite cloning and sequencing

Given that most of the primers we used were originally developed for chicken, and had not been used before for *C. coturnix*; one homozygote of each species was selected for each locus in order to be cloned and sequenced. This procedure allowed us to certify the correct amplification of all loci and to perform the fragment analysis with the correct repeat pattern. Amplification of the selected loci was performed according to conditions described in Table 1. PCR products were purified using commercial kits for PCR purification (Jetquick purification spin kit, Genomed, Löhne, Germany) following manufacturer's instructions. The cloning of the PCR products was performed using a commercial cloning kit pGEM T- vectir system (Promega, Madison, WI, USA). Cloning products were amplified using universal primers T7 e SP6. PCR products were checked in agarose 1% gel and purified with a commercial kit and diluted in ultrapurified water. Products were directly sequenced in both directions using the same amplification primers and the ABI Dye Terminator Sequencing Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). Sequencing was performed on an ABI 310 automated sequencer (Applied Biosystems, Foster, CA, USA).

Data analysis

Mitochondrial DNA

Sequences were edited using the BIOEDIT software v. 7.0.1 (Hall 1999) and aligned using accessory application ClustalW (Thompson et al. 1994). Haplotype (*h*) and nucleotide (π) diversity (Nei 1987) were estimated with DnaSP v4.10.4 (Rozas et al. 2003). Pairwise F_{ST} were calculated between samples and departures from zero were tested using 1000 permutations as implemented in the Arlequin 3.1 (Excoffier et al. 2005).

Phylogenetic trees were generated using maximum-parsimony and neighbour-joining methods, as implemented in the PAUP 4.0d software (Swofford 1998). Cyt *b* gene sequences for *C. coturnix africana* (Acc. No. U90641 in Bloomer & Crowe 1998)

Table 1. Primer sequences, repeat motifs , PCR conditions and references of the eight *G. gallus* microsatellite loci.

Locus	Primer (ρmol/μl)	$MgCl_2$ (mM)	T_a (ºC)	Primers (5' – 3')	Chicken Repeat	Repeat in *C. coturnix*	Repeat in *C. japonica*
MCW 135	0,3	1,5	49	F: ATATGCTGCAGAGGGCAGTAG R: CATGTTCTGCATTATTGCTCC	$(CA)_{25}$	-	-
MCW118	0,2	1,5	53	F: ATGATGAAGCATTTAGTCTAAG R: CAATTTACTCAGAGATGCAGTG	$(TG)_3TA(TG)_5TT$ $(TG)_5(TA)_4TG(TA)_7$	$(CA)_6TG$ $(TA)_2TGTATC(TA)_3$	$(CA)_6TG$ $(TA)_2TGTATC(TA)_3$
MCW225	0,2	1,5	49	F: AACGGACTCTTCTGTCTATAG R: TGCTTTGCTCCTCATTAAAGG	$(TG)_{13}$	$(TG)_7(CG)_2CA(CG)_2$ $(TG)_{11}$	$(TG)_7CGCAC(TG)_{15}$
MCW252	0,2	1,5	3	F: CTGCTCAAGCCCATCAAATGG R: CGATAACATCTGACACTGCC	$(TG)_{14}$	$(TG)_8$	$(TG)_8$
MCW276	0,3	2,0	53	F: ACTCTGAGTGGAATTACCT R: TTTCTGTTAGAAGCAGCTGC	$(TG)_8(AG)_5$	$(GT)_4GC(GT)_3GA$	$(GT)_4GC(GT)_3GA$
MCW280	0,1	1,5	53	F: TGAATGGTTTTATGCATTGT R: AGCAACATATCCATAAGTGT	A_{18}	$A_5TAT_5(AT)_9$	$A_5TAT_5(AT)_9$
UBC0004	0,06	1,5	50	F: TCCTTGGGCAGTAGTTTCAA R: CTCCCATGTTGCTCCTTTAG	$(CT)_6(CA)_5$	$(CT)_5(CA)_6$	$(CT)_5(CA)_6$
UBC0005	0,3	0,3	53	F: GGAACATGTAGACAAAAGC R: AGTAGTCCATTTCCACAGCCA	$(AC)_9$	$(CA)_3CG(CA)_5$	$(CA)_{10}$

