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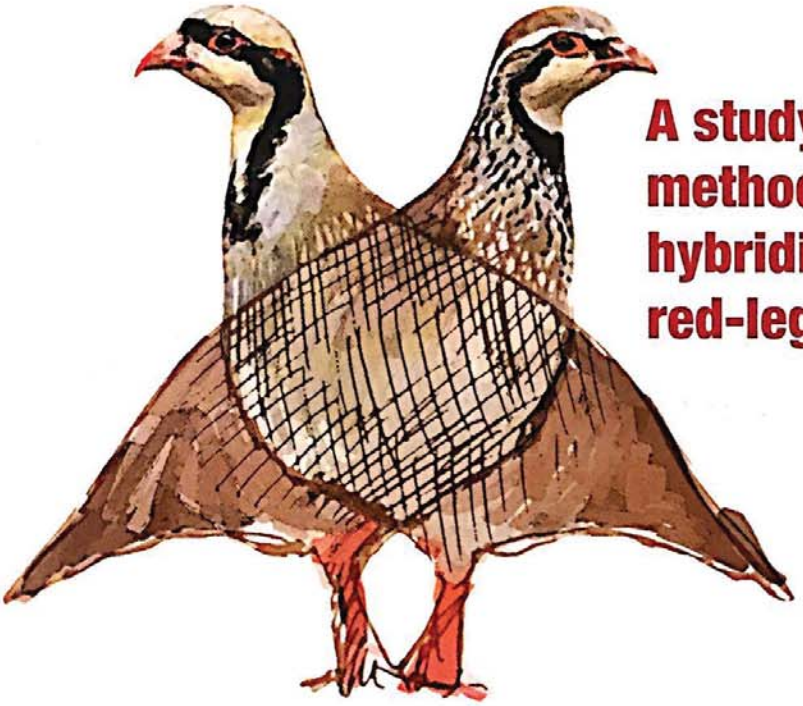

A study of genetic methods to detect hybridization in the red-legged partridge

Consortium *Perdiz Roja*

FEDENCA-Laboratorios de Genética®

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1. Presentation

The booklet set out below is a summary of one of the most important contributions of the Spanish federated hunters and some European Laboratories for the defence of biological, genetic and wild values of the red-legged partridge, the iconic and most popular game species.

The initial project "Study of genetic methods to detect hybridization in the red-legged partridge", developed by the foundation FEDENCA, has been an essential tool for the management and control of red-legged partridge populations by governments, hunters, managers, partridge breeders and any public entity responsible for wildlife care. For the first time, unifying the criteria and method for hybrids detection among the main European laboratories dedicated to the partridge study provides an effective tool to control Turkish introgression into red-legged partridges.

The European Directives related to wildlife and environment conservation are the 79/409/EEC of 2 April 1979 on bird conservation, and the 92/43/EEC of 21 May 1992 on habitat and wildlife conservation. These two directives apply to all European citizens and every EU member state must obey them and legislate according to them.

In Spain, Law 42/2007 of 13 December 2007 on Natural Heritage and Biodiversity includes those two European directives, and regional laws are subjected to it. Its article 52 ensures the conservation of wild autochthonous species: "The competent public authorities will ban the introduction of allochthonous species, subspecies or breeds if they are capable of competing against wild native species or altering their genetic purity or ecological balances".

Despite the existence of these laws, genetic introgression in partridges is frequently detected in Spain and other European countries.

To perform the application of the genetic method uniformly agreed by the laboratories that completed the project, the Consortium Perdiz Roja FEDENCA-Laboratorios Genética® has been created; this is a multidisciplinary entity and a quality warranty brand, and operates under public regulations and agreements.

With the project summarized here, we have taken a firm step in wildlife defence. Now, authorities and wildlife police can immediately act in compliance with the aforementioned laws and legislate on the genetic control of the red-legged partridge. We understand that regulations and requirements of genetic quality should be identical throughout Spain, thus enabling the exchange and marketing of partridges across the country. We hope that the government will progress in the application of the law and responsible hunters will demand it.

The Consortium, which owns the rights to carry out the genetic testing through the participant laboratories, will apply in accordance with the regulations established in this

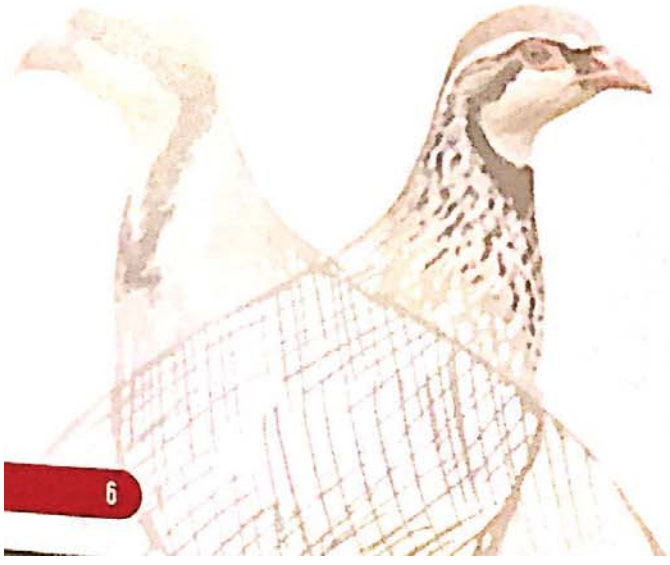
project. Also, the Consortium, in addition to provide an effective genetic tool to control partridge genetic quality, has also submitted comprehensive recommendations for public and private entities interested in its application. This project is currently being implemented in Spain.

