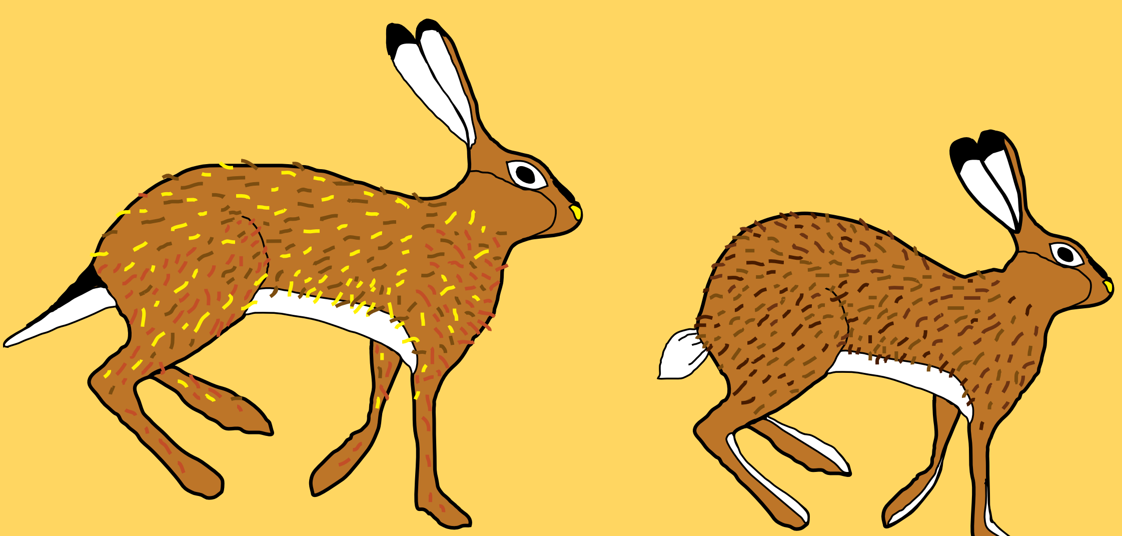
GHOST HARES

The Evolution of Hares in the Iberian Peninsula



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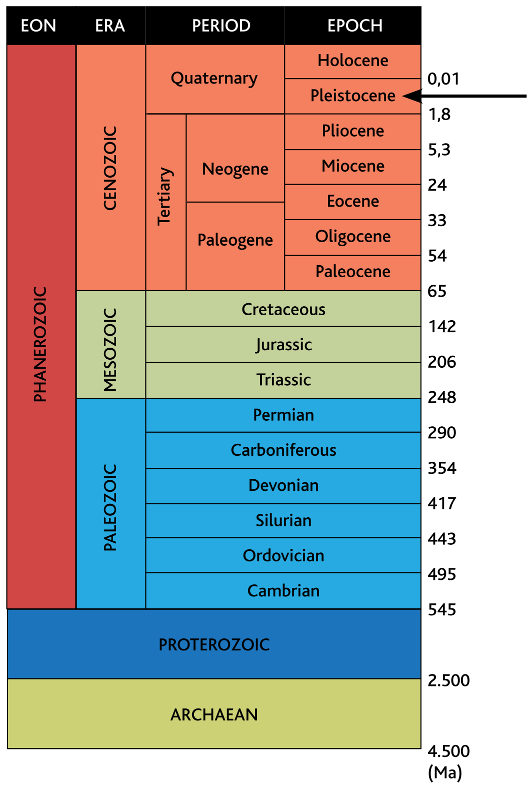
- A lot, a lot of snow!
- Really?
- And ice. Everything was frozen! Well… not everything; there were areas without snow, where there was even food and places to eat.
- Refuges?
- That! There were refuges. It was good for those who were cold. The others lived well in the snow. And there was a lot, a lot of snow!

