LIFE PERDIX

REDISCOVER BEAUTY IN RARITY

Realizzato con il contributo dello strumento finanziario LIFE dell'Unione Europea

Progetto LIFE PERDIX: dalla selezione dei riproduttori al monitoraggio della variabilità genetica negli individui rilasciati

(C. Tabarroni, C. Greco, N. Mucci)

Coordinatore beneficiario

ISPRA
Istituto Superiore per la Protezione e la Ricerca Ambientale

Sistema Nazionale per la Protezione dell'Ambiente

Beneficiari associati

CARABINIERI

Federazione Italiana della Caccia

Fédération Nationale des Chasseurs

LEGAMBIENTE

PARCO DELTA DEL PO
EMILIA-ROMAGNA

Cofinanziatore

Contents

1. Useful Knowledge: molecular markers

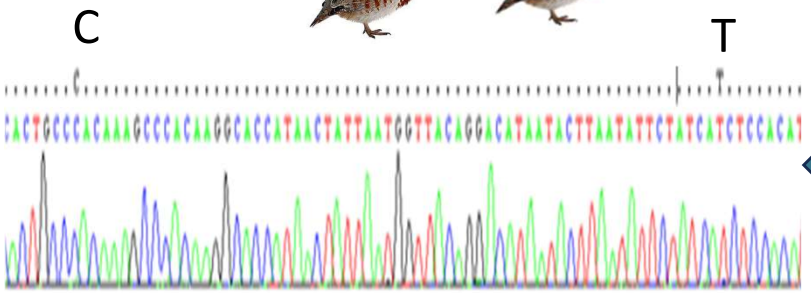
Mitochondrial DNA – d-Loop

What it is used for: Identifying the origin of *phylogenetic lineages and population differentiation*

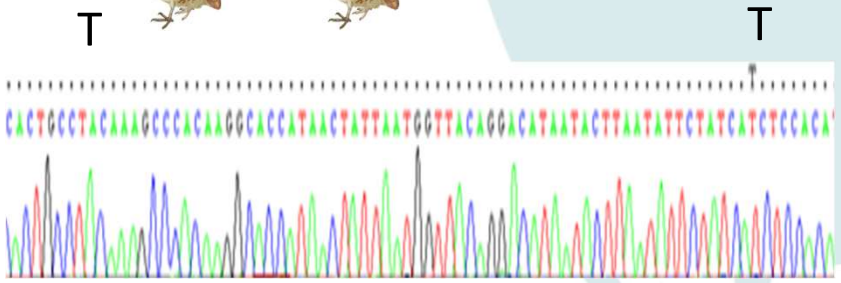
Characteristics: Haploid, not affected by recombination, uniparental maternally inherited, D-Loop - non-coding; high mutation accumulation rate

What we obtain: *Haplotypes* => specific combinations of variants in the DNA sequence

Individuals



Haplotypes

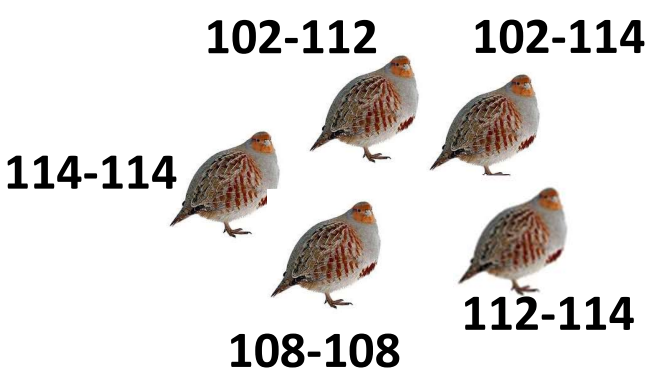


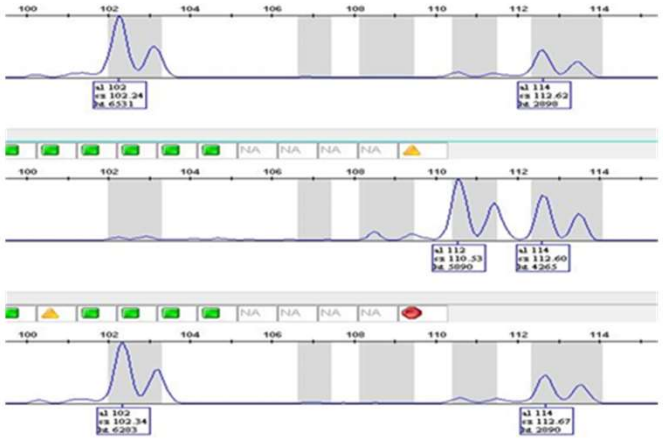
Nuclear DNA – microsatellites (STR)