José Luis Garrido

FEDENCA's General Director

Andrés Gutiérrez

FEDENCA and Royal Spanish Hunting Federation's President

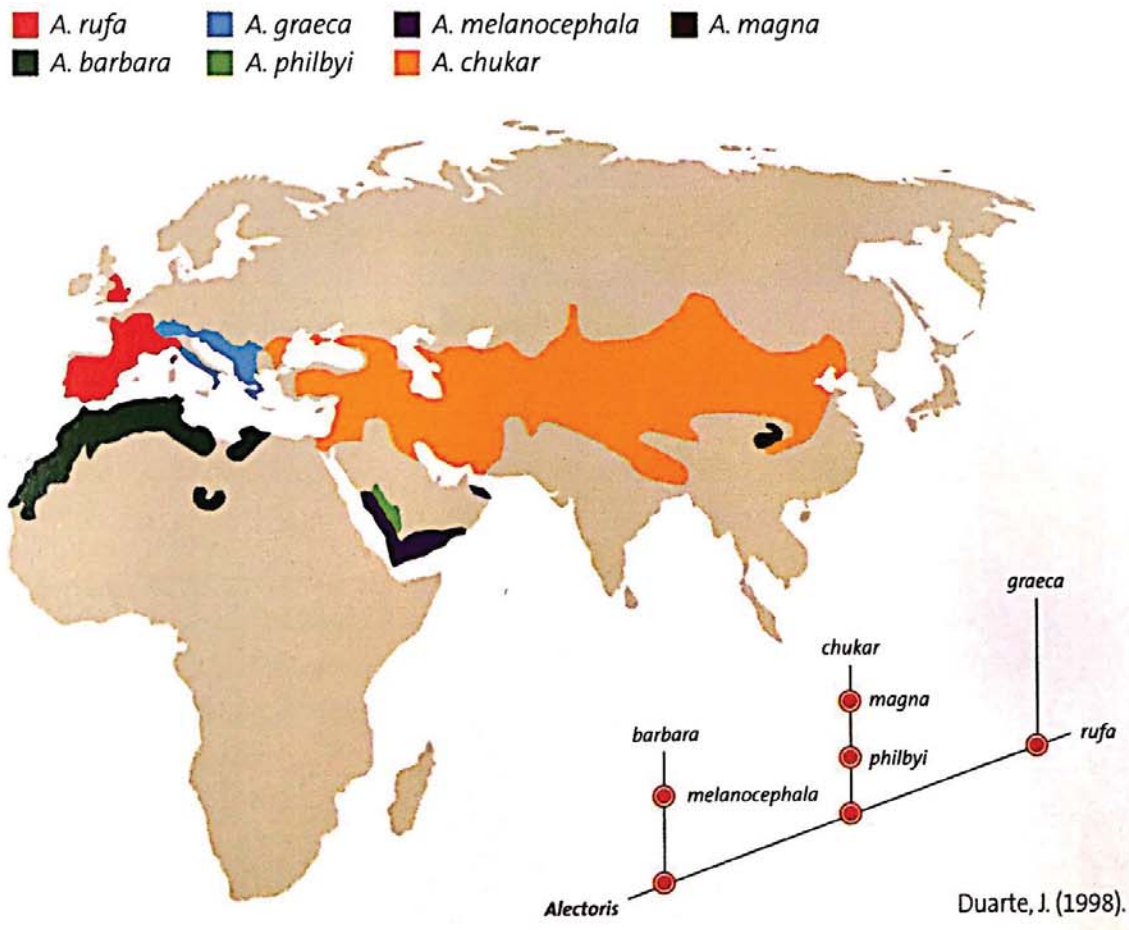


2. Background

The red-legged partridge *(Alectoris rufa)* is the most important small game bird in Spain, country where many game reserves are spread across. Partridge breeding and hunting represent important sources of both directly and indirectly income in rural areas.

From the ecological point of view, the red-legged partridge also plays a key ecological role since it is the prey of several predator species, many of them considered as endangered. However, despite its widespread distribution along the Spanish territory (figure 1), red-legged partridge populations have decreased, especially since the 60's. Some authors have suggested loss of habitat quality and over-hunting as the main causes of its decline, but also intensification of agriculture, predation pressure and uncontrolled restocking have contributed to it. All these facts have led to the classification of the red-legged partridge as Species of European Conservation Concern (SPEC).

Figure 1. Geographical distribution of the *Alectoris* genus



Duarte, J. (1998).

During the last few decades, the heavy hunting pressure has far exceeded natural resources and many stocking farms have emerged in order to sustain hunter's demands. So, millions of captive-bred partridges are released every year throughout Southern European Countries this way. According to recent estimations, 4.5 millions of partridges are annually released, although it is very difficult to know accurately the exact number as a significant number of individuals issued from other European countries is introduced in the Spanish land and their rate of survival, spread and fitness are largely unknown.

One of the problems caused by the release of farm animals is the hybridization of red-legged partridges with foreign species that have a better growth rate and adaptation to captivity as is *A. chukar,* which reproductive use has promoted the release of different degrees of hybridized individuals. However, the introduction of hybrids in wild populations is not legal and could result in severe environmental and health problems, as alien species can displace native ones from their habitats and/or widespread new diseases to local wildlife. In addition, interbreeding between non-native and native species may lead to the introgression of foreign genes in locally adapted populations.