About 21 thousands years ago the Iberian Peninsula was very cold and most of Europe was even covered by snow! This was at the end of the Pleistocene, the geologic epoch from the Quaternary period that predated the current, the Holocene. And it was during this epoch that most of the planet suffered many glacial events and that's why the Quaternary is also known as the glacial period. The modern glacial period, because previously there were another four! One of the major consequences of these climatic oscillations was changes in the geographic distribution and genetic variability of species, especially those that inhabited regions most affected by glaciations.

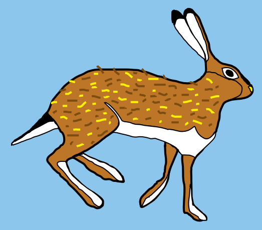
But why are we talking about Quaternary, Pleistocene, Iberian Peninsula, Europe and snow? Because we will be talking about hares!



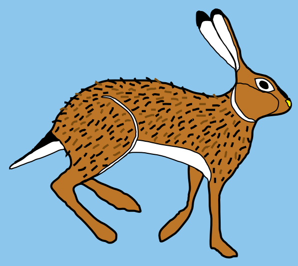
The history of Earth can be divided in several moments. During the Pleistocene, identified with an arrow, there were profound climatic oscillations that influenced the evolution of many living beings, including the Iberian and European hares.

SCIENTIFIC DESIGNATION OF HARES

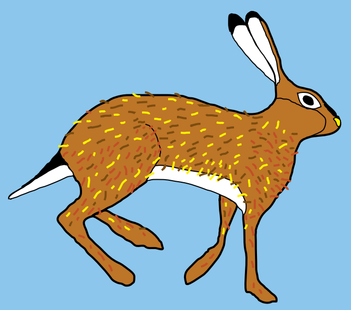
Hares are lagomorphs, which mean that hares are mammals that belong to the order Lagomorpha. There are two recognized families within Lagomorpha: the ochotonids (Ochotonidae) and the leporids (Leporidae). Hares, as rabbits, belong to this last family. And this is a big family: it has 11 genera. Hares belong to genus *Lepus* that is also very rich in species: there are currently 32 hare species! The protagonists of this story belong to species *Lepus granatensis* (the Iberian hare), *Lepus castroviejoi* (the broom hare), *Lepus europaeus* (the European hare) and *Lepus timidus* (the mountain hare).