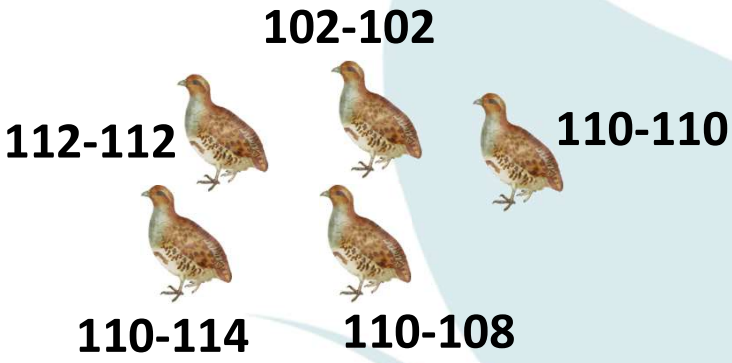
What it is used for: Describing the *genetic variability, individual genotyping, family relationships*

Characteristics: Diploid, subject to recombination, biparental inheritance, non-coding loci *very high mutation accumulation rate*

What we obtain: *Genotype* => combination of alleles at different loci (homozygous-heterozygous)







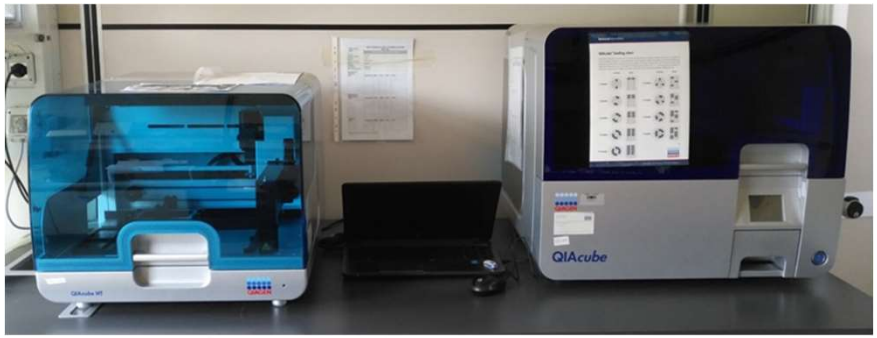
2. Methods: workflow

Samples management

- ✓ Protocol and materials for sampling
- ✓ Support for sample activities
- ✓ Database development and optimization

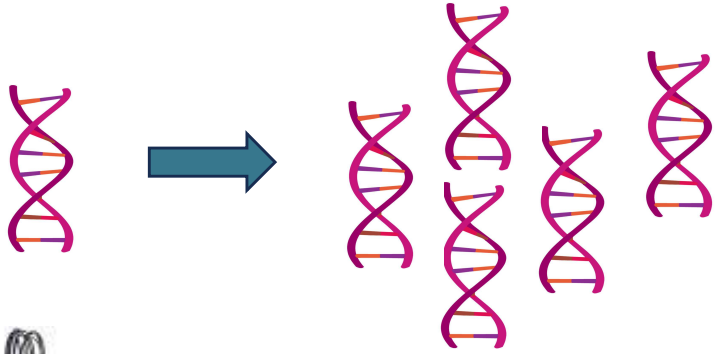
DNA extraction

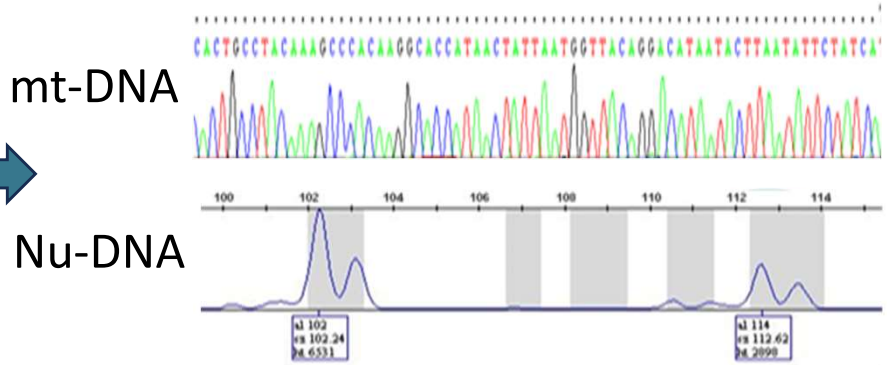
- ✓ Feathers stored dry or in 96% ethanol at -20°C, using the "DNeasy Blood and Tissue Kit" (Qiagen).
- ✓ Extraction on automated robotic stations "QIAcube" and "QIAcube HT" (Qiagen).