and *C. japonica* (Acc. No. AF119094) were also included in the analysis. The published sequence for *Gallus gallus* (Acc. No. X52392 in Desjardins & Morais 1990) was used as an outgroup for tree rooting. The maximum-parsimony analysis used heuristic search, random stepwise addition and tree bisection-reconnection methods. For the neighbour-joining tree, sequence divergence was calculated according to a nucleotide substitution model implemented in Modeltest software v.3.0 (Posada & Crandall 1998). The Tamura & Nei (1993) model with different base frequencies for nucleotide (freqA = 0.2607, freqC = 0.3527, freqG = 0.1342, freqT = 0.2523) was selected according to the Akaike information criterion with a proportion of invariable sites of 0.7440. Genetic distances among samples using the model described above were calculated with PAUP version 4.0d (Swofford 1998). Support for nodes was assessed by bootstrap resampling using 1000 replicates (Felsenstein 1985) as implemented in PAUP 4.0d.

Microsatellites and genetic diversity

Allelic frequencies, number of alleles, observed and unbiased expected heterozygosity (H_o, and H_e, respectively) (Nei 1978) were calculated for each population. Wright's F statistics were calculated according to Weir & Cockerham (1984). Genetic differentiation between populations was assessed by calculating an average F_{ST} and by estimating pairwise F_{ST} values. Deviations from Hardy-Weinberg (HW) were measured by computing F_{IS} values. Significance of these values was assessed by a permutation approach as implemented in GENETIX 4.05 (Belkhir et al. 2001). Deviations from linkage equilibrium were tested using the probability test implemented in GENEPOP 3.4 (Raymond & Rousset 1995). Patterns of differentiation were also visualized by Factorial Correspondence Analysis (Benzécri 1973) of individual multiscores computed using GENETIX 4.05 (Belkhir et al. 2001). A nonparametric analysis of variance (Mann–Whitney U test; Sokal & Rohlf 1995) was performed to test for differences in the average of H_o, and H_e between the studied populations using R language (CRAN 2005).

Since wild common quail individuals were captured by hunters and admixture with *C. japonica* has been recently detected in wild common quails in genetic studies of this species in Spain and Italy (Barilani et al. 2005), misidentification of individuals could not be excluded. In order to determine whether genetic data can be used to separate the two species and hence detect and quantify recent admixture we used the method of Pritchard et al. (2000) as extended by Falush et al. (2003) and implemented in the STRUCTURE 2.1 software. This clustering method aims to determine, within a particular set of multilocus genotypes, whether it is possible to identify K groups of individuals that are at HW and linkage equilibrium. The method (hereafter referred to as the Pritchard and Falush method, for simplicity) is typically run for different values of K and an *ad hoc* method proposed by Pritchard et al. (2000) is used to select the most "likely" K value. Recently Evanno et al. (2005) have proposed a new method for selecting K. This method has been tested on extensive simulations under different models of population structure and it appears to work better than the original method for cases where the population structure is hierarchical. An algorithm implementing Evanno et al.'s (2005) method in the R language (CRAN 2005) was thus also used and both approaches were used for comparison to select the most "probable" K value. The R code is available from the authors upon request. The method of Pritchard and Falush can be used either assuming admixture or not. In the no admixture mode, it is assumed that the individuals (i.e. the multi-locus genotypes) can only come

from one of the K populations, whereas in the admixture mode, the individual's genome can be the result of admixture between individuals from different populations. The latter case seemed more appropriate for our case and we hence used the admixture model, setting the parameters as follows: 20 runs with K=1-10 were done with a burn-in of 100,000 steps, followed by 1,000,000 steps of the MCMC algorithm.