The red-legged partridge belongs to the Alectoris genus, which comprises seven partridge species. Along with *A. rufa,* the main Alectoris species of the Mediterranean area are the rock partridge *(A. graeca)* and the Turkish partridge *(A. chukar),* which show small overlapping areas and close genetic relationship between them (Figure 1). The red-legged partridge is distributed across the Iberian Peninsula, southern France, and in the Balearic Islands, and has successfully been introduced in Great Britain and parts of Italy. *A. graeca* is distributed in Italy, southeaster of France and western Balkans, including Greece, while *A. chukar* occupies a wider area, from the Greek islands and Turkey to China. Natural hybridisation is known to occur between *A. rufa* and *A. graeca* in Italy and southeastern France as do *A. graeca* and *A. chukar* in Bulgaria and Greece. Although natural hybrids between *A. rufa* and *A. chukar* do not occur, since their geographical distributions do not overlap (Figure 1), farmers have crossed these two species as Turkish partridges show a better adaptation to captivity and produce higher performances than red-legged partridges.

The Turkish partridge is not used directly for restocking purposes because it can be distinguished quite easily from the autochthonous red-legged partridge. However, from the first hybrid generation on, differences are not that evident. Although early studies suggested *A. graeca* introgression in *A. rufa* during captive rearing, no *A. rufa* × *A. graeca* hybrids were identified in such conditions and recent data clearly point to extended hybridization of *A. rufa* with *A. chukar* in restocked areas and on farms, despite release of foreign species or hybrids is strictly forbidden by law.

2.1. DETECTION OF INTROGRESSION USING MOLECULAR METHODS

The red-legged and the Turkish partridges have a common ancestor and as a result their genomes are relatively close. However, diverse evolutionary processes have generated genetic differences that can be detected by appropriate genetic tools, such as the use of molecular markers.

There are several types of markers available, but the most reliable ones are DNA markers, which provide results starting from small and easily obtainable samples. Moreover, they allow to directly genotype individuals and, currently, many molecular databases are available in different species to sustain these studies, including whole-genome libraries.

Single-nucleotide polymorphisms (SNPs) are molecular markers which allow identification of hybrid individuals based on DNA profiling. These markers are a DNA sequence variation occurring when a single nucleotide in the genome differs between individuals, populations or species. SNPs are the last generation of markers and show advantages that make them ideal for hybrid detection due to their high occurrence, and to their bi-allelic condition, easy to validate and to genotype with medium and high-throughput technologies which lead to the easy analysis of large amounts of animals in a short time.

2.2. PROJECT "HYBRIDIZATION DETECTION IN THE RED-LEGGED PARTRIDGE"

The main Spanish and foreign partridge genomic laboratories worked together to undertake a project which aimed to detect hybridization in the red-legged partridge. The objective was to develop a cost-effective medium-throughput genotyping method to allow easy and fast introgression detection of *A. chukar* into wild *A. rufa* populations that could be used by stocking farmers and public and private institutions as an effective tool to control the genetic purity of individuals both in hunting areas and on farms. For this purpose, each laboratory contributed with different molecular markers.

The goal of the present booklet is present the project, as well as the phases that led to the final results.

3. Description and development

The aims of the project "Study of genetic methods to detect hybridization in the red-legged partridge" were conducted in two phases: 1) evaluation of the diagnostic methods applied by the participant laboratories through the analysis of blind control samples of the species *A. chukar* and *A. rufa*; 2) development and consensus of a method and criterion for hybrids detection among the laboratories.

3.1. PHASE I: EVALUATION AND SELECTION OF THE DIAGNOSTIC METHODS APPLIED BY THE PARTICIPANT LABORATORIES

In this first phase, each laboratory applied their own markers and methodology to analyse 266 anonymous DNA partridge samples detailed in Table 1.

The participant laboratories contributed with a total of 57 molecular markers: 55 nuclear (34 SNPs, 1 INDEL and 19 microsatellites) and 3 mitochondrial SNPs. The number of markers used by each laboratory ranged from 2 to 19. The results obtained by the participants showed clear differences in hybrids detection power, specificity and sensitivity. Although specificity and sensitivity concepts are used here, we defined three alternatives –*A. rufa, A. chukar* and hybrid–, instead of the classical two.

Wild Spanish and French red-legged partridges (n = 68) have been regarded as *A. rufa* references to calculate specificity (probability of correct identification of a red-legged partridge as red-legged). Museum samples were finally not used because of their poor DNA quality and quantity, corroborated by all laboratories. All participants, except one, showed high specificity values (Figure 2).

Turkish partridges (n = 38) were used to calculate sensitivity (probability of correct identification of a Turkish partridge as Turkish). Figure 3 shows high sensitivity values for all laboratories except one.

Known hybrids, which were produced and provided by the French participants, were used to calculate the hybrid detection power. Figure 4 shows the detection power of each laboratory for the different hybrid types – B1, B3 and B4 refer to the number of backcrosses with red-legged partridge. B2 detection power is not shown due to the fact that only one sample was available. It is worth highlighting that the number of hybrids in each group is small, thus deviations between the theoretical detection power of a set of markers and the real one may be produced.