- ***DNA amplification and sequencing***.

✓ **mt-DNA** => around 350 bp following Randi et al., 1998
✓ **Nu-DNA** => 8 microsatellite from Ferrero et al. 2007, Bechet al 2010

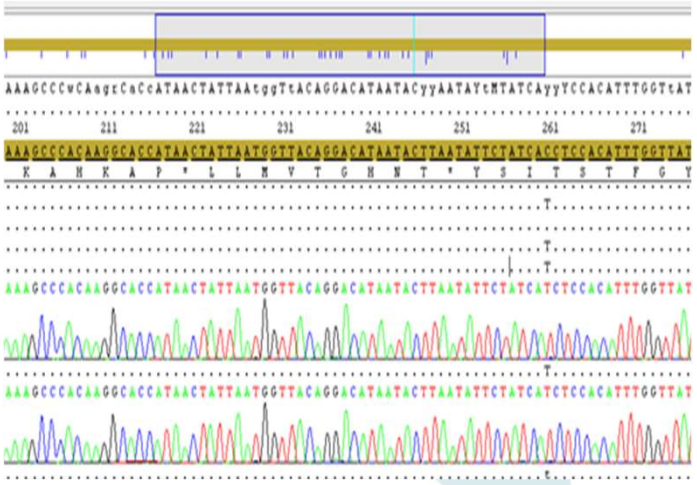




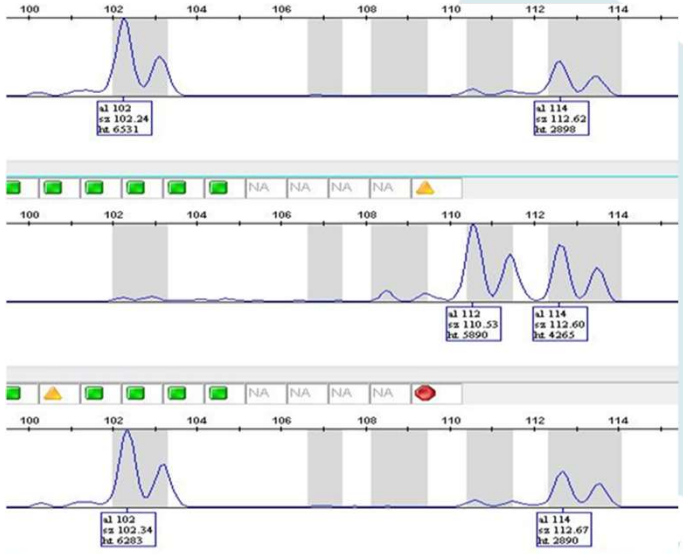
Liquid Handlers robotic stations, PCR machine and Automatic sequencer 3500xL Thermo-Fisher for activities and sequencing

Data correction and quality control using dedicated software

- ✓ **mt-DNA d-loop**
  - Visualization, editing and quality base calls in SEQSCAPE (Thermo Fisher Scientific)
  - DNA alignment in BIOEDIT (Hall 1999).

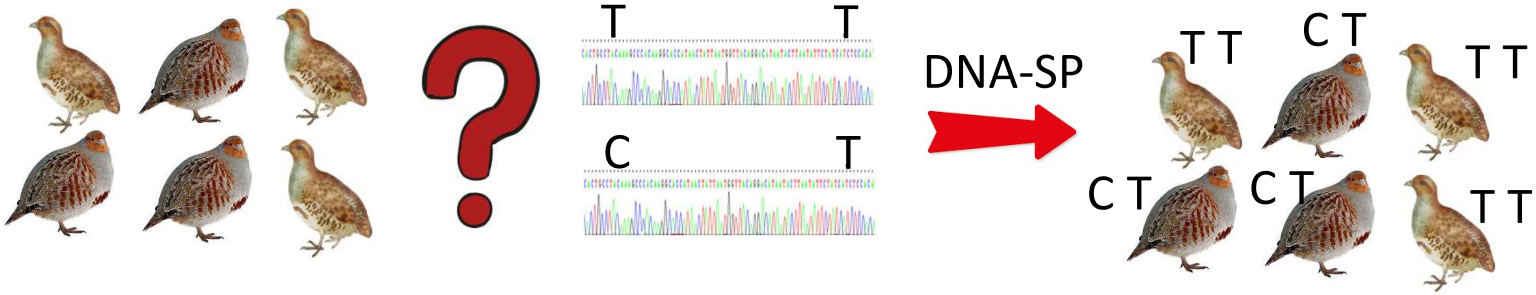


- ✓ **Nuclear DNA microsatellites**
  - Visualization, editing, sizing and quality allele calls in GENEMAPPER (Thermo Fisher Scientific)