We also used assignment tests to determine whether it was possible to assign the different multilocus genotypes to particular groups (see below). In particular we wanted to determine (i) whether assignment rates were very different between the three types of quails and (ii) whether individuals identified by STRUCTURE as potential hybrids were assigned to *C. japonica*. To do this we used the Bayesian method of R a n n a l a & M o u n t a i n (1997) and applied the resampling approach of P a e t k a u et al. (2004) as implemented in the GENECLASS v2.0 software (P i r y et al. 2004). Contrary to the method of Pritchard and Falush, assignment tests require the use of reference populations. Since the two natural groups of interest are the Japanese and common quail, the reference data file only contained data of these two groups. The individuals to be assigned contained all the data set, namely common, Japanese and hybrid quails. One thousand simulations were performed to estimate for each individual the probability of originating in any of the two reference populations. These probabilities are the two likelihoods L_J and L_C for the Japanese and common quail respectively. These values were used to determine whether individuals were more likely to be assigned to their population of origin and whether hybrids were indeed intermediate. As a way of representing these data we also produced a plot where the x axis corresponds to the sum of the likelihoods ($L_J + L_C$) and the y axis is the ratio $L_J / (L_J + L_C)$.

Results

C y t o c h r o m e *b* s e q u e n c e a n a l y s i s

The alignment of the cytochrome *b* gene sequences allowed us to detect 16 haplotypes with 21 polymorphic sites 17 of which were parsimony informative. Twenty base changes correspond to transitions and one to a transversion. The amplified fragment corresponds to the region between the positions 15002 and 15613 from the chicken reference mtDNA sequence (Acc. No. X52392). Sequences were translated into amino acids and two insertions were detected when compared to the chicken reference sequence. The total nucleotide variation induced nine amino acid changes. The obtained sequences revealed a similar content in base composition, adenine (~26.70%), cytosine (~34.0%), guanine (~13.20%) and thymine (~26.0%). The sequences generated by the present work were deposited in GeneBank under Accession Nos. DQ515805-DQ515818 and DQ987599. The F_{ST} values between common and Japanese quails (0.93) and between common quails and the hybrids (0.922) were very high and showed significant differentiation. Differentiation was not significant between Japanese and hybrid quails (F_{ST} ~-0.021). Interestingly Southern and Northern common quails present considerable and significant differentiation (F_{ST} ~0.126).

The high levels of mtDNA differentiation are particularly clear when one notices that the *C. coturnix* individuals did not share haplotypes with *C. japonica* individuals (Table 2) and that nine of the 21 polymorphic sites appear to be species specific (Table 2). Altogether, we were able to identify 16 haplotypes (H1-H16), 4 of which were only observed in *C. japonica*, namely H5, H6, H10 and H11. All the hybrid individuals analyzed here had

Table 2. Nucleotide substitutions in the Cytochrome *b* gene of the 16 haplotypes identified. The nucleotides position numbers in the top of the table are according to the *C. c. africana* sequence (Ac. No: U90641) denoted as CcRef and corresponding to haplotype H17 in the text. Dots indicate homology with sequence U90641. CcSouth and CcNorth correspond to wild *C. coturnix* sampled in the South and North, respectively; Cj stands for *C. japonica*; Hyb for hybrids. Hapl..- Haplotypes. Values of haplotype diversity (*h*) and nucleotide diversity (π) are shown with its respective standard deviation values.

Hapl.	Polymorphic sites																					Haplotype frequency				
	005	030	066	096	111	183	213	234	237	240	267	300	306	357	375	435	465	492	513	600	609	Cc South	Cc North	Cc total	Cj	Hyb
CcRef	A	T	C	G	T	C	A	C	C	A	T	C	T	G	T	C	T	T	T	T	T					
H1	.	.	.	A	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	9	3	12		
H2	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	4	9	13		
H3	.	.	.	A	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	1		1		
H4	.	.	.	A	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	1		1		
H5	.	C	T	.	.	T	.	T	.	.	C	T	C	A	C	.	C	.	.	A	.				35	20
H6	.	.	T	.	.	T	G	T	.	G	C	T	C	A	C	.	C	.	.	A	.				2	2
H7	.	.	.	A	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.		1	1		
H8	.	.	.	A	C	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	.		1	1		
H9	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C		1	1		
H10	.	C	T	.	.	T	.	T	.	.	C	T	C	A	C	.	C	.	.	A	.				1	
H11	G	C	T	.	.	T	.	T	.	.	C	T	C	A	C	.	C	.	.	A	.				1	
H12	.	.	.	A	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		2	2		
H13	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		1	1		
H14	.	.	.	A	.	T	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.		1	1		
H15	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.		1	1		
H16	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.		1			
h																						0,600 ± 0,113	0.800 ±0.089	0.775 ±0.052	0,196 ±0,084	0,173 ±0,098
π																						0,00122 ±0,0003	0.0023 ±0.0004	0.00193 ±0.0003	0,00091 ±0,0004	0,00085 ±0,0005