In summary, these results showed disagreements between laboratories on the classification of a large number of samples, especially those showing a low percentage of foreign genome such as hybrids belonging to B4 and farm samples. These discrepancies could be

Table 1. Partridge samples analysed by the participant laboratories

TYPE	SUBTYPE	SOURCE	Nº OF SAMPLES	TISSUE
Captive-reared *A. rufa*	High producing farm (A)	Spain	40	Pectoral muscle
	Small size farm (B)	Spain	40	
Wild *A. rufa*	Spain (C)	Vejer (Cádiz)	10	Tongue
		Berceruelo (Valladolid)	10	
		Mués (Navarra)	10	
		Pozondón (Teruel)	9	
	France (D)	Córcega	29	Lengua, hígado
Museum *A. rufa* (E)	Museum of Natural Sciences (Madrid)		9	Plantar pad
	Doñana Biological Station (Sevilla)		31	
A. chukar (F)	Wild	Lebanon	31	Tongue
	Restocked	Cyprus	7	
Hybrids (G)	B1: *A. rufa* x F1[1]	ONCFS	10	Liver
	B2: *A. rufa* x R1		1	
	B3: *A. rufa* x R2		10	
	B4: *A. rufa* x R3		19	
Total	–	–	**266**	–

[1]F1: *A. rufa x A. chukar.*

explained, on the one hand, by the small number of markers used by some of the participants, and, on the other hand, by the different reference samples used by each laboratory to search and/or test their markers.

These facts support the need to unify the method and criterion for hybrids detection to avoid discrepant results between laboratories and to increase the hybrid detection power by increasing the number of markers.