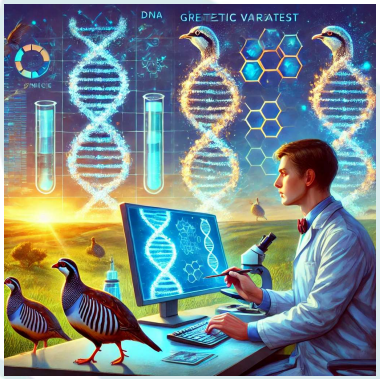


Statistical analysis

- ✓ **Sequence analyses (mt-DNA - haplotype)**
  - Haplotype identification in DNA-SP software (Rozas, et Al. 2017)



- ✓ **Analysis of genetic variability (Nu-DNA – genotype)**
  - Indices of genetic variability: Number of alleles (Na), Observed Heterozigosity (Ho), Expected Heterozigosity (He) => GenAlEx (Peakall and Smouse 2006, 2012
  - Principal Component Analysis => GenAlEx (Peakall and Smouse 2006, 2012)
  - Correspondence Factor Analysis (AFC) => GENETIX (Belkir et al 1996-2004)
  - Analysis of relatdness (ML-RELATE (Kalinowski et Al 2006)



3. Preliminary work: search of historical haplotypes

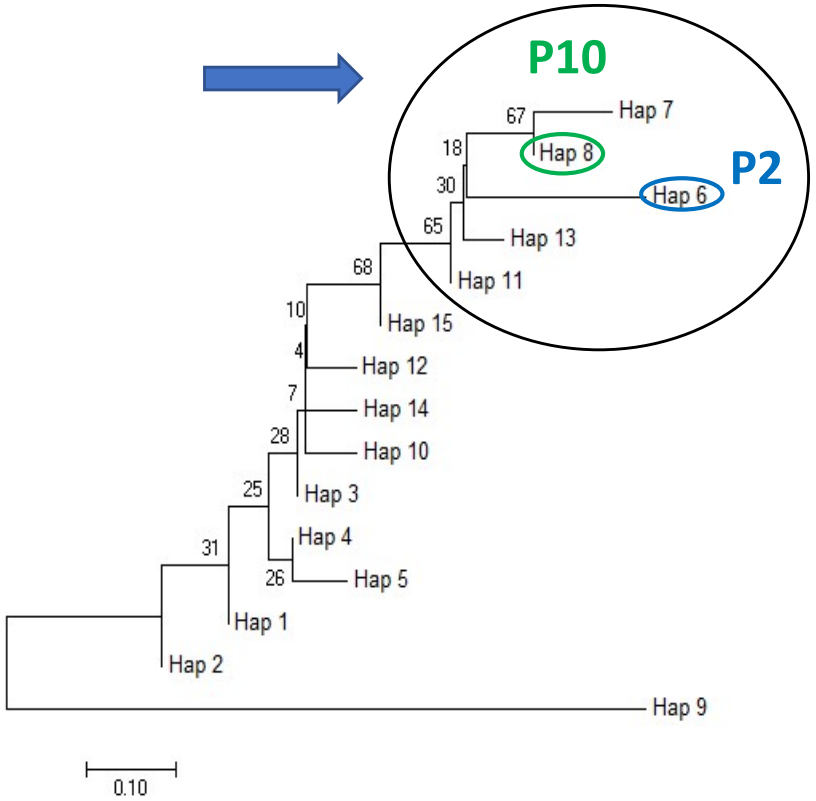
- ***Identification of Italian historical haplotypes in breeding centers following the described workflow***

✓ 3 farms declaring presumed historical native individuals tested

✓ Search for the presence of historical haplotypes Pdx_W2 (P2), Pdx_W7 (P10), Pdx_W1 (MW)