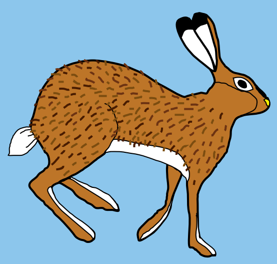
IBERIAN HARE



BROOM HARE



EUROPEAN HARE



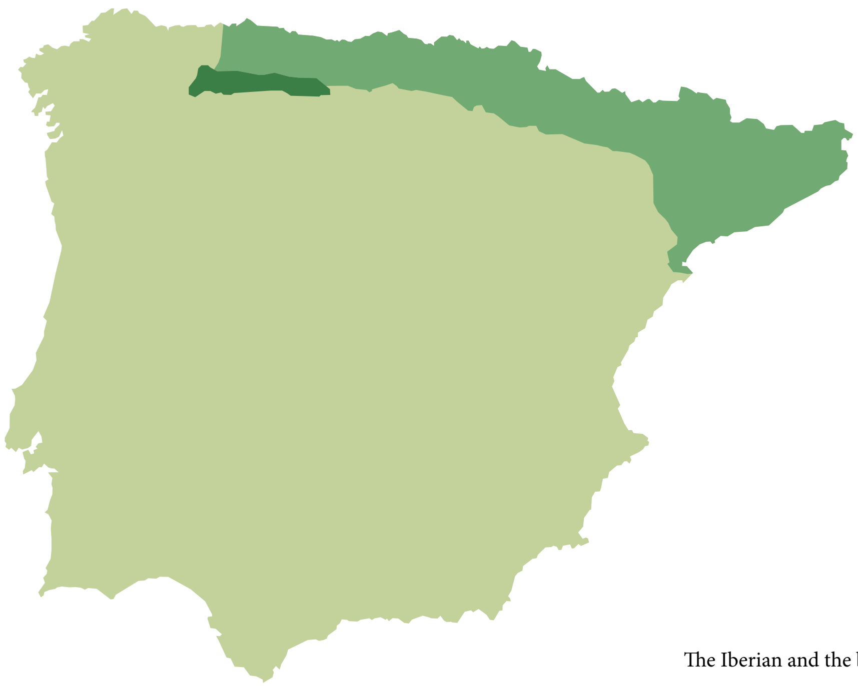
MOUNTAIN HARE

LAGOMORPHA

- OCHOTONIDAE
  - PIKAS
    - *Ochotona*
- LEPORIDAE
  - HARES
    - *Lepus*
  - RABBITS
    - *Brachylagus*
    - *Bunolagus*
    - *Caprolagus*
    - *Nesolagus*
    - *Oryctolagus*
    - *Pentalagus*
    - *Poelagus*
    - *Pronolagus*
    - *Romerolagus*
    - *Sylvilagus*

Nowadays three species of hares inhabit the Iberian Peninsula: the Iberian hare, the broom hare and the European hare. The European hare also occurs in other European and Middle Eastern countries, such as Germany or Iraq, but the Iberian and the broom hares can only be found in Iberia. The Iberian hare occupies almost the entire Peninsula, except its Northern part between Asturias and Catalonia where the European hare lives, whereas the broom hare only occurs in the Cantabrian Mountains.

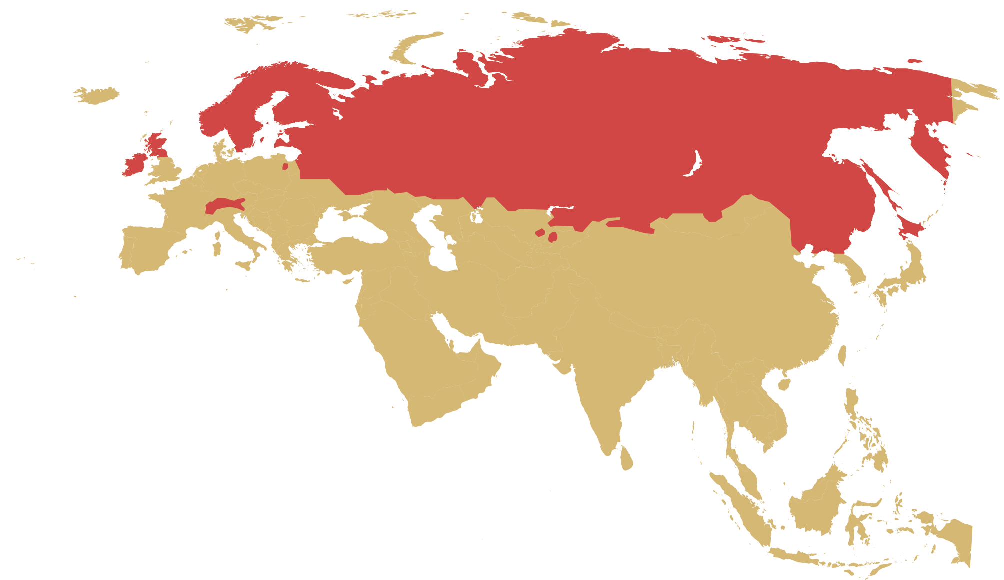
These are the species that inhabit Iberia today. But many years ago – 21 thousand years! – another species lived in the Iberian Peninsula: the mountain hare.



The Iberian and the broom hares are endemic to the Iberian Peninsula, that is, they only inhabit this region. The Iberian hare (light green) is distributed across most of the Peninsula while the broom hare (dark green) inhabits only the Cantabrian Mountains region. The European hare (median green) occupies the Northern part of Iberia.