Figure 2. Specificity values obtained by the participant laboratories

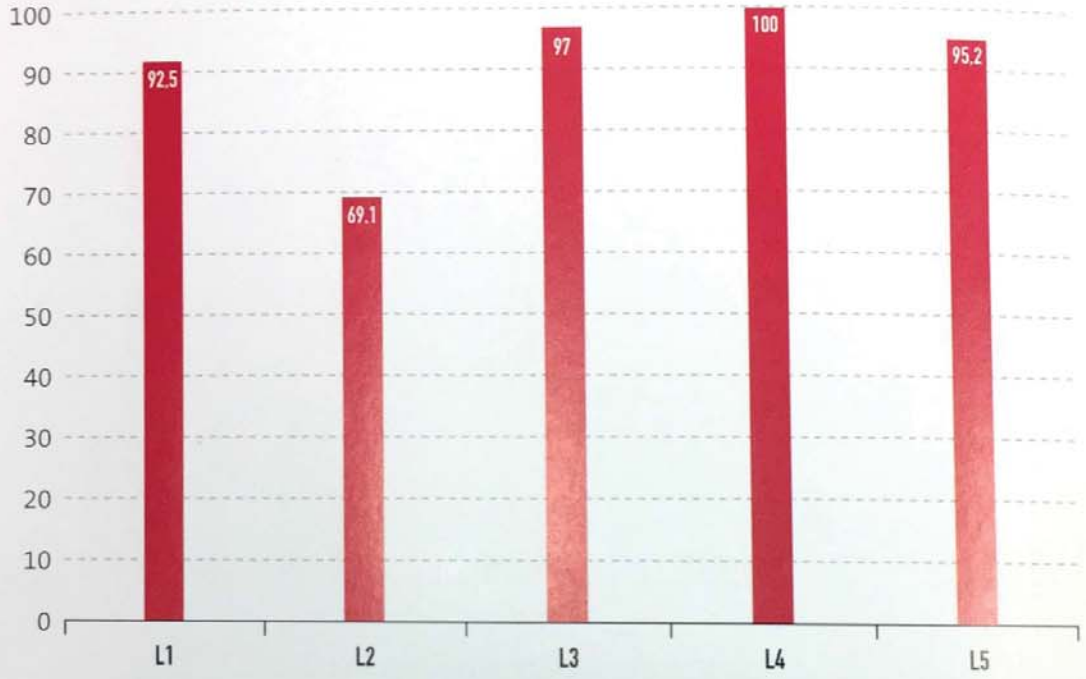


Figure 3. Sensitivity values obtained by the participant laboratories

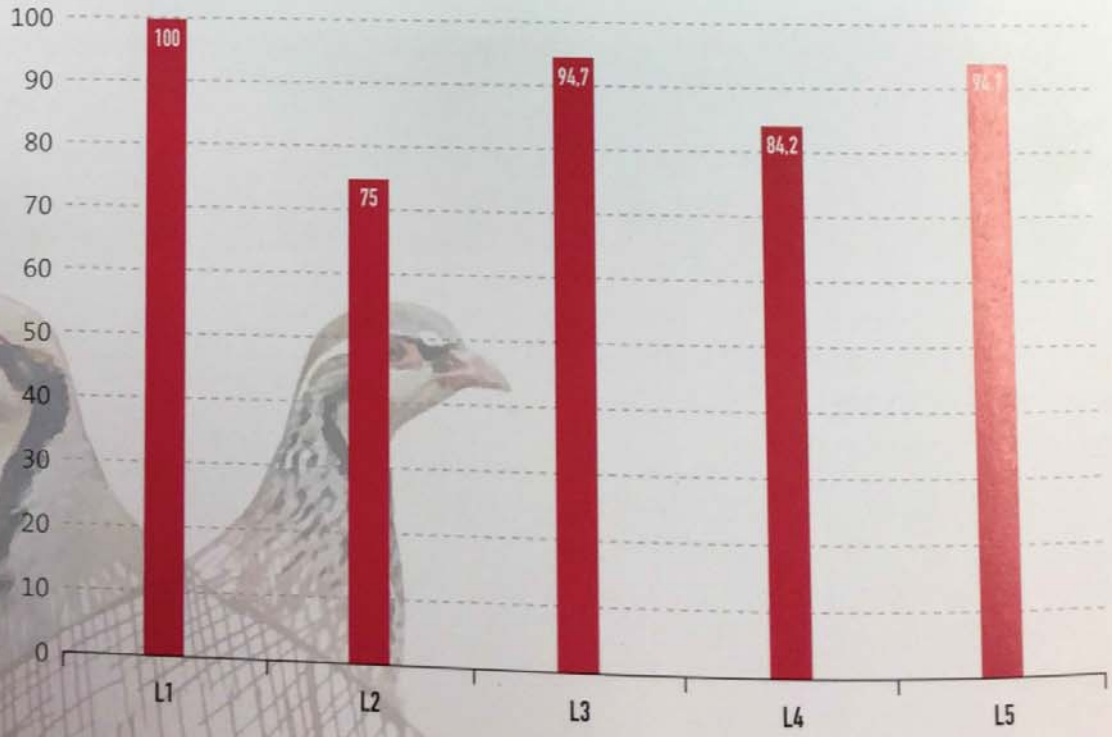
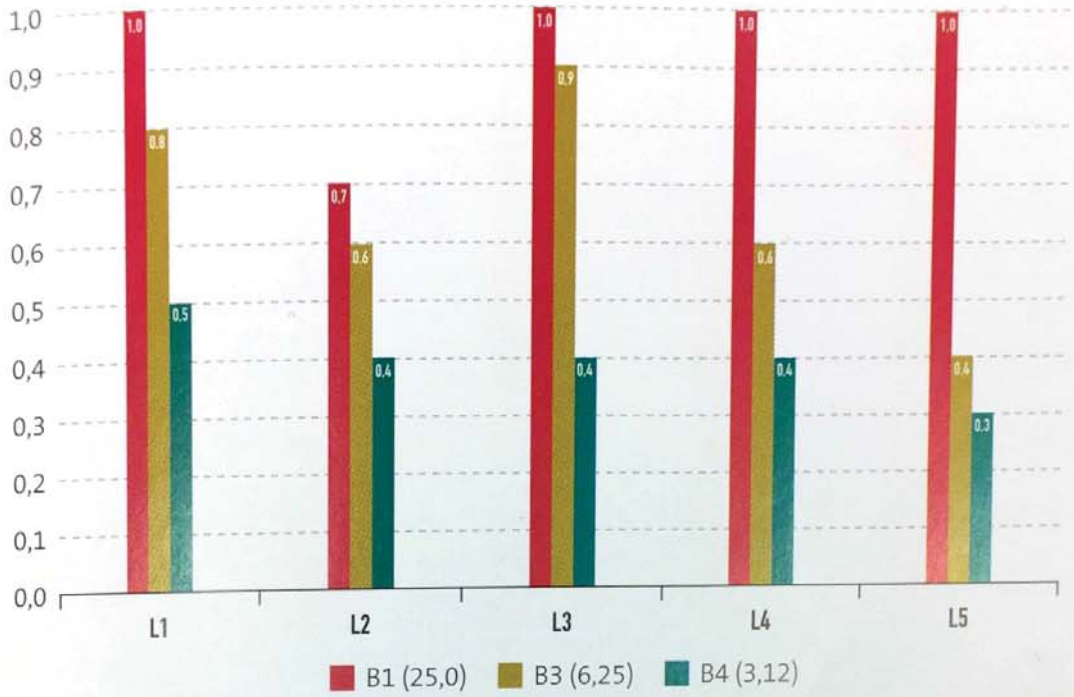


Figure 4. Probability of detecting the different hybrids types by each laboratory. In parenthesis, percentage of Turkish genome in the hybrid



3.2. PHASE II: DEVELOPMENT OF THE METHODOLOGY AND THE DIAGNOSTIC CRITERION TO DETECT *A. CHUKAR* INTROGRESSION INTO *A. RUFA*

In order to develop a cost-effective method for introgression detection in *A. rufa*, we used diagnostic (unique) – or almost unique- SNP markers (i.e. the marker has an allele in one species and the alternative in the other species).

Among the total of markers provided by each laboratory 23 polymorphisms (SNPs) were validated and included in a Multiplex-PCR-Primer Extension system (PE) (Table 2).