Breeding centers	Positive %
Emilia Romagna	**3%**
Umbria	0%
Sicilia	0%

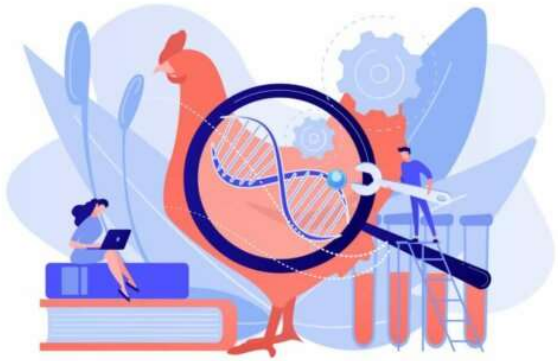
Exclusive Italian haplotypes



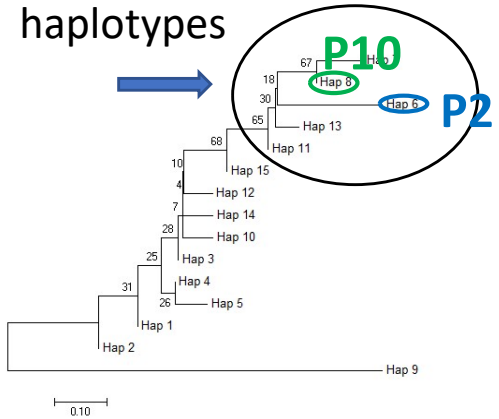
4. Action A.4: The genetic selection of reproductive pairs

- ***Aim***

  Selection based on historical native haplotypes



Exclusive Italian haplotypes



- ***Foreseen***
  - ✓ Genetic analysis of 1,750 individuals (first year)
  - ✓ Genetic analysis 500 new individuals (second year)
  - ✓ Obtaining 200 breeding pairs in the 1st year and 200 in the 2nd year, to be reproduced

Result summary

Years	2019	2020
Type of analysis	mt dna	
foreseen	1750	500
collected in db	4028	746
total analized	2905	699
aplotipe mw	1212	479
aplotipes P2-P10	124	212
total aplotipe (P2-P10 + MW)	1336	691

Deviation from planned activities

- ✓ *Analysis of a higher number of individuals* due to the scarcity of historical haplotypes (*2905 VS 1750, 699 vs 500)*

- ✓ A non-foreseen analysis of **individual genetic variability** (Nuclear-DNA) was carried out **to reduce the effects of inbreeding** typical of breeding farms. A total of 360 were analysed **for microsatellite loci.**

5. Action D.2: Genetic monitoring of reproductive stock and reintroduced population

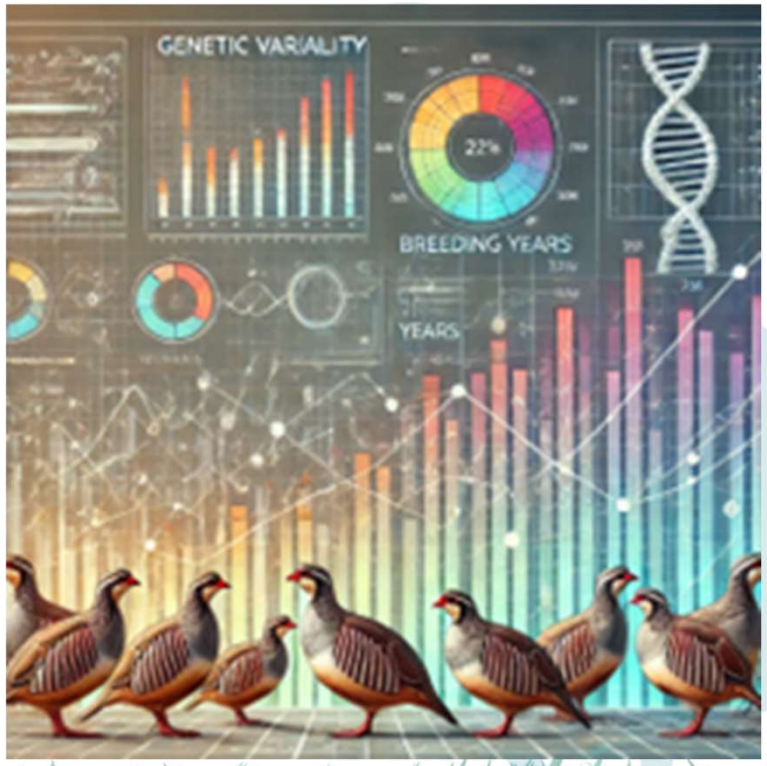
- ***Aim***

  Indirectly provides *information on the fitness and adaptability* of released individuals and the potential for sustaining a long-term viable population.

- ***Foreseen***

  Analysis of genetic variability on:

  - ✓ 400 specimens each year, individuals released in summer (over 3 years).
  - ✓ 30 from captures (over 3 years).
  - ✓ 100 from samples found on the ground (feathers) (over 2 years).



Result summary

Years	2021-2024			
Tipe of sampling	reintroduced	On-site monitoring		total
		recapture	field collection	
foreseen	1200	90	200	1490
obtained	1820	28	56	1904
analyzed	1458	28	56	1542
genotype obtained	1356	23	13	1392

- ✓ **Difficulty encountered:** finding feathers to recapture and on the ground
- ✓ **Solution adopted:** Analysis of an increased number of samples from releases and collection of feces instead of feathers.
- ✓ The total number of analyzed samples was higher than the foreseen

Deviation from planned activities

- ✓ Additional samples collected in *2018 and 2019 were also analyzed* at STRs (microsatellites) with the aim of:
  - Selecting mating couples to *reduce the risk of inbreeding* and maximise genetic diversity
  - *Characterizing the genetic variability at the beginning of the project* and monitoring any deviations during the project.

2021-23

2018

2019

- ✓ Analysis of an *increased number* of samples *from releases* and collection of *feces* instead of feathers, due to difficulty in finding feathers to recapture and find on the ground

Results of the analyses on variability

- ✓ *No significant differences in genetic variability* (Na, Ho, He) across the years

- ✓ the lowest values of **RIC** due to the poor numerical consistency
- ✓ **RIC**: samples obtained from recapture or found at release sites

Na= n alleles, Ho = observed Heterozigosity, He = expected Heterozigosity

Seasons		N	Na	Ho	He
2018	Average	99	**7,143**	**0,614**	**0,624**
	Standard error		1,870	0,099	0,082
2019	Average	229	**7,143**	**0,627**	**0,644**
	Standard error		1,957	0,071	0,074
2021	Average	244	**7,000**	**0,625**	**0,634**
	Standard error		2,000	0,080	0,077
2022	Average	372	**7,286**	**0,637**	**0,640**
	Standard error		2,090	0,074	0,075
Ric 2022	Average	**13**	4,857	0,579	0,606
	Standard error		0,986	0,088	0,078
2023	Average	390	**7,429**	**0,605**	**0,633**
	Standard error		2,170	0,076	0,075
Ric 2023	Average	**19**	5,714	0,662	0,635
	Standard error		1,426	0,090	0,062
Ric 2024	Average	**5**	3,143	0,533	0,535
	Standard error		0,404	0,123	0,065

Results of Analysis of Molecular Variance

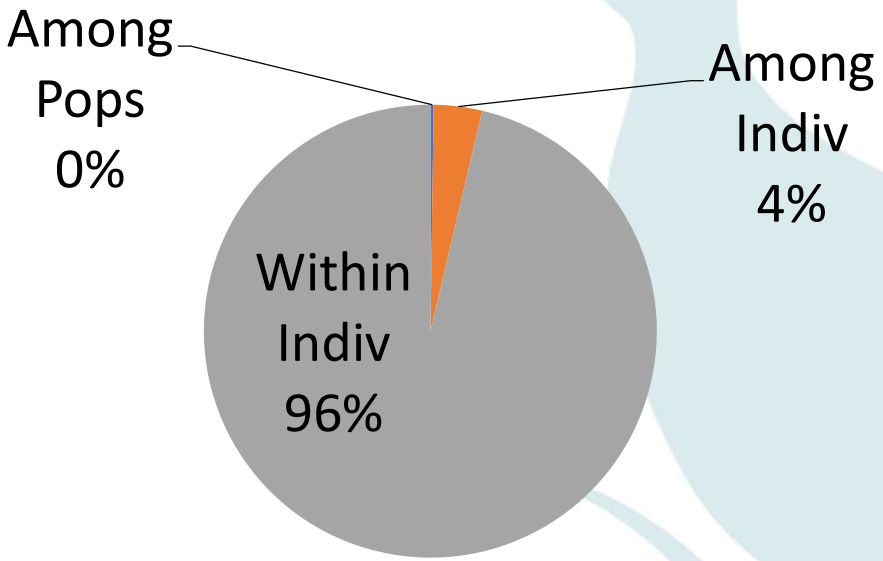
- ✓ The percentage of variance among populations is 0, *there are no differences among years*

Summary AMOVA Table

Source	Est. Var.	%
Among Pops	0,005	0%
Among Indiv	0,078	3%
Within Indiv	2,164	96%
Total	2,246	100%

F-Statistics	Value	P(rand >= data)
Fst	0,002	0,001
Fis	0,035	0,001
Fit	0,037	0,001