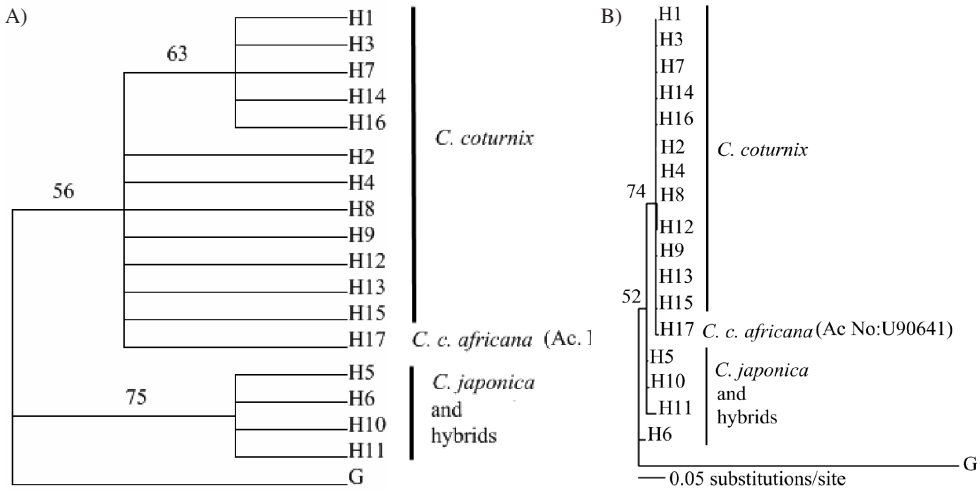

Fig. 2. (A) Maximum parsimony tree (B) Neighbour-Joining tree of *Coturnix* sp. cyt *b* haplotype*s*. Numbers of internal nodes indicate values of bootstrap (BP) over 50%. G- outgoup *Gallus gallus* (Acc. No. X52392).

haplotypes H5 and H6 which were only observed in *C. japonica* explaining the low F_{ST} value between these two groups. Also, the *C. japonica* published sequence (Acc. No. AF119094) is very similar to haplotype H5, which was at high frequency in both *C. japonica* and hybrid individuals (35/39 and 20/22, respectively). The *C. c. africana* published sequence had a

Table 3. Summary of the observed range of alleles per microsatellite locus, expected (H_e) and observed heterozygosity (H_o) and F_{IS} and Sign. (% <) values for the F_{IS} permutation test. Cc- wild *C. coturnix* ; H- hybrids; Cj- *C. japonica*.

		MCW118	MCW252	MCW280	UBC004	UBC005	All loci
CcSouth	H_e	0.600	0.402	0.494	0.370	0.699	0.513
	H_o	0.400	0.333	0.266	0.467	0.800	0.453
	F_{IS}	+0.341	+0.176	+0.469	-0.273	-0.151	+0.120
	Sign.(%<)	92.20	71.90	99.30	0.000	9.10	89.80
CcNorth	H_e	0.621	0.145	0.638	0.550	0.659	0.523
	H_o	0.450	0.150	0.750	0.500	0.700	0.510
	F_{IS}	+0.280	-0.036	-0.180	+0.093	-0.0644	+0.024
	Sign.(%<)	91.70	0.000	2.30	58.70	23.50	59.80
Cc total	H_e	0.522	0.612	0.260	0.577	0.485	0.668
	H_o	0.4857	0.429	0.229	0.543	0.486	0.743
	F_{IS}	+0.067	+302	+121	+0.060	-0.009	-0.114
	Sign.(%<)	86.20	98.00	71.0	66.90	42.70	9.20
H	H_e	0.502	0.741	0.430	0.333	0.575	0.516
	H_o	0.182	0.727	0.455	0.318	0.636	0.464
	F_{IS}	+0.643	+0.019	-0.058	+0.045	-0.109	+0.104
	Sign.(%<)	99.60	49.10	17.50	34.20	20.80	91.20
Cj	H_e	0.251	0.683	0.441	0.281	0.368	0.405
	H_o	0.180	0.564	0.307	0.180	0.333	0.313
	F_{IS}	+0.288	+0.176	+0.305	+0.365	+0.097	+0.230
	Sign.(%<)	93.0	94.30	98.30	93.80	72.00	100.0

