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Rapid differentiation of *Alectoris rufa* L., 1758 and *Alectoris chukar* (Gray, 1830) (Galliformes: Phasianidae) by melting curve analysis of a parathyroid hormone gene SNP

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Resumen

Diferenciación rápida de Alectoris rufa *Linnaeus, 1758 y* Alectoris chukar *(Gray, 1830) (Galliformes: Phasianidae) mediante curvas de disociación de un SNP del gen de la hormona paratiroidea*

Los métodos actuales basados en ADN para identificar los niveles de introgresión en especies cercanas implican la manipulación posterior a la PCR, aumentando el tiempo y coste en el procesado de las muestras. El análisis de curvas de disociación es una técnica que utiliza cebadores especie-específicos. En este trabajo se demuestra que tal análisis resulta muy preciso para la diferenciación de individuos puros de *Alectoris chukar* y *A. rufa*, utilizando un SNP del gen de la hormona paratiroidea de una manera simple, rápida y eficiente. Se analizaron setenta y ocho muestras (*A. chukar*, n=15; *A. rufa*, n=63), y no se detectaron híbridos. Este método podría ser aplicado en políticas de gestión basadas en el control genético de las perdices reproducidas en granjas y utilizadas en repoblación u otras actividades cinegéticas.

Palabras clave: Perdices, Introgresión, Diferenciación de especies, Cebadores especie-específicos.

Abstract

Current DNA-based methods for identifying introgression levels of closely related species imply post-polymerase chain reaction manipulations that requires additional time and expenses for sample processing. Melting curves analysis is a technique that uses species-specific primers. Here we show this method to be highly accurate for the rapid differentiation of pure individual partridges of *Alectoris chukar* and *A. rufa* species, using a SNP of the parathyroid hormone gene in a simple, rapid and efficient way. Seventy eight individuals were analyzed, (*A. chukar*, n=15; *A. rufa*, n=63), and no hybrids were detected. This method should find wide use for the management polices based on strict genetic controls for partridge stocks reproduced in farms and used in restocking or other hunting activities.

Key words: Partridges, Introgression, Species differentiation, Species-specific primers.

Introduction

The red-legged partridge (*Alectoris rufa* L., 1758) is one of the most important small game animals in Europe, with a high socio-economic value (Martínez et al. 2002). Natural populations of this species have suffered a marked decline of 95% in its distribution area (Cramp & Simmons 1980, Aebischer & Lucio 1997), habitat destruction, interaction with other species and hunting activities being some of the reasons which could explain this decline (Blanco-Aguiar et al. 2003). In Spain, in 2010, around three millions partridges were hunted, and near two millions were released into the field (Annual Statistical Hunting, Ministry of Agriculture, Food and Environment, Government of Spain). Lack of scientific criteria when performing reintroductions and restocking has lead to the introduction of hybrid partridges in the field. In the last decades, *Alectoris chukar* (Gray, 1830) introgression in *A. rufa* genome has frequently been documented in the Iberian Peninsula (Días 1992, González et al. 2005, Henriques-Gil et al. 2005, Tejedor et al. 2007, Martínez-Fresno et al. 2008), France and Italy (Baratti et al. 2004, Barbanera et al. 2005, 2009, Barilani et al. 2007). *A. rufa* is endemic of southwestern Europe (Spain, Portugal, France, northwestern Italy, Elba and Corsica (Johnsgard 1988), whereas *A. chukar* is distributed from Bulgaria to northern India and from Mongolia to Manchuria (Madge & McGowan 2002). These species do not cross in nature as they do not share habitats and therefore hybridization is related to human management (McCarthy 2006, Randi 2008). Several papers have dealt with the introduction as a game bird of *A. chukar* in areas where *A. rufa* is naturally distributed, and with the genetic and fitness consequences of the intensive management (Barbanera et al. 2009, 2010, Casas et al. 2012, 2013) and the associated risk of genetic contamination in local populations (Casas et al. 2012).