Percentages of Molecular Variance

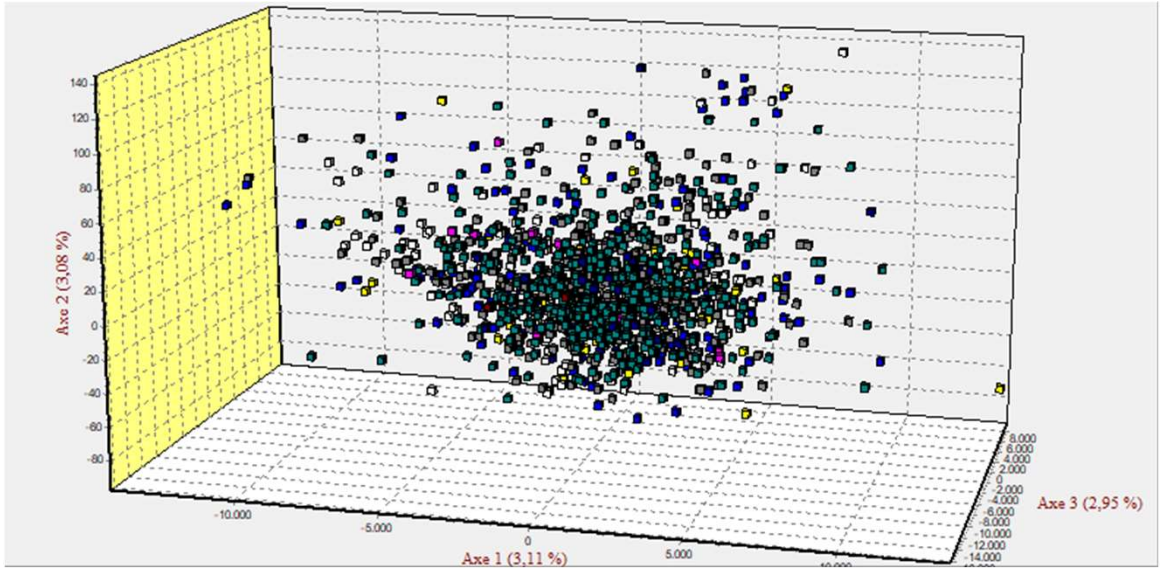


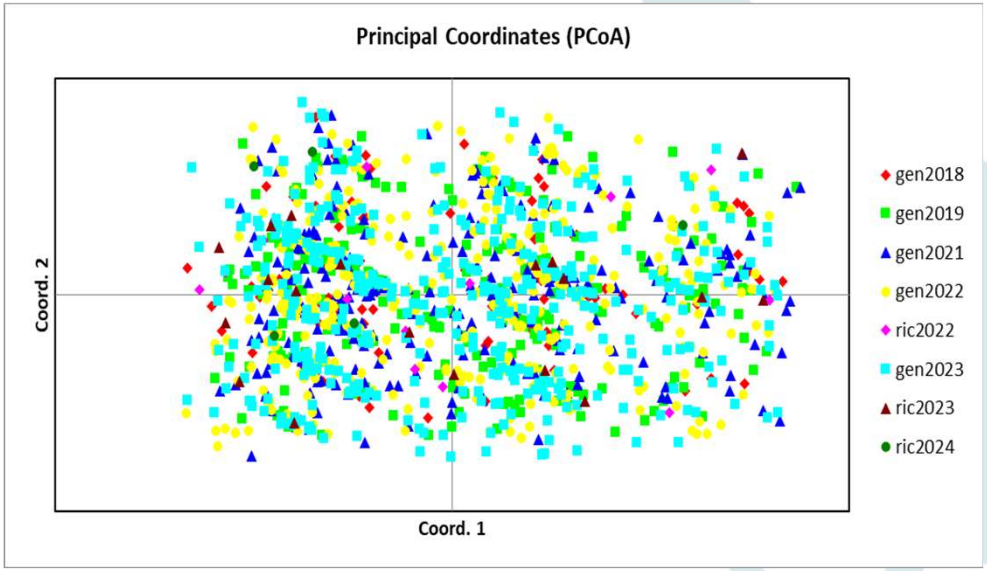
Comparison between genetic variability of the different breeding years

✓ **Statistical methods:** Factorial Correspondence Analysis (**FCA**) and Principal Component Analisys (**PCoA**) (software GENETIX and GENAIEX).

dataset: 1371 genotypes **points:** individuals **colours:** different breeding years

The graphical overlap of the points shows that the *variability among generations does not display any differences* between the breeding years. *No genetic drift during the project.*





- ✓ Genetics is a *very useful tool* in wildlife conservation, crucial during a species reintroduction

- ✓ The A4 and D2 actions allowed the *reintroduction* of specimens *genetically compatible* with those *historically present in Italy*

- ✓ *Some deviations from planned activities*

  A4:
  - *Higer number of analyzed samples* due to the rarity of historical haplotypes
  - *Non-foreseen* analysis of STRs *(Nuclear-DNA)* to reduce the risks of inbreeding

  D2:
  - *Collection of feces* instead of feathers due to difficulty in recapturing individuals and finding feathers on the ground

- ✓ *No differences in genetic variability were found across the years*

Many thanks to all contributors

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LEGAMBIENTE

Parco Delta del Po Emilia-Romagna

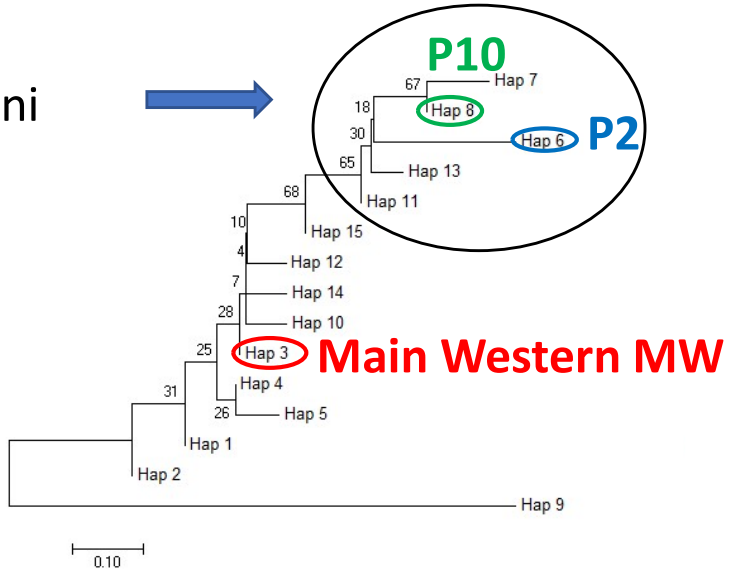
Cofinanziatore

formazione delle coppie utilizzando l'aplotipo

Ordine preferenziale per la scelta

- Femmina P2 o P10 e Maschio P10 o P2.
- Femmina P2 o P10 e Maschio MW
- Femmina MW e Maschio P2 o P10 (in percentuale ridotta).
- Femmina MW e Maschio MW (in percentuale ridotta).

Aplotipi esclusivi italiani



Work carried out

- Gestione dei campioni e redazione database

1. Predisposizione ed invio del materiale adeguato alla collezione dei campioni;
2. Supporto e tutorial per le attività di campionamento;
3. Creazione di un database excel dei campioni biologici dedicato;

- genetic analyses - aplotipe + genotipe (mt DNA + nuclear DNA)

-Identificazione aplotipi idonei alla riproduzione I e II anno

-analisi variabilità genetica per costituzione coppie riproduttrici II anno

1. Analisi di laboratorio: estrazione amplificazione e elettroforesi capillare del DNA;
2. Analisi dei dati genetici;
3. Stesura di indicazioni e linee guida per la corretta formazione delle coppie in allevamento.

Primo anno:
Scelta dei riproduttori mediante aplotipo (mt DNA)

Selezione 2019

- ✓ Analisi DNA mit => ricerca aplotipi storici MW, P2 e P10
- ✓ Oltre **4000** campioni archiviati
- ✓ **2905 campioni analizzati**. (in parte con il supporto di un laboratorio esterno, Bio-Fab Research).
- ✓ 352 aplotipi scartati
- ✓ 124 aplotipi p2-10
- ✓ 1217 non venuti togliere?
- ✓ 1212 aplotipi mw

Tenere solo il summary???

Secondo anno: Scelta riproduttori mediante aplotipo (mt DNA) e genotipo (nuclear DNA)

Selezione 2020

- ✓ Verifica degli aplotipi mitocondriali di oltre **700 individui** per conferma, su 750 inseriti in database.
- ✓ Valutazione della **variabilità genetica**, 8 loci microsatellite, su circa 360 esemplari per ridurre il rischio di consanguineità mediante l'utilizzo di software. (non prevista in questa fase)
- ✓ Costituite **274 coppie**

Tenere solo il summary???

Work carried out

- Gestione dei campioni e redazione database

1. Predisposizione ed invio del materiale adeguato alla collezione dei campioni;
2. Supporto e tutorial per le attività di campionamento;
3. Creazione di un database excel dei campioni biologici dedicato;

- genetic analyses - genotipe (nuclear DNA)

1. Analisi di laboratorio: estrazione amplificazione e elettroforesi capillare del DNA;
2. Analisi dei dati genetici;

Deviation from planned activities

- ✓ *Many more samples* than expected *were analyzed* due to the rarity of historical haplotypes *2905 VS 1750*

- ✓ In addition to selection based on haplotypes (mt-DNA), an analysis of **individual genetic variability** (Nuclear-DNA) was carried out **to reduce the effects of inbreeding** typical of breeding farms.