haplotype (H17) that was not observed in our samples but is closer to *C. coturnix* haplotypes (Table 2, and Fig. 2). Genetic diversity was much higher in *C. coturnix* than in *C. japonica* with nucleotide diversity being at least an order of magnitude higher (Table 2). Within the common quail samples, genetic diversity appeared to be higher in Northern sites, but more samples would be needed to confirm this observation.

The reconstructed trees using the *cytochrome b* sequences are shown in Fig. 2. They clearly show that the *C. japonica* haplotypes do not cluster with the *C. coturnix* haplotypes, but it is worth noting that the bootstrap values are not very high. The highest values are around 75% either in the NJ tree or using Maximum parsimony. However, in both trees, this value corresponds to a split between *C. coturnix* and *C. japonica* haplotypes. It is worth mentioning that the *C. c. africana* haplotype (H17) is within the *C. coturnix* clade in both trees.

Microsatellites genetic diversity and differentiation

The repeat sequences of the microsatellite loci used in the study were successfully assessed for all loci except MCW135 which we did not manage sequencing (Table 2) and were deposited in GeneBank under Accession Nos. EF014433-EF014440. While the repeat sequences are similar across species they show differences when compared to the chicken repeat sequence in all loci except in MCW252 and UBC0004 loci. Moreover, the two *Coturnix* species are slightly different at loci MCW225 and UBC005. Altogether three of the eight loci analyzed, presented fragment sizes which don't match with the obtained sequences (MCW135, MCW225 and MCW 276) and therefore were excluded in all the following analysis. The analysis of LD (linkage disequilibrium) did not detect significant (p>0.01) departure from equilibrium and the remaining five loci could thus be used for all calculations.

The five microsatellite loci analysed (Table 3) are moderately polymorphic with H_e values being much higher in common (H_e=0.52) than in Japanese (H_e=0.41) quail. In hybrids (H_e=0.50) as well, H_e is higher than in Japanese quails. No strong and significant departure from Hardy-Weinberg equilibrium was observed in common and hybrids quails but departures were significant in Japanese quail, with H_O values being lower than H_e values (H_O=0.49, 0.46 and 0.31 in common, Hybrid and Japanese quails, respectively). Comparing common quails sampled in North and South, differences in H_O and H_e were not significant (p>0.01). Out of a total of 26, five alleles were found that presented important differences in frequency in *C. coturnix* compared to hybrids and *C. japonica*, or alleles that were only found in one of these two groups. These alleles, found at loci MCW252, MCW280 and UBC0005 are shown in Table 4. These differences are particularly important for alleles 272 of locus MCW252 and 174 of locus MCW280, suggesting that there is significant differentiation between the two species and that these and other similar loci could be useful for species identification. The average F_{ST} between common and Japanese quail is indeed

Table 4. Frequencies of alleles exhibiting differences in the two species. Cc- wild *C. coturnix*; H- hybrids; Cj- *C. japonica*.

Locus	Private allele (bp)	*Cc* (N=34)	*H* (N=22)	*Cj* (N=40)
MCW252	272		0.23	0.45
MCW280	174	0.01		0.22
UBC0005	106	0.01		
	108	0.03		
	110	0.06		

quite high (~0.18), but the differentiation between hybrids and *C. coturnix* or between hybrids and *C. japonica* is not as high and the two F_{ST} values are rather similar (0.07 and 0.06, respectively). Significant differentiation was not found between Southern and Northern quails (0.005).

The pattern of genetic differentiation is represented in Fig. 3, where the three types of genotypes (*C. coturnix, C. japonica* and hybrids) can be identified. The figure shows that most *C. coturnix* are on the left of the figure whereas most *C. japonica* are on the right, with the hybrids being located in an intermediate position on the first axis (representing 83% of the inertia).