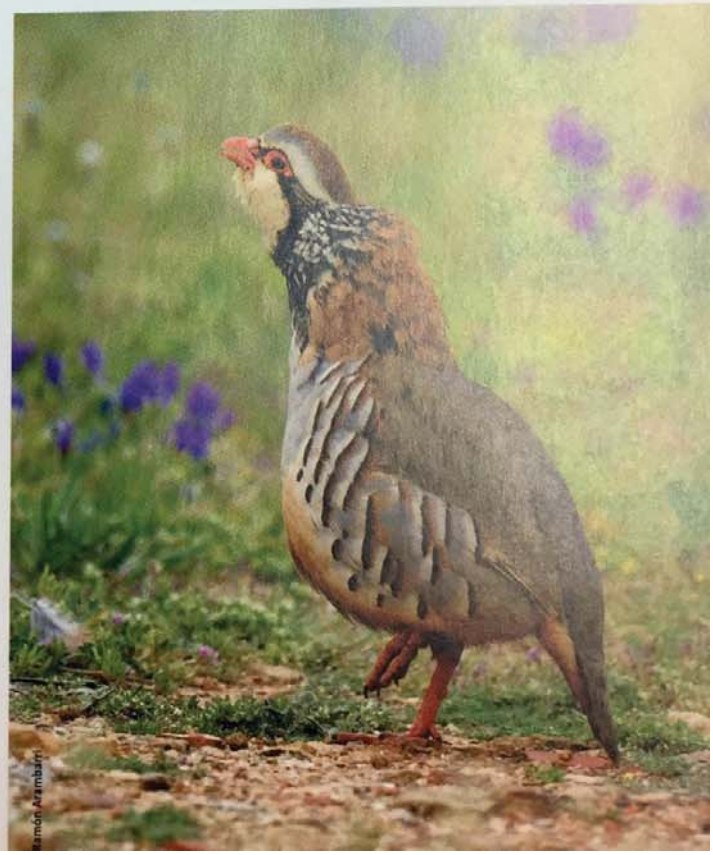


Table 2. Names and symbols of the genes including the validated SNPs for *A.chukar* introgression in *A. rufa* detection

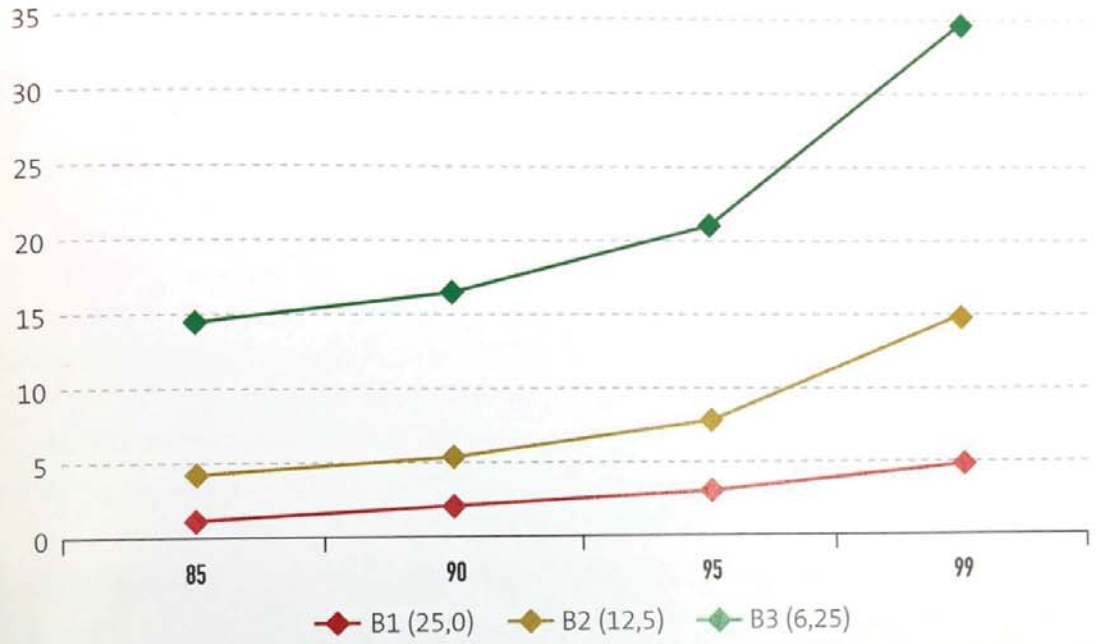
GENE NAME	SYMBOL	GENE NAME	SYMBOL
Aru1I68	Aru1I68	*Ribosomal Protein L7A*	RPL7A
Aru1F32	Aru1F32	*Creatine Kinase 6*	CKB
Aru1V16	Aru1V16	*Cell Division Cycle 2-like1*	CDC2L1
ATP-citrate lyase	ACLY	*ß-Cristalline*	CRYB
Adenylate Kinase 1	AK	*Glyceraldehyde -3-P dehydrogenase*	GAPDH
Alpha-enolase	ENO	*Recombination activating gene 1*	RAG1
Beta Fibrinogen	FGB	*Aggrecan 1*	AGC1
Hepatocyte nuclear factor 1 alpha	HNFA1	*Parathyroid hormone*	PTH
Myelin proteolipid protein	PLP	*Thrombospondin 1*	THBS
Rhodopsin	RHO	*Pterin-4-alpha-carbinolamine dehydratasE 2*	TCF1
Mitochondrial marker	DLOOP/CitB	*Interleukina 12b*	IL12B
Vimentine	VIM	–	–

Although arrayed different techniques have been developed in the last years for simultaneous SNP detection, the economic value of a partridge does not justify the application of expensive procedures and PE assays are a good alternative as it 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 of an expensive infrastructure.

3.2.1. Calculation of the theoretical hybrid detection power

Figure 5 reflects the relationships between the level of introgression, the power of hybrid detection and the number of exclusive markers needed to detect it.

Figure 5. Number of unique markers needed to detect hybrids with different detection powers. In parenthesis, percentage of theoretical Turkish genome



A theoretical power[1] of 100, 99.8 and 94.7% to detect hybrids with 25, 12.5 and 6.25% of foreign genome respectively can be performed with the current number of validated nuclear markers is 22 (the remainder is a mitochondrial marker with a non-Mendelian inheritance pattern). When Turkish partridges are used on the maternal side to obtain hybrids, the mitochondrial SNP increases the power to detect hybrids.