Differentiating second generation hybrid individuals from the pure species at phenotypic level is difficult (Negro et al. 2001) and it is only possible using genetic techniques. Identifying admixed individuals is intended to assess the degree of introgressive hybridization and to evaluate possible biodiversity loss due to genetic homogenization (Blanco-Aguiar et al. 2008). Several studies have used microsatellites (Baratti et al. 2004, González et al. 2005) or SNPs (Sevane et al. 2010) to differentiate these species. Due to the economic value of partridge (2€/partridge), it would be convenient to obtain a low-cost method that permits genotyping numerous individuals in a short time (Dixon et al. 2005, Sánchez et al. 2003). The use of species-specific primers and melting curve analysis (MCA) has previously been used for polymorphism detection, including long deletions (Sangkitporn et al. 2003) and SNPs (Akey et al. 2001, Ye et al. 2010, Yu et al. 2005), and in the last years has also been used for species identification (Berry & Sarre 2007, Dalmasso et al. 2007, Kochan et al. 2008, Pietila et al. 2000, Yu et al. 2005), because it requires no post–PCR manipulation, among other advantages. PCR amplification was followed by a melting profile consisting on an initial annealing followed by a temperature ramping. The method makes use of SYBR Green, a double stranded DNA specific dye, which is monitored during the temperature cycles, showing a rapid loss of fluorescence when the DNA reaches the melting temperature (Tm). Melting curves depend on the DNA sequence, its GC content and its length. Different melting peaks are observed when the negative first derivative of fluorescence curves is represented with respect to temperature. Specific primers can be designed to obtain suitable PCR products that could be differentiated by their melting curves, when product temperature differences are closed to 2 °C (Rieri et al. 1997).

The aim of the present study is to evaluate the applicability of MCA to differentiate *A. chukar* from *A. rufa* partridges and putative hybrids. Thus, a set of species-specific primers were designed from a SNPs of the parathyroid hormone gene previously described by Sevane et al. (2010), that has a characteristic allele in each species (with a G in *A. chukar* and a C *in A. rufa*). This primer pair was tested to optimize a rapid, efficient and reasonably priced method based on real-time PCR and melting curve analysis and was applied to individuals of both species collected in the natural range of distribution.

Materials and methods

Samples

This work has been carried out in species without

legal protection (*A. chukar* and *A. rufa*). A total of 78 samples were collected in 2009 and 2010. Of them, 63 individuals were morphologically identified as *A. rufa* (Spain, n=58; France, n=5). Muscle tissue samples were provided by Spanish hunters from wild hunted animals (Albacete, n=14; Alicante, n=1; Cuenca, n=1; Granada, n=1, Jaen, n=1; Murcia, n=8;), feathers were collected from released partridges (Albacete, n=4; Burgos, n=2; Córdoba, n=1; Jaen, n=1; Murcia, n=4, Soria, n=1; Toledo, n=1) and also from captive birds from a farm that guarantees genetic purity of *A. rufa* individuals (Albacete, n=18), performing a non invasive sampling. We also included feathers from five farm-bred individuals from France. Additionally, muscle tissue from 15 individuals of *A. chukar* (Turkey; n=5 and Iran; n=10) were obtained from local markets. Biological tissues were immersed in a tube with 1,5 ml of absolute ethanol and stored at -20 ºC. DNA was extracted from feathers and muscle using the Invisorb® Spin Tissue Mini Kit according the manufacturer's instructions. DNA samples were stored at -20 ºC until analysis.

Marker selection and primer design

The parathyroid hormone (PTH) SNP (Sevane et al. 2010) was selected due to the diallelic condition of SNP markers that makes easier genotyping a large number of individuals (Sánchez et al. 2006). One common reverse (PTH-R-5'-cagctcacctcgaacaagcatgc-3') and two species-specific forward primers (PTH-FC-5'-caagcgtggctgtgggctg-3' and PTH-FR-5'-ggggggggggcaagcgtggctgtgggctc-3') were manually designed on the basis of the available parathyroid hormone SNP sequences (Genbank accession codes FI166042 and FI166079). Sequences were aligned with MEGA 4.0.2 (Tamura et al. 2007) in order to identify a short PCR fragment that included the variable position (G/C), located at the position 74 of the alignment. The differences between the two allele-specific forward primers consisted in the base that defined the SNP in the 3' end, and the addition of a 12 bp guanine tail in the 5' end of *A. rufa* specific primer, which resulted in different sizes of PCR products with varying Tms to facilitate greater resolution of fragments during MCA analysis (Fig. 1).

The theoretical Tm value of the amplicons were calculated by the method of the nearest neighbor (NM) (Breslauer et al. 1986) and with the basic method (BM) using the OligoCalc software (Kibbe 2007), in order to calculate the temperature differences between amplicons (*A. chukar* amplicon, 183pb: BM Tm; 87 ºC, NM Tm; 88.17 ºC and *A. rufa* amplicon, 195pb: BM Tm: 88.2 ºC, NM Tm: 88.34 ºC).

The effectiveness of the primers was confirmed by regular PCR reactions, trialing the pair of species-specific primers on positive controls of both species, analyzing products by 2% agarose gel electrophoresis. PCR conditions were modified until obtaining the optimal conditions.

q-PCR Amplification

The amplification, melting curve, and data analyses were run in a 7500 Real Time PCR System (Applied Biosystems). All reactions were carried out in a total volume of 12 µl. Each reaction mix contained 6 µl de Master mix SYBR Green (Applied Biosystems), 0.25 µmol L^{-1} each primer and 2 µl (≈10ng) template DNA. According to the suggestion made by Berry & Sarre (2007), several variables were considered to standardize the technique. We conducted melt curve assays on five individuals of each species to test the intraspecific variability, and we repeated the assay for the five examples of each species five times to evaluate individual repeatability. Additionally, to establish the optimal concentration of DNA, we ran assays on a dilution series of template DNA (1:5 and 1:10 with ≈10 µgm^{-1} as the initial concentration).

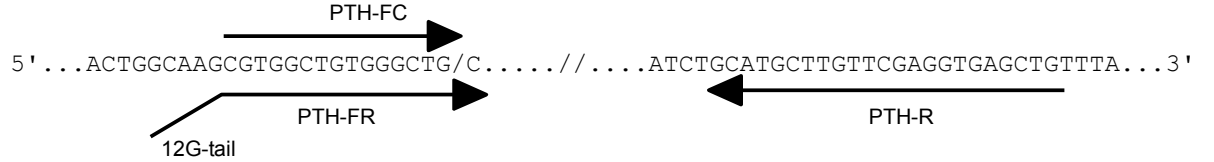

Figura 1: Diagrama de los cebadores utilizados para detectar el marcador SNP PTH. Se añadió una cola de 12-G al extremo libre del cebador PTH-FR para aumentar la longitud del producto y el valor de su Tm.

Figure 1: Diagram of the primers used to detect PTH SNP marker. A 12-G tail was added to the free end of PTH-FR primer to increase the length of the product and its Tm value.

Finally the rate of temperature transition was modified between 0.5 to 0.1 °C per step with a 30s pause at each step. When the technique was standardized, to confirm results, positive and negative (no DNA template) controls were included in each run and each sample was re-amplified. Data were analyzed using 7500 System v2.0 software (Applied Biosystems).

Results

Parathyroid hormone (PTH) primers were able to amplify each sample and produced single product band when were checked by gel electrophoresis. The real-time PCR profile was as follows: denaturation at 96 ºC for 2 min, followed by 30 cycles of denaturation at 96 ºC for 30 s, annealing at 65 ºC for 30 s, and extension 72 ºC 1 min, and a final extension at 72 ºC for 10 min. As far as melting curves results is concerned, no relation between the DNA template concentration and the Tm variation was found. The extent of intraspecific variation and of repeatability are shorter when the temperature transition rate is smaller, the standard deviation decreased from 0.36 to 0.29 when the dissociation ramp profile was changed from 0.5 °Cs^{-1} to 0.1 ºCs^{-1}, establishing the last one as the final condition. Melting curves are acquired by initial heating to 95 ºC for 15 s, subsequently cooling at 57 ºC for 45 s and slowly ramping the temperature from 57 ºC to 95 ºC at a rate of 0.1 ºCs^{-1}, with 30 s pause between different steps, and a final holding for 30 s at 95 ºC.

Three analyses were run with the final conditions, and all the samples were analyzed twice. Temperature differences among replicas ranged between 0 and 0.4 °C. Differences of Tm of the same sample in the different runs ranged between 0.03 and 0.54 SD. Mean Tm values of *A. chukar* and *A. rufa* and the corresponding coefficients of variation of each run are shown in Table 1.

For SNPs, heterozygotes are defined by the presence of two temperature peaks and the homozygous mutation is defined by the presence of one temperature peak (Fig 2). Seventy eight samples were analyzed for the PTH marker, 15 of them showed Tm corresponding with *A. chukar* and 63 samples were associated with *A. rufa.*

		Test 1	Test 2	Test 3
A. rufa	Mean Tm (°C)	86.15	86.31	86.46
	Standard deviation	0.29	0.19	0.21
A. chukar	Mean Tm (°C)	84.87	85.06	85.44
	Standard deviation	0.19	0.21	0.12

Tabla 1: Tm media y desviación típica para cada uno de los análisis realizados con el marcador PTH en las especies *Alectoris rufa* y *A. chukar*.

Table 1: Mean Tm and standard deviation for each one of the analysis run with PTH marker in *Alectoris rufa* and *A. chukar* species.

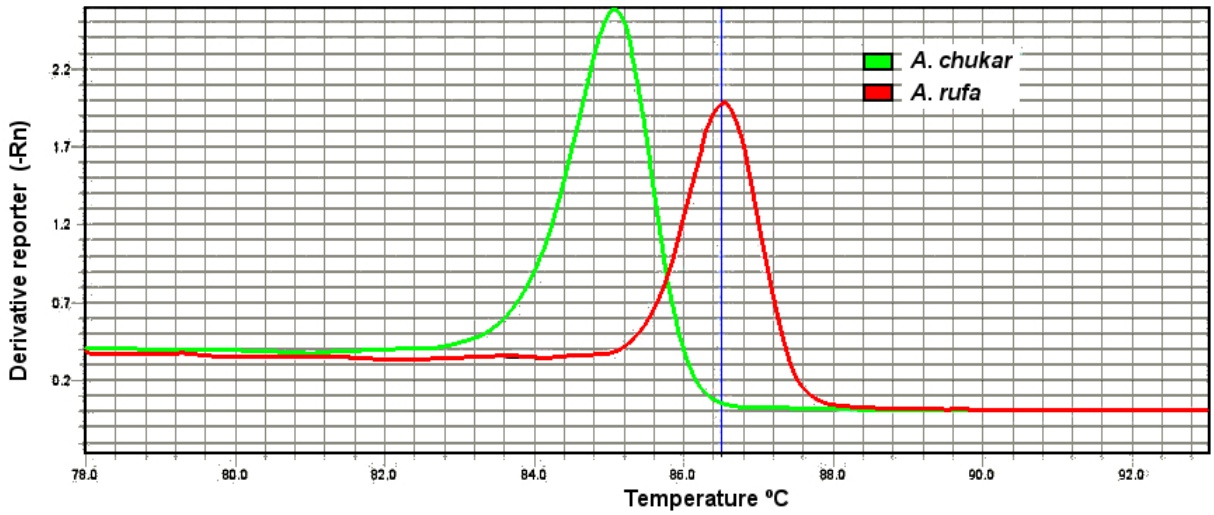

Figura 2: Curvas de disociación para el marcador PTH. Se representa la derivada de la fluorescencia con respecto a la temperatura, frente a la temperatura. La curva correspondiente a un individuo *Alectoris rufa* se muestra en rojo y la curva de un individuo *A. chukar* en verde.

Figure 2: Fig 2. Melting curves for the PTH marker. The derivative of fluorescence with respect to temperature vs temperature is represented. The curve corresponding to an *Alectoris rufa* individual is shown in red, and that of an *A. chukar* individual in green.

Double peaks indicative of heterozygous individuals were not found in any sample.

Discussion

The results obtained in this survey showed that the species-specific primers of the parathyroid hormone SNP together with melting curve analysis can be used to differentiate *A. rufa* from *A. chukar* partridges. The effectiveness of allele-specific real time PCR method for SNP detection had been previously documented (Feligini et al. 2005, Ye et al. 2010). This survey supposes the first attempt which allows high accuracy for individual identification, with less cost and time effort, to identify and to differentiate partridge species (*A. rufa* and *A. chukar*). The main advantages are the elimination of post amplification procedures (agarose gel and ethidium bromide), the possibility of genotyping 96 samples at the same time (Sander-Sevall 2001, Ye et al. 2002) and the reduced cost associated. The price difference between MCA and PCR is approximately 0.10€/sample, being considered the reagents and consumable costs and taking into account that the conventional method requires two PCR reactions to obtain the proper genotyping.

The success of the melting curve analysis depends on the design of the primers, so that they result in different peaks for each species-specific amplicon. The addition of C tails increased the Tm 1 ºC per every five nucleotides, according to Berry & Sarre (2007) and Ye et al. (2002). In this study, the increase of the Tm was not as pronounced, but the addition of G tails was necessary to differentiate the sizes of the PCR fragments.

Tm values can vary depending on several factors such as the DNA quality, the SYBR Green concentration and the temperature transition rate employed in the analysis (Rieri et al. 1997). The ramp profile was established in a transition rate of 0.1 $ºCs^{-1}$ to reduce the standard deviation. Due to the fact that Tm were not repeatable between the different runs the addition of positive controls is necessary to establish the benchmark for each species (Berry & Sarre 2007); additionally negative controls are useful to detect and to distinguish unspecific products that can be amplified although those generally show lower temperatures.

Melting curves of the individuals analyzed for PTH marker in this work found no heterozygous individuals, meaning lack of introgression of this polymorphism at nuclear level in the examined individuals. To detect the full extent of introgression in the genome of an individual it is necessary to develop a panel of adequate markers to determine an acceptable probability of exclusion (95%) for a given degree of introgression. A careful selection of markers with different Tm profiles would potentially allow multiple analyses (Ye et al. 2002), without increasing the reaction cost. This technique allows identifying putative introgression level and will be useful for the management polices based on strict genetic controls for partridge stocks reproduction in farms, restocking or other hunting activities. It will also be useful to ascertain the distribution of natural red-legged partridge populations.

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