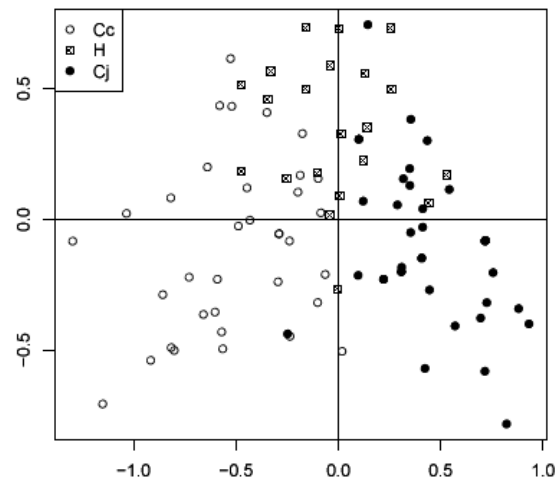

Fig. 3. Factorial Correspondence Analysis of individual quail microsatellite genotypes. Each dot on the graphic corresponds to one individual. Cc- *C. coturnix; H- hybrids;* Cj- *C. japonica.*

Bayesian clustering

Using the clustering approach implemented in the STRUCTURE software our results indicate that two most likely K values are K= 2 when we apply the Pritchard et al.'s (2000) criterion (Fig. 4B), and K=3 when we follow the approach of Evanno et al. (2005) (Fig. 4A). In both cases, the analysis shows a significant difference between *C. coturnix* and *C. japonica*, whereas hybrids appear to be either closer to *C. japonica* or to *C. coturnix*. Despite the existence of a high and significant differentiation between the two species, the STRUCTURE outputs did not identify homogeneous groups. Indeed, the individuals belonging to *C. coturnix* or *C. japonica* all appear to present different proportions of genes originating from the clusters reconstructed by STRUCTURE. Choosing the "right" K value is thus not trivial and to some extent arbitrary. From a biological point of view, however, K=2 seems to make more sense as it corresponds to the two species that are involved in the introgression.

Considering K=2 the STRUCTURE analysis allows to quantify the proportion of genes coming from either of the two groups. Barilani et al. (2005) identified as hybrids common quail individuals which showed an individual proportion lower then 80%. Therefore in this study, individuals for which the most important contribution is lower than 80% were considered as hybrids. Our results indicate that eleven individuals of wild common quails and seven of Japanese quails appeared to be admixed. Concerning hybrids, six individuals revealed a genotype profile similar to wild common quails and four to Japanese.

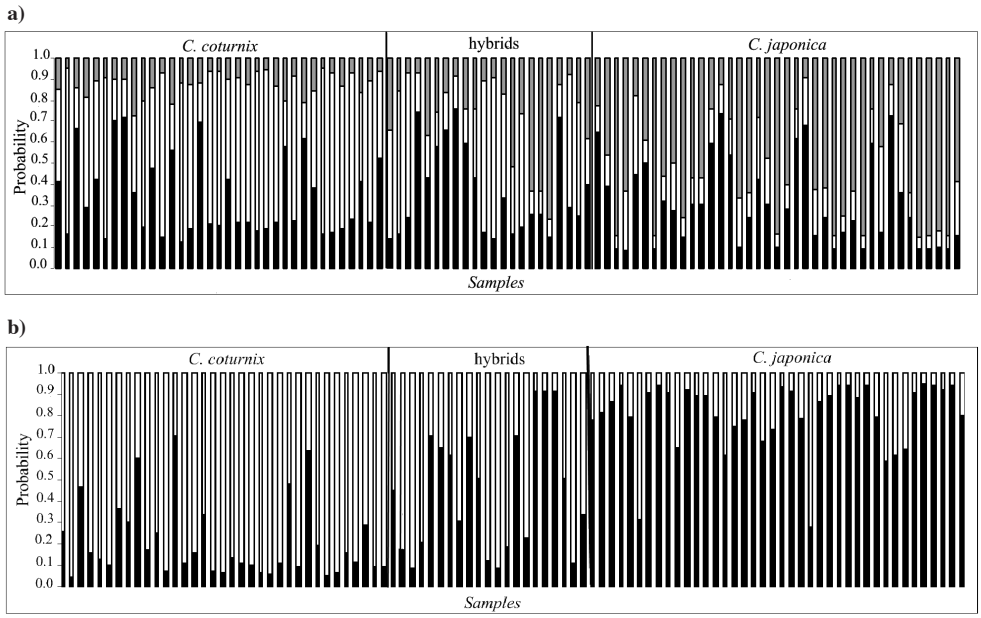

Fig. 4. Individual proportion of membership (q_i) using Structure 2.1 with K=3 (a) and K=2 (b).

The assignment test calculated according to Rannala & Mountain (1997) combining with Monte-Carlo resampling (Paetkau et al. 2004) allowed us to assign individuals of common and Japanese quails with an average of probability of only 50 % and 52.2 %, respectively. Despite these similar and not very high average values, the probability of assigning an individual to the Japanese quail group was very different between the

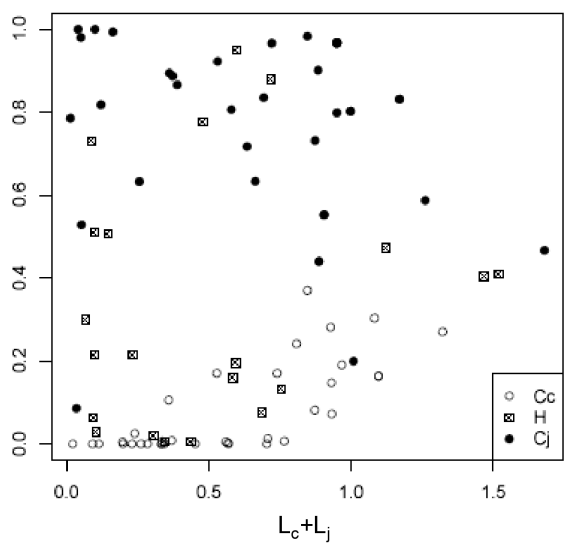

Fig. 5. Graphic display of the probability of each individual to be common or Japanese quail. X axis: Sum of likelihood (Lc+Lj); Y axis: Ratio Lj (Lc+Lj).

three groups. For instance, 24 of the 35 common quails showed a likelihood below 10 %, whereas 30 Japanese quails had a likelihood greater than 70 % (Fig. 5). Hybrids quails were equally assigned to both parental species, with assignment probabilities of 30 % and 21 % to common and Japanese quails, respectively. These patterns can be seen in Fig. 4B where12 and 10 hybrids are identified by STRUCTURE as having a higher contribution from common and Japanese quail, respectively. The STRUCTURE analysis also identified five common and six Japanese quails as potentially admixed that were assigned to the other species with a greater probability by the assignment approach.

Discussion

The analysis of the microsatellite and mtDNA data clearly showed a higher level of genetic diversity in *C. coturnix* samples compared to *C. japonica*. This is not surprising as we are comparing wild populations with introduced individuals that are likely to represent only part of the original *C. japonica* diversity. Also, the fact that both hybrids and Japanese quails were captive animals and coming both from a single farm may have contributed to this low diversity. Comparing our results with those of B a r i l a n i et al. (2005) it appears that they found much higher H_e values (H_e~0.82) in *C. coturnix* populations, however these authors only provided the heterozygocity values obtained by pooling individuals from Spain, France, and Italy. When H_o values are considered the two studies appear more comparable as these authors found values around H_o~0.55. It thus appears that their higher values are most likely due to a Wahlund effect generated by the pooling of differentiated populations. This is consistent with our findings in which the difference between H_e and H_o is very small in the *C. coturnix* natural populations, indicating no significant departure from Hardy-Weinberg equilibrium.

The Northern common quails showed higher levels of genetic diversity in cyt *b* when compared to the Southern population. These differences were also found in microsatellite data although not significant. This could be related to the fact that the Southern samples are made of resident populations whereas the Northern samples are both from resident and migrating populations C a b r a l et al. (2005). Migrating individuals may have introduced new alleles through gene flow, contributing to the increase of variability in Northern common quails. However the data are too limited to make a strong claim at this stage.

The level of genetic differentiation between *C. coturnix* and *C. japonica* was quite high with both mtDNA and microsatellites. However, as discussed below, it was with mtDNA that the two species were most easily identified as they shared no haplotypes. Compared to the study of B a r i l a n i et al. (2005), we found a much higher level of genetic differentiation using microsatellites: we found a F_{ST} of 0.18 against 0.05 in their study. This may be caused by the fact that they pooled all *C. coturnix* samples together. Also, if their *C. japonica* were indeed hybrids, as they suggested that would fit with the much lower F_{ST} value that we found when hybrids were compared to both species (F_{ST} on the order 0.07). The fact that all the hybrids had *C. japonica* mtDNA haplotypes strongly suggests that hybridization is not symmetrical, involving mostly (if not only) *C. japonica* females and *C. coturnix* males. These results are in agreement with studies on mating call discrimination of female quail. Previous studies have shown that common quail females responded more to calls from conspecific males than to mating calls from hybrids and Japanese male quails. On the contrary females of Japanese quail did not show such selectivity (D e r é g n a u c o u r t & G u y o m a r c h 2003). These authors suggested that the domestication of Japanese quail may have led to an increased sexual activity, making *C. japonica* females less selective

and more receptive to mating. This has two different consequences. First the artificial production of hybrids in captivity is more likely to use Japanese females, as they are easier to manipulate. Second, hybridization in the wild might be easier to detect if *C. coturnix* females do not reproduce with *C. japonica* and hybrids. At this stage it is difficult to provide a firm conclusion but our admixture and assignment analyses provide some worrying results. Indeed, both these approaches identified common quail individuals that were most likely assigned to the Japanese quail group. We find two reasons for these results. First these individuals may correspond to offsprings from crosses between wild *C. coturnix* females and introduced *C. japonica* or hybrid males. Second, since Japanese and common quail are taxonomically close species, may present similar allelic frequencies and shared alleles and thus more loci would be needed to separate both species. It may be worth noting that introgression of Japanese quail has been reported in wild common quails from Spain and France (B a r i l a n i et al. 2005) but these authors did not observed admixture in their Portuguese samples. Our results showed that cyt *b* may be a very promising gene to detect hybrids, as long as hybridization remains asymmetrical and that male hybrids do not mate with female common quails. However, these results should be verified in future studies using common quails from other regions and Japanese quails from several farms and if possible from pure lines. In the wild, *C. coturnix* females in the absence of *C. coturnix* males can, in principle, breed with *C. japonica*, or hybrids. In this case the identification of hybrids requires besides mtDNA, information about nuclear loci. The set of microsatellite loci used in this study did not allow the detection of such events with high certainty. Therefore, it is thus urgent to provide means to develop new nuclear markers. Indeed, the study of B a r i l a n i et al. (2005) demonstrated higher levels of admixture in other European samples, and we might be able to limit introgression in Portugal, if actions are rapidly taken. Currently, Portugal forbids releases of any kind of quails for restocking. While this seems to have had a positive effect our results demonstrated that hybrids were produced in a farm selling them as common quail. Also, the fact that in Spain and France releases for restocking are allowed (D e r é g n a u c o u r t et al. 2005a) and that many hybrids were detected by B a r i l a n i et al. (2005) is alarming. Indeed, D e r é g n a u c o u r t et al. (2005b), detected migratory behavior in hybrid quails, which combined with the diminishing of long-migratory phenotypes of common quail, may affect seriously the natural population of this species, across borders.

This is thus urgent since several are the examples of endemic bird species which have suffered a severe population decline due to the introduction of exotic species and subsequent hybridization and introgression: the white-headed threatened by the introduction of the North American ruddy duck (M u ñ o z - F u e n t e s et al. 2007); the American black duck populations by the introduction of mallards (M a n k et al. 2004). Furthermore, some examples have been reported in other galliform game species like the rock and the red-legged partridges threatened by the introduction of chukars (B a r a t t i et al. 2004, B a r i l a n i et al. 2007).

The unequivocal identification of hybrids requires the study of mtDNA haplotypes and the analysis of biparental inherited nuclear markers. Due to the limited reliability of the analyzed set of nuclear markers to detect hybrids with wild common quail maternal lineage we advise the maintenance of the current legal protection and also the application of the same measures to all the other countries where *C. coturnix* has its natural distribution. In parallel, the use of more nuclear loci and samples of individuals identified according to calling patterns are highly recommended for future studies.

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