This analysis is conservative as if we do not detect any Turkish allele in the sample, we assume absence of hybridization. On the other hand, the rejection of the purity of an individual is not probabilistic but categorical, so the presence of foreign alleles in a sample allows to reject its genetic integrity, ruling out any custody or process error.

No further backcross levels were checked due to the very low percentage of Turkish genome present (3% in a B4 backcross, for example). The benefits of a power increase to detect animals with such a low hybridization level would not compensate the cost of setting up a battery of markers large enough to accomplish it.

1 As the molecular markers used are unique –or almost unique–, the expression applied to calculate the theoretical total exclusion power (P_{TE}) was:
$P_{ET} = 1 - (1 - P_E)^n$
n: number of unique markers
$P_E = 2p$
p: proportion of Turkish genes in the autochthonous population

3.2.3. Hybridization index

Although the method developed fullfils all the quality requirements, we must be aware that there are two major assumptions underlying the methodology: 1) the markers must be totally unique, i.e., all the markers used have an allele in one species and the alternative in the other species; and 2) there are no genotyping errors – but the technique involves the analysis of a relatively large number of genes in each individual. Under these conditions, it is important to be very rigorous when interpreting the overall results of hybridization and, for this reason, an individual is classified as hybrid only when more than one Turkish allele is detected. Otherwise we could generate high levels of "statistical" hybridization.

Taking this into account, we have defined the **hybridization index** as the ratio between the number of Turkish alleles in a sample or a population, and the whole number of alleles analyzed, expressed as a percentage. From a management perspective, the goal of a full-cycle farm would be to gradually reduce this index. For the next 4 years, the Consortium criteria that should be applied to rank the partridge stocks (farms, territories, hunting areas, etc.) are:

1. Hybridization index = 0%: category **Absence of Hybridization**.
2. Hybridization index > 0 and < 2%: category **Very Low Hybridization** –only if the percentage of partridges with one or more foreign alleles was ≤ 5%. If it is > 5%, the category will be Low Hybridization.
3. Hybridization index ≥ 2 and < 5%: category **Low Hybridization**.
4. Hybridization index ≥ 5 and < 10%: category **Medium Hybridization**.
5. Hybridization index ≥ 10%: category **High Hybridization**.

The categories **Low Hybridization** and **Medium Hybridization** would require to gradually removing hybridized breeding individuals from the stock and monitoring the hybridization level evolution during the following 4-5 years.

High Hybridization would involve more rigorous actions, including the possibility to restrict the release of animals and an intensive plan for analyzing breeders in 3-4 years in order to reduce the current hybridization level.

4. Conclusions

Taking into account that recent data clearly point out to an extended hybridization of *A. rufa* with *A. chukar* across the entire *A. rufa* range –with the exception of Corsican–, as well as in stocking farms from France, Portugal and Spain, the development of a molecular tool for detecting *A. rufa* x *A. chukar* hybrids as the one presented in this booklet by the Consortium *Perdiz Roja* FEDENCA-Laboratorios Genética® seems justified.

This molecular tool fullfils all the technical and quality requirements to be applied massively under field conditions. Its application will allow, on the one hand, to acquire an accurate knowledge of the red-legged partridge genetic hybridization situation, both on the wild and on captive-reared stocks, and, on the other hand, to prevent introgression consequences on wild red-legged partridge populations by carrying out controls on stocking farms and restricting the use of hybrid individuals.

5. Practical applications

Genetic studies are increasingly used to make practical decisions on wildlife management, especially for the protection of endangered species and for the management of fishery and stocking resources. The impact of these genetic studies do not only affect the involved populations, but also human activities related to these populations or species.

After the "Genetic methods to detect hybridization in the red-legged partridge" study several practical applications can be envisaged and the success of genetic recommendations will depend mainly on (1) the experimental design, including the appropriate number and origin of samples according to the question stated, (2) the sampling and shipment protocols, and (3) on the analysis performed and the appropriate interpretation of the results. Of course, the quality of the genetic data obtained must be adequate to support the conclusions reached and it must also be understandable by other scientists, managers, hunters, courts and authorities.

5.1. ABOUT THE SAMPLING

The first step in any genetic study is to perform an adequate sampling. Whenever possible, collect blood (0.1 to 0.5 ml) from partridges. Other type of samples such as feathers or other tissues can also be analyzed. Blood and other tissues should be immersed in some solution to preserve DNA at room temperature.

The procedure and the number of partridges sampled vary depending on the kind of partridge stock analysed-hunting reserves, stocking farms or transport vehicles.

5.2. SAMPLING ON HUNTING RESERVES

Sample sizes for testing the absence of hybridization in the hunting reserve should ensure that a negative result (Absence of Hybridization) implies that the percentage of hybrid partridges is less than 5% with a probability of 95 or 99% (58 and 90 samples, respectively). These sample sizes are considered for each sampling unit determined by the technician.

Sample sizes for testing the average percentage of hybrid partridges in the hunting reserve depend on several factors that we ignore beforehand. Apart from the criterion of the technician, a general recommendation which considers a wide range of situations would be to collect between 100 and 250 random samples.

Anyway, if the technical documentation needed to determine the number of sampling units is not available, 10% of the hunted individuals should be sampled.

