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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

New single nucleotide polymorphisms in *Alectoris* identified using chicken genome information allow *Alectoris* introgression detection

N. SEVANE, O. CORTÉS, D. GARCÍA, J. CAÑÓN and S. DUNNER
Department of Animal Production, Veterinary Faculty, Universidad Complutense, 28040 Madrid, Spain

Abstract

Using the chicken genome, 114 polymorphisms (109 SNPs and 5 INDELs) were identified in the *Alectoris* genus by polymerase chain reaction–single strand conformation polymorphism. Using these, a panel of SNPs is described, which allows easy detection of introgression of *Alectoris chukar* in wild *Alectoris rufa* populations, when used with a primer extension protocol. The selected polymorphisms were genotyped and their allelic frequencies estimated on 98 *A. rufa* partridges sampled from nonrestocking Spanish areas, and 63 *A. chukar* partridges from Greek and Spanish farms. Power calculations to determine an optimum subset of markers for a given significance level were performed.

Keywords: hybridization, partridge, primer extension, SNPs

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The main *Alectoris* species of the Mediterranean area are the red-legged partridge (*Alectoris rufa*), the rock partridge (*Alectoris graeca*), and the chukar partridge (*Alectoris chukar*), which show a small overlapping area (Johnsgard 1988). As a consequence, natural interbreeding between A. *rufa* and *A. graeca* (Bernard-Laurent 1984) and *A. graeca* and *A. chukar* (Dragoev 1974) can occur, but hybridization between *A. rufa* and *A. chukar* partridges should not. However, several studies have recorded cases of artificial genetic pollution of *A. rufa* and *A. graeca* by *A. chukar* (Randi *et al.* 2003; Barilani *et al.* 2006; Barbanera *et al.* 2007). *Alectoris* genus includes species with important cynegetic characteristics in European countries (Vargas *et al.* 2006) and, as wild partridge populations decrease (Negro *et al.* 2001; González 2004), numerous hunting areas across the countries are periodically reinforced with millions of captive-bred individuals (Negro *et al.* 2001). These captive individuals are the result of wild *A. rufa* and non-native species that have a better growth rate and adaptation to captivity as a result of artificial selection like *A. chukar* (Baratti *et al.* 2004; Barbanera *et al.* 2005), and uncontrolled restocking of hybrids may lead to a widespread introgression of foreign species in locally adapted partridge species (Allendorf *et al.* 2001; Barilani *et al.* 2007; Barbanera *et al.* 2009). Identification of hybrids by phenotypic characteristics (e.g. plumage) appears to be difficult beyond the first cross generation (Negro *et al.* 2001; Barilani *et al.* 2007). However, the detection of these advanced hybrids is crucial to avoid introgression by removal of hybrids or by a captive-breeding programme that allows strict control of farming and restocking with captive-reared birds (Allendorf *et al.* 2001). Although some efforts have led to the development of microsatellites (Baratti *et al.* 2004; González *et al.* 2005) or randomly amplified polymorphic DNA (RAPD) markers (Cortés *et al.* 2001; Negro *et al.* 2001), the identification of SNPs in the *Alectoris* genome is crucial for the easy analysis of large amounts of animals in a short time (Sanchez *et al.* 2006), because of the biallelic condition of these markers, which makes them easy to validate and to genotype with high-throughput technologies. The difficulty of developing a hybridization technique based on SNPs in the *Alectoris* species lies in the fact that there is rather scarce information about the partridge genome. To be able to use a better alternative to STR or RAPD for *Alectoris* hybrid identification, here we identify 109 SNPs and five INDELs located in 35 different genes in the partridge genome by polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) of fragments chosen on the basis of the *Gallus gallus* genome. *Alectoris rufa* and *A. chukar* samples were used to detect polymorphism and to estimate subsequent allele

Correspondence: S. Dunner, Fax: +34 91 3943772; E-mail: dunner@vet.ucm.es

frequencies. Although some commercial applications are available (e.g. SNPlex or Veracode), which allow screening of a few mutations (a minimum of 48) in a reduced number of individuals, here we have developed a medium-throughput method for which we chose the 23 most informative polymorphisms of the 114 genomic polymorphisms, together with one located in the mitochondrial genome. Several PCR multiplexes were built and resolved in an SNP detection system based on two sets of primer extension (PE) reactions, in which an unlabeled primer that immediately anneals 5′ to the relevant SNP is extended with a single fluorochrome-labelled dideoxynucleotide (ddNTP) complementary to the template strand at the site of interest (Sokolov 1989), with 11 and 13 markers analysed in two capillary electrophoresis runs. Theoretical allelic frequencies of A. *rufa* – *A. chukar* hybrids belonging to successive backcrossed offspring to *A. rufa* were estimated and used to calculate powers and Type-I error probabilities.

For SNP detection, we defined primers with the Primer 3 program v.0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) from the chicken sequence for 110 highly conserved genes (Tables 1 and S1) in multiple species including mammals (Aitken *et al.* 2004) and obtained from GenBank®. These were used to amplify DNA extracted from an initial reference panel consisting of seven *A. rufa* partridges from different wild areas of Spain, which had historically never been restocked, and two *A. chukar* partridges from a Greek farm. The polymorphisms obtained were validated on 161 samples (98 *A. rufa* from wild Spanish areas historically never restocked, and 63 *A. chukar* from Spanish and Greek farms). All individuals were previously checked for purity through STR, RAPD and mitochondrial markers (data not shown). The latter sampling was performed on farms and commercial meat markets based on the consideration that hybridization occurs with farmed animals and not with wild ones. PCR reactions were composed of 0.75 mM $MgCl_2$, 0.25 U of Taq Polymerase (Biotools), 0.3 mM dNTPs, 0.5 μM of each primer and 10 ng of DNA in a total volume of 10 μL. To select the optimal annealing temperature of each primer (Table 1 and S1), we started with a gradient cycle programme ranging from 53 to 63 °C. Reactions started at 94 °C for 4 min followed by 34 cycles of 50 s at 94 °C, 50 s at six different annealing temperatures (53, 54.7, 57.3, 59, 61.5 and 63 °C), 50 s at 72 °C, and final extension at 72 °C for 10 min. PCR fragments were visualized in 1.5% ethidium bromide-stained agarose gels.

The PCR products showing a strong band at the highest annealing temperature were subjected to SSCP electrophoresis in 16% acrylamide-bisacrylamide 29:1 gels, using the technique described by Barroso *et al.* (1998), and silver stained, following the procedure described by Bassam *et al.* (1991). Polymorphic bands were extracted from gels, purified (High Pure PCR Product Purification Kit; Roche) and sequenced [Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems)] in an ABI3130 to identify the polymorphism.

Mitochondrial polymorphisms (ss107795934-39) were detected by partially sequencing the D-loop fragment (from 16731 to 829 nt.) (Desjardins & Morais 1990), using primers 5′-AGGACTACGGCTTGAAAAGC-3′ and 5′-TATGTCCGACAAGCATTCAC-3′. The sequences from 36 non-restocked *A. rufa* and 21 farm *A. chukar* partridges were aligned with ClustalW (Thompson *et al.* 1994) and polymorphism was inferred.

Twenty-four target sequences (23 genomic and 1 mitochondrial DNA polymorphisms) showing clear discrimination were chosen and amplified in two multiplex reactions, one 11-plex (Multiplex 1) and one 13-plex (Multiplex 2). The SNP multimix for each amplification reaction consisted of oligonucleotide primers at varying concentrations ranging between 0.5 and 1 μM (Table 1), 1.25 μL of QIAGEN® Multiplex PCR (Izasa, Spain) and 10 ng of DNA with a final volume of 3 μL. The thermal cycling consisted of a first denaturation step at 95 °C for 15 min followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min 30 s, and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min.

PCR primers and unincorporated dNTPs were removed by incubating 2.5 μL of PCR product at 37 °C for 15 min with 5 U of Exonuclease I (ExoI) and 1 U of Shrimp alkaline phosphatase (SAP) (USB Corporation, Germany), followed by enzyme inactivation through heating at 80 °C for 30 min.

Following Sanchez *et al.* (2003) guidelines, different lengths of PE primers ranging from 22 to 70 bases were designed. Above 44 nt length a variable number of nucleotides and/or a neutral oligonucleotide region *TAAACTAGGTGCCACGTCGTGAAAGTCTGACAA* were totally or partially added at the 5′ end to generate longer products following a test with BLAST to avoid any match with other *Alectoris* sequences present in the multiplex. All PE primers were desalted and purchased from INVITROGEN™ (Groningen, The Netherlands). The PE reaction was carried out in a 5 μL final volume containing 0.75 mM $MgCl_2$, different concentrations of PE primers (Table 1), 0.2 U of Thermo Sequenase (Amersham Biosciences Inc.), 225 μM ddNTPs (Perkin Elmer) and 2 μL of cleaned PCR multiplex product. The thermal cycling programme consisted of 1 min at 96 °C followed by 34 cycles of 96 °C for 15 s, 58 °C for 15 s, and 60 °C for 15 s. Unincorporated ddNTPs were degraded by adding 0.33 U of SAP to 5 μL of the extension products, diluted 1:2 with Dilution Buffer. Two microlitre of multiplex cleaned extension product was added to 15 μL Hi-Di™

Table 1. Genes tested in *Alectoris* genus based on *G.gallus* gene information, polymorphisms identified (SNP and INDEL) with flanking sequence in *Alectoris* genus. For the chosen genes, multiplex and primer extension primers based on *Alectoris* sequences are shown

GenBank accession *G. gallus*[1]	Gene *G. gallus*[2]	*G. gallus* primers (5′–>3′)[3]	G. gallus length[4]	Annealing Temp (°C)	GenBank Acc.No *A. Rufa*[5]	GenBank Acc.No *A.chukar*[6]	Number of SNPs/ INDELs	dbSNPs accession[7]
NW_001471428	AGC1	F_CAGAGCAGTTCACCTTCCAA	210	59	FI166058	FI166021	2	ss105106807
		R_GCTGGTAGATGGTCCTCACA						ss119759578
NW_001488884	ALDOB	F_TCAGACATTGCTCAGAGGAT	220	59	FI166060	FI166023	3	ss105106809-10
		R_TGTTCTTCCACTTTAGATGC						ss119994664
NW_001471519	ARSA1	F_CCTCCACTCAGCACTTCTAC	229	61	FI166061	FI166024	1	ss105106811
		R_TAAGCCTTGTTTCCCTTTAG						
NW_001471532	CFRT	F_CGGTTTACCCTGACATTTTA	293	55	FI166062	FI166025	1	ss105106812
		R_ACTTGTTCTTTTCCCTCTCC						
NW_001471572	CG3869_1	F_TGCCCTCTACTGGTCTAAGT	260	55	FI166063	FI166026	5	ss105106813
		R_GACTCTCATCCTTGTGGGTA						ss119994665-68
NW_001471673	CLU	F_CAACCCTAATACGAGACAGC	350	55	FI166064	FI166027	2	ss105106814
		R_ACTTCACCGACAAACATTTC						ss119994669
NW_001471449	GMCSF	F_GCAGTAAGTTCCTCTTGGTG	232	55	FI166065	FI166028	2	ss105106815-16
		R_AATACCTTTCCCCAGATTTC						
NW_001471503	GSN	F_ATCGTGCCTACCAGAAACTA	320	55	FI166066	FI166029	8	ss105106817-23
		R_CACCTCCTTACCATCCATAA						ss105106881
NW_001471556	HBB	F_ACACCTTCTCCCAACTGTC	266	59	FI166067	FI166030	8	ss105106824-31
		R_CCCTTCATTCCTTTCTCACT						
NW_001471737	LAMC1	F_CTCTTACTGACCCTGTGCTC	273	57	FI166068	FI166031	2	ss105106832
		R_CCTGGTGATTCTATGATGCT						ss119994670
NW_001471681	MNK	F_CTTGTGCCTCAGGTAAAGTC	237	61	FI166069	FI166032	2	ss105106833
		R_ATCTGTAGGTTGGGTTGTTG						ss119993302
NW_001471508	MPO	F_CCCACCAAGTATGTCACTCT	234	53	FI166070	FI166033	1	ss105106834
		R_TGAATCCTACCCAGTTTTTG						
NW_001471681	NID	F_GCTCTTCTGCTCAGGACTT	324	59	FI166071	FI166034	4	ss105106835-37
		R_CCCAGTTCACTTCTCTTCTG						ss119994671
NW_001471534	OTC	F_CCAGTCTCTTCCTTGTTTTG	228	57	FI166073	FI166036	3	ss105106842
		R_TTAGCCTTATTTGCCACAGT						ss119994672-73
NW_001471688	OXT	F_TATCCCACAGAGAGCACTTC	332	55	FI166074	FI166037	2	ss105106843-44
		R_CAACCTCAGTTTTTCCTCAG						
NW_001488875	PDE6B	F_TTTATCCACCAAATAGTCCAA	221	55	FI166075	FI166038	3	ss105106845
		R_GCCATTCAGGTTTATGTGAT						ss119759589
								ss119759592
NW_001471423	PKM2	F_CTTGGGAGGTGACAGACAT	303	57	FI166077	FI166040	3	ss105106847-48
		R_CAATAGGTTCAGCAGGGTAG						ss119994675
NW_001471518	PTHLH	F_GCAGACTGTGAGTTACCTGAA	344	59	FI166079	FI166042	6	ss105106851-54
		R_ATGGCATTTCGTATTACCTG						ss107938223-24
NW_001471714	RET	F_AGTGGTTCAGTCCAGTTCCA	249	57	FI166082	FI166045	6	ss105106862-65
		R_TACACCTCCCGTAATCCTCA						ss119994676-77

Table 1. *Continued*

GenBank accession *G. gallus*[1]	Gene *G. gallus*[2]	*G. gallus* primers (5′–>3′)[3]	G. gallus length[4]	Annealing Temp (°C)	GenBank Acc.No *A. Rufa*[5]	GenBank Acc.No *A.chukar*[6]	Number of SNPs/ INDELs	dbSNPs accession[7]
NW_001471675	SPTBN1	F_GCTTCTTGTTCCCACTCTCA R_TTCCCGACTGAATCAATGTT	249	53	FI166084	FI166047	2	ss105106870-71
NW_001471449	PCBD2	F_GGATTTCAGTGAGGTCAGGA R_CAGTGCTTTGGTCTGTGTGT	293	57	FI166085	FI166048	4	ss105106872-73 ss119759582 ss119759586
NW_001471710	THBS1	F_GTGAGGGTGAAGCAAGAGAA R_ACAGAATGGCAGCCGTAAT	267	61	FI166086	FI166049	1	ss105106874
NW_001471733	TNFAIP6	F_CTGTGTTGAGCAGGAAGAGA R_AGCGTGAGCCTACCCTAAAT	350	59	FI166087	FI166050	9	ss105106875-79 ss105106882-84 ss119993306
_	_	_	_	_	AJ586225	_	6	ss107795934-39
NW_001471744	AHCY	F_TAACTGAGCACACAGCAAAC R_ACAGAAATGGGATAAACTGG	345	59	FI166059	FI166022	1	ss105106808
NW_001471722	OAT	F_TTTCCAAGGATACACCAGTC R_ACTCTTCTGCTTTTCAACCA	268	55	FI166072	FI166035	4	ss105106838-41
NW_001472038	PFKM	F_GCACCTACTGGAAGTCTGTC R_CCTGCTGCTCTATTTCAAAG	225	59	FI166076	FI166039	6	ss105106846 ss119994674 ss119994678 ss119993303-05
NW_001471715	PLAU	F_CCCAACATAGAGGTAAGCAT R_GCTGAAGAAGCGATAGGTAA	235	55	FI166078	FI166041	2	ss105106849-50
NW_001471454	PVALB	F_CCATCACTGACATCCTTTCT R_GCTCTTACTGCCACTCTGTT	257	55	FI166080	FI166043	2	ss105106855-56
NW_001471554	RB1	F_TGTCTGGCTTTTCCTCTGTC R_CAGTCCCTGCCCTATCTTTT	329	55	FI166081	FI166044	5	ss105106857-61
NW_001471668	SOD2	F_TCTGACAGTCGTTTGACCTG R_AACCTACTCCCCAAGACCAC	336	55	FI166083	FI166046	4	ss105106866-69
NW_001471686	DCUN1D4	F_TAGTCTGATGGGTTGAGTCC R_TGGAAACTGAGATGTTAGGAA	224	55	FI166088	FI166051	1	ss105106880
NW_001471572	CG3869_2	F_CACTGCTGACTGGGAGATAC R_GGATTATTTCCTGATGTCCA	324	59	FI569716	_	1	ss119994681
NW_001471572	CG3869_4	F_CATCAGCATTTATCTGCCAGT R_GGGAGGATTGTTTCTGTGTG	289	59	FI569715	_	2	ss119994679-80
NW_001471681	F9	F_CTACCTGAGCCTTGAAACAC R_TTTGTGGGTGAAGTTCTGA	244	55	FI569711	_	1	ss119994682
NW_001471695	FSHB	F_TTCATACACAACCTCCTTGA R_TTGATTTCAGTGGGATTTTC	200	55	FI569712	_	1	ss119994683
NW_001471633	GLB1	F_TACAGCCACAGAAACCATAG R_TGGGAAGAAGGTAAGAAGGT	212	61	FI569713	_	1	ss119994684
NW_001471508	GUSB	F_TTTTAGTGCGGTATGACCTT R_ATGTAACCAAGCAGCAGAAT	300	55	FI569714	_	3	ss119994685-87

Table 1. *Continued*

GenBank accession *G. gallus*[1]	Gene *G. gallus*[2]	Multiplex primers (5′–> 3′)[8]	Final primer conc (μM)[9]	Multiplex[10]	dbSNPs accession[11]	Poly (dNTP)[12]	Neutral Sequence (5′ –> 3′)[13]	Target specific sequence[14]	Mutation[15]	Final primer conc (μM)[16]	Primer size[17]
NW_001471428	AGC1	F_CAGAGCAGTTCACCTTCCAA R_GCTGGTAGATGGTCCTCACA	0.5	1	ss105106807	None	None	F_GCGGTACCCCATT GTGAGCCCCCGGCCC GCCTGTGG	G/C	0.5	36
NW_001488884	ALDOB	F_TCAGACATTGCTCAGAGGAT R_CACTTTAGATGCAGCTCTGG	1	2	ss105106809	None	None	R_CACTTTAGATGCAG CTCTGGGT	A/G	1	22
NW_001471519	ARSA1	F_CCTCCACTCAGCACTTCTAC R_GAGGTCCTGTACCCCAGTAA	0.5	1	ss105106811	None	None	F_CATCGGGCTCCTGT CCATATCC	A/G	0.5	22
NW_001471532	CFRT	F_CATCTAGGTTCTGTCTGTTTTG R_TTTAGGACTGAAGAAAGTGCAT	0.5	2	ss105106812	None	None	F_CAGAAATAAGACAT TTATAAGATTTTACTTT GGAAGAAAG	T/C	0.5	30
NW_001471572	CG3869_1	F_TGCCCTCTACTGGTCTAAGT R_GACTCTCATCCTTGTGGGTA	1	1	ss105106813	12(dC)	TAGACAGTAG AGCAGATAGA CC	R_TAGATGACTTGGAT GACTTCTGGAAG	A/G	1	60
NW_001471673	CLU	F_CAACCCTAATACGAGACAGC R_CAACACCAACTCATCCCTCT	0.5	1	ss105106814	None	None	F_ATGGGTTTTGGGGT GGTTTTTGCC	C/T	0.5	24
NW_001471449	GMCSF	F_GCAGTAAGTTCCTCTTGGTG R_TCTGGAGCAAATGCAGTG	0.5	1	ss105106815	None	None	F_TTGCCTCAGCTCCC AGATTTCTTTTCTG	C/T	0.5	28
NW_001471503	GSN	F_ATCGTGCCTACCAGAAACTA R_AGCAGTGGGAGCTGTACTTC	0.5	2	ss105106817	12(dC)+ 4(dCT)	TAGACAGTAG AGCAGATA	F_CCCTTTCCTTTACC ACTGCA	A/T	0.5	58
NW_001471556	HBB	F_ACCTTCTCCCAACTGTCTGA R_CCCTTCATTCCTTTCTCACT	1	1	ss105106824	None	GAGCAGATAG ACCAAGA	F_GCTGTGCTCTTGG CCAGGTCCCATCCCA ACG	T/C	1	48
NW_001471737	LAMC1	F_CTCTTACTGACCCTGTGCTC R_CCTGGTGATTCTATGATGCT	0.5	2	ss105106832	None	None	F_GCAGCTTCACCTGT GTTCTCTGTA	T/C	0.5	24
NW_001471681	MNK	F_CTTGTGCCTCAGGTAAAGTC R_TGGGTTGTTGGTTGGACT	1	1	ss105106833	None	None	R_GGTTGGGTTGTTG GTTGGACTTGATGATC GTGGAGG	T/A	1	36
NW_001471508	MPO	F_CCCACCAAGTATGTCACTCT R_TGAATCCTACCCAGTTTTTG	0.5	2	ss105106834	None	None	R_ATCGTCGACCTTAT TTGAAGTGTGAATGCA CAA	A/T	0.5	33
NW_001471681	NID	F_GCTCTTCTGCTCAGGACTT R_GCTGTGAACTTGCACATAACAA	0.5	1	ss105106835	None	None	F_TGGAGGGGATGTT TTCTGGTAATCCCCAG AGCTA	T/C	0.5	34
NW_001471534	OTC	F_CCAGTCTCTTCCTTGTTTTG R_GGTGGATTAGCATGGCATA	0.5	2	ss105106842	None	None	F_TCAGGGCGTGATTA AAACAGAAATGCAGA GAA	A/T	0.5	32
NW_001471688	OXT	F_TATCCCACAGAGAGCACTTC R_CAAGATCCCTTCCTGTTGG	1	1	ss105106843	None	None	F_TTTTCTGCATATGCTGC AGATAAACAAT	T/G	1	28

Table 1. *Continued*

GenBank accession *G. gallus*[1]	Gene *G. gallus*[2]	Multiplex primers (5′–> 3′)[8]	Final primer conc (μM)[9]	Multiplex[10]	dbSNPs accession[11]	Poly (dNTP)[12]	Neutral Sequence (5′ –> 3′)[13]	Target specific sequence[14]	Mutation[15]	Final primer conc (μM)[16]	Primer size[17]
NW_001488875	PDE6B	F_TTTATCCACCAAATAGTCCAA R_TCTGAGGAGTATGAGGGGTAAT	0.5	2	ss105106845	6(dCT)	None	F_CTCTTTCAGCAGAC ATAGTGCAGTGCAATA CTGA	C/G	0.5	46
NW_001471423	PKM2	F_CAATAGGTTCAGCAGGGTAG R_CTGCCTCTTCCTGTTCTACA	0.5	2	ss105106847	None	None	F_GCCTCTTCCTGTTC TACACAGCACCTCTG CCAGCAG	C/A	0.5	29
NW_001471518	PTHLH	F_GCAGACTGTGAGTTACCTGAA R_CTGCAGCAGCCCGATGAAA	1	1	ss105106851	None	None	R_GCCCAAACCGTGC CCAGAGCACCTGAGG CAGG	G/C	1	32
NW_001471714	RET	F_AGTGGTTCAGTCCAGTTCCA R_TACACCTCCCGTAATCCTCA	0.5	2	ss105106862	15(dTC)	TAGACAGTAG AGCAGATAGA	F_GTGGTGTGTACAAT TAAATA	T/C	0.5	70
NW_001471675	SPTBN1	F_GCTTCTTGTTCCCACTCTCA R_TTCCCGACTGAATCAATGTT	1	2	ss105106870	18(dCT)	AGACCAAGA	R_GAGAGGTGCAAGC TAGAAAGGC	A/G	1	67
NW_001471449	PCBD2	F_GGATTTCAGTGAGGTCAGGA R_GTACACAGACCACAGGAAGG	0.5	2	ss105106872	None	None	F_AATCACCACCCAGA ATGGTTTAACGTCTAC AGCAAGGTAA	T/C	0.5	40
NW_001471710	THBS1	F_GTGAGGGTGAAGCAAGAGAA R_GGCTCCACAGAAAACTCG	0.5	1	ss105106874	None	None	F_GCTGCCATGTACTG GACTTCTAGTGGTCTT TAAAC	G/A	0.5	35
NW_001471733	TNFAIP6	F_CTGTGTTGAGCAGGAAGAGA R_AGCGTGAGCCTACCCTAAAT	0.5	2	ss105106875	None	None	R_GTGTCCCAGAGCT GTGGATGTGGCACTG	C/T	0.5	28
_	_	F_AGGACTACGGCTTGAAAAGC R_TATGTCCGACAAGCATTCAC	0.5	2	ss107795934	None	TAAACTAGGT GCCACGTCGTG AAAGTCTGACAA	R_TACGACTGGCATAA CCA	A/G	0.5	51

[1]GenBank Accession numbers for *Gallus_gallus-2.1* sequences used for primer design. [2]*Gallus_gallus-2.1* gene symbols used for primer design. [3]Primer sequence based on *Gallus_gallus-2.1* gene information. [4]Sequence lengths according to *Gallus_gallus-2.1* genome information. [5]GenBank Accession numbers for *A. rufa* sequences obtained in this study. [6]GenBank Accession numbers for *A. chukar* sequences obtained in this study. [7]dbSNPs accession number for the polymorphisms obtained in this study. [8]Primer sequence based on *Alectoris* sequences for multiplexing. [9]Final primer concentration (μM) in PCR multiplex systems. [10]Multiplex that included each amplicon. [11]dbSNP accession number of the interrogated polymorphisms. [12]Variable number of nucleotides added at the 5′ end to allow longer runs. [13]Neutral oligonucleotide region added at the 5′ end to allow longer runs. [14]PE primer sequence based on *Alectoris* sequences. Detection orientations are indicated by F (forward) and R (reverse). [15]Polymorphism interrogated in each *Alectoris* sequence. [16]Final primer concentration (μM) in primer extension reactions. [17]Complete PE primer size including poly(dNTP) region, neutral sequence and target specific sequence.

Formamide (Applied Biosystems) and 0.25 μL of GeneScan™-120 LIZ™ internal size standard (Applied Biosystems), before injection in an ABI 3130 sequencer using POP-7® (Applied Biosystems). Data were analysed using GeneMapper v4.0 (Applied Biosystems).

Allele frequencies were calculated and used to perform analytical Type-I error probability and power calculations for the 23 nuclear markers, and to select the most powerful subset for a given significance level, when testing $H_0 \equiv$ *A. rufa* vs. $H_1 \equiv$ hybrid from the *k*th backcross. In this test, an individual is rejected, i.e. identified as a hybrid, when the number of markers showing the presence of their so-called chukar allele exceeds a given threshold. This is calculated to keep the Type-I error probability below a certain level, set here to 0.1 to allow for greater power in the detection of hybrids.

Starting from 112 sequences belonging to 110 different *G. gallus* nuclear genes, a total of 109 SNPs and five INDELs (dbSNP numbers ss105106807–84; ss107938223–24; ss119759578; ss119759582; ss119759586; ss119759589; ss119759592; ss119994664–87; ss119993302–06) located in 35 different genes (corresponding to chicken genes updated in *Alectoris* and corresponding to GenBank Accession numbers FI166021–FI166094; FI569711–FI569716) were identified in the partridge genome (Table 1). The search for SNPs in the rest of the genes (75) was unsuccessful, either because no polymorphism was found through SSCP (38 genes), because of sequencing being impossible to perform although polymorphisms appeared in SSCP (6 genes), or because chicken based primers mismatched the *Alectoris* sequence (31 genes) (Table S1). For the development of a medium throughput method for *Alectoris* introgression detection by multiplex-PE, we chose one SNP at each gene sequence (Table 1) with reasonably acceptable behaviour when multiplexed, avoiding expected repetitive information from linked markers, and, whenever possible, with fixed alleles in both species. As introgression in *A. rufa* is

Table 2 Allele frequencies of 24 SNPs in *Alectoris rufa* and *Alectoris chukar* and their chromosomal allocation and relative position in the *Gallus gallus* genome

	Position in *G. gallus*					Allele frequencies				
						A. rufa		*A. chukar*		
SNP locus	Chromosome[1]	Position[2]	Distance[3]	dbSNPs accession[4]	Mutation (Allele 1/ Allele 2)[5]	Allele 1	Allele 2	Allele 1	Allele 2	Reproducibility[6]
AGC1	10	14.7	13.7	ss105106807	G/C	0.990	0.010	0.071	0.929	98
ALDOB	Z	63.7	11.3	ss105106809	A/G	0.552	0.448	0.000	1.000	95
ARSA1	1	0.1	74.7	ss105106811	A/G	0.845	0.155	0.040	0.960	96
CFRT	1	101.4	15.0	ss105106812	T/C	0.958	0.042	0.058	0.942	98
CG3869_1	21	5.7	—	ss105106813	A/G	0.918	0.082	0.085	0.915	100
CLU	3	108.0	105.3	ss105106814	C/T	0.371	0.629	0.000	1.000	95
GMCSF	13	17.2	1.1	ss105106815	C/T	0.773	0.227	0.008	0.992	99
GSN	17	9.1	—	ss105106817	A/T	0.907	0.093	0.025	0.975	97
HBB	1	199.4	83.0	ss105106824	T/C	1.000	0.000	0.210	0.790	100
LAMC1	8	7.8	—	ss105106832	T/C	0.903	0.097	0.038	0.962	100
MNK	4	13.0	8.6	ss105106833	T/A	0.990	0.010	0.524	0.476	100
MPO	19	0.4	—	ss105106834	A/T	0.947	0.053	0.000	1.000	100
NID	4	4.4	8.6	ss105106835	T/C	1.000	0.000	0.302	0.698	100
OTC	1	116.5	15.0	ss105106842	A/T	0.500	0.500	0.355	0.645	95
OXT	4	92.0	79.0	ss105106843	T/G	0.825	0.175	0.024	0.976	97
PDE6B	Z	52.4	11.3	ss105106845	C/G	0.929	0.071	0.009	0.991	100
PKM2	10	1.0	13.7	ss105106847	C/A	0.897	0.103	0.000	1.000	97
PTHLH	1	74.8	26.6	ss105106851	G/C	1.000	0.000	0.016	0.984	100
RET	6	5.9	—	ss105106862	T/C	1.000	0.000	0.740	0.260	98
SPTBN1	3	2.7	105.3	ss105106870	A/G	0.976	0.024	0.009	0.991	100
PCBD2	13	16.1	1.1	ss105106872	T/C	0.974	0.026	0.024	0.976	96
THBS1	5	31.9	—	ss105106874	G/A	0.964	0.036	0.008	0.992	100
TNFAIP6	7	36.8	—	ss105106875	C/T	0.933	0.067	0.025	0.975	96
Dloop SNP1-R	MT	—	—	ss107795934	A/G	1.000	0.000	0.000	1.000	100

[1]*Gallus gallus* chromosome in which the sequence obtained in this study matched when tested with BLAST. [2]Position on *G. gallus* chromosome expressed in Mb. [3]Distance (in Mb) from closest SNP used in multiplex. The symbol '—' means that no other SNP is allocated in the chromosome. [4]GenBank accession nº for SNP in *Alectoris*. [5]Polymorphism in *Alectoris* sequence. [6]Reproducibility percentage in duplicate PCRs for each sample.

mainly caused by *A. chukar*, 23 nuclear SNPs and one mitochondrial polymorphism (inferred by sequencing of D-loop region), resolved in two capillary runs of two PCR multiplex previously hybridized by primer extension (Sokolov 1989) were selected as the most powerful battery to detect introgression only by accounting for the presence of chukar alleles. Reproducibility was tested by performing PCR in duplicate for all samples. Those SNPs not showing at least 94% reproducibility were discarded, due either to a low primer affinity with the target sequence, or to the existence of some interaction adversely affecting PE primer binding.

Allele frequencies were calculated (Table 2) to perform analytical Type-I error probability and power calculations. The power of hybrid detection was calculated for a given significance level of $\alpha = 0.1$ and several degrees of backcrossing. First, the whole 23 nuclear marker battery was tested. Then five markers (ALDOB, CLU, GMCSF, MNK, and OTC) were excluded on the basis of their highly intermediate frequencies. Power analysis was performed again with the reduced battery of 18 markers, and finally an optimum subset of markers was chosen to maximize power by checking all the possible subsets of any size. This check discarded three of the 18 (ARSA, OXT, and PKM2, all of them showing reasonably extreme frequencies for the chukar allele, but not so extreme for the *A. rufa* allele), and thus the final set of 15 markers with maximum B3 (an F1 *A. rufa* × *A. chukar* backcrossed three consecutive times with *A. rufa*) detection power was established. This marker subset shows a detection power of 1; 0.99567; 0.84296, and 0.52355 for an F1 *A. rufa* × *A. chukar*, and three consecutive backcrosses with *A. rufa* B1, B2, and B3, respectively, with an associated Type-I error probability of 0.0984. No further backcross levels were checked because of the very low percentage of *A. chukar* present (3% in a B4 backcrossing, for example).

Evidence from isolated populations suggested that linkage disequilibrium extends to a few hundred kilobases (Collins *et al.* 2001). In our study, the shortest distance in *G. gallus* between two multiplexed SNPs (GMCSF vs. PCBD2) was 1.1 Mb, while the rest of the pair-wise distances among SNPs were >8 Mb (see Table 2). Consequently, the assumption of independence was reasonable with regard to physical linkage.

Although arrayed PE assays (Deshpande *et al.* 2005; Pullat & Metspalu 2008) are being developed with more recent state-of the art technology, the economic value of a partridge does not justify the application of expensive procedures. Our aim was to obtain a low-cost system that allowed analysis of a large number of individuals in a short time (Sanchez *et al.* 2003; Dixon *et al.* 2005). Primer extension is a simple, flexible and low-cost technique for fast genotyping of few SNPs in a few hundred individuals at a reasonable price with no need for an expensive infrastructure.

In conclusion, we show here that a domestic species, like *G. gallus*, can be used effectively to develop SNPs in a divergent genus, such as *Alectoris*. Moreover, the number of newly identified polymorphisms in the *Alectoris* genome and the simple and efficient SNP typing assay developed in the present study, compared with STRs and RAPDs, can be applied to the genetic control of reproductive-bred individuals in hunting areas and on farms before restocking, thus limiting any harm to wild populations.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Unsuccessful genes tested for polymorphism detection in *Alectoris* genus based on *Gallus gallus* gene information

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