5.3. SAMPLING ON STOCKING FARMS

In all stocking farms, regardless of the productive cycle, a representative number of samples from each breeding line should be obtained. To determine appropriate sample sizes, the criteria aforementioned can be followed, along with the technician's advice. If the technical documentation needed to determine the number of sampling units is not available, 10% of individuals of each production level-breeding animals, eggs, chicks and young partridges- should be sampled. In any case, reports or certificates will refer exclusively to the sampled farm situation.

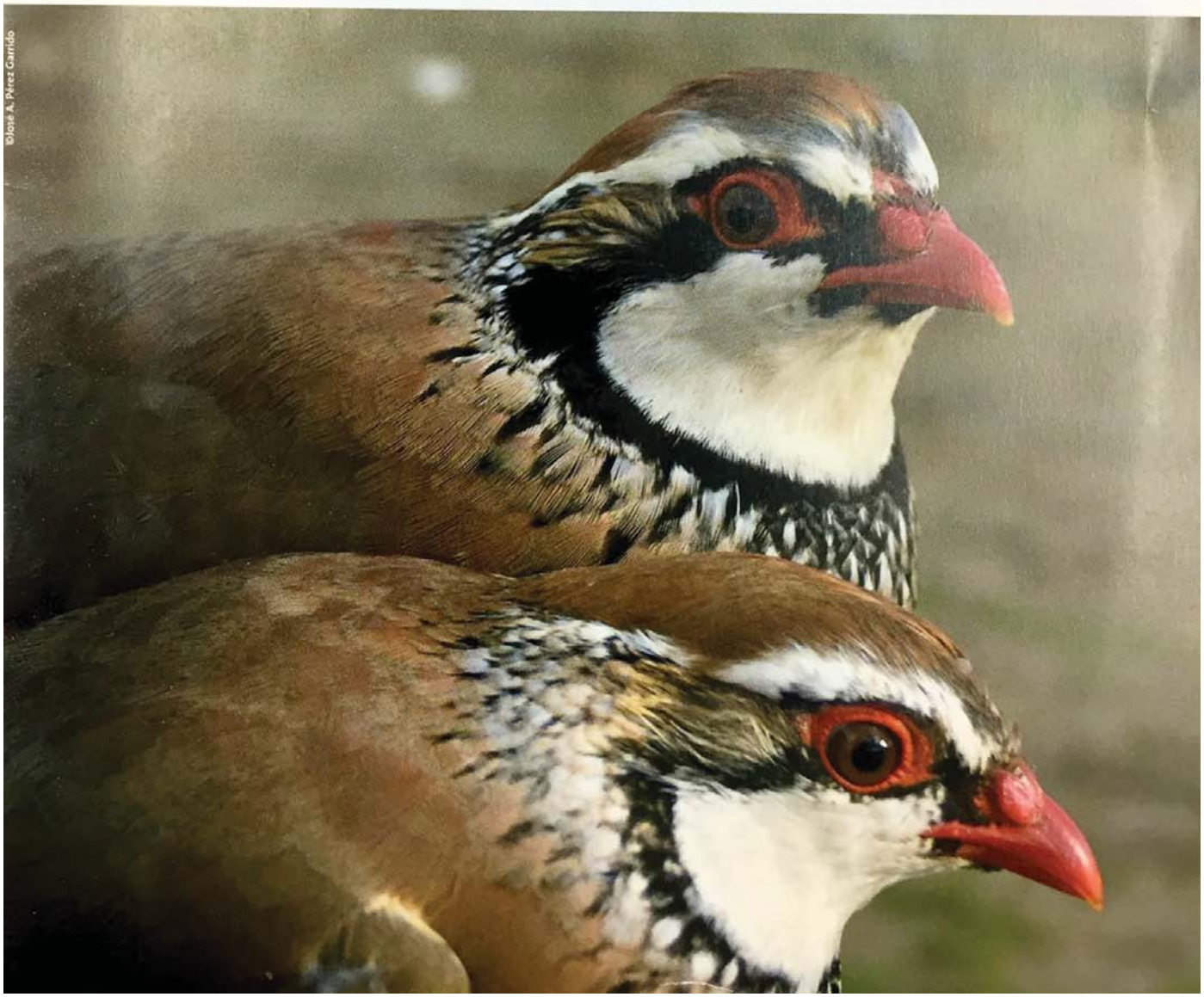
5.4. SAMPLING ON TRANSPORT VEHICLES

In transport vehicles the competent authority (different police forces) must verify the compliance with current legislation on marketing, transportation and release of live game animals. The technician will determine the appropriate number of samples following the same criteria than in previous sections. Sample traceability and custody will be guaranteed by the government or the competent entity, ensuring the impossibility of manipulation of biological samples and documentation.

Regulations regarding sampling and custody of samples for genetic analysis are decided by the different administrations. This booklet provides only basic recommendations on the way genetic studies should be performed.

6. Genetic purity map of the Spanish red-legged partridge *(Alectoris rufa)* and study of the Turkish *(Alectoris chukar)* introgression level in Spain

This project is a practical application of the study "Genetic methods to detect hybridization in the red-legged partridge" with the aim to assess the genetic quality of partridges in Spanish hunting reserves. This project is going to be developed throughout four hunting seasons (2010/2011, 2011/2012, 2012/2013 and 2013/2014), and the main aims are: (1) to establish the Spanish map of red-legged partridge genetic purity by regions; and (2) to determine the possible genetic and morphological differences between partridges from different Spanish latitudes. This study will allow the knowledge of the current situation of this bird on the wild and, in the future, to follow the evolution of wild populations under genetic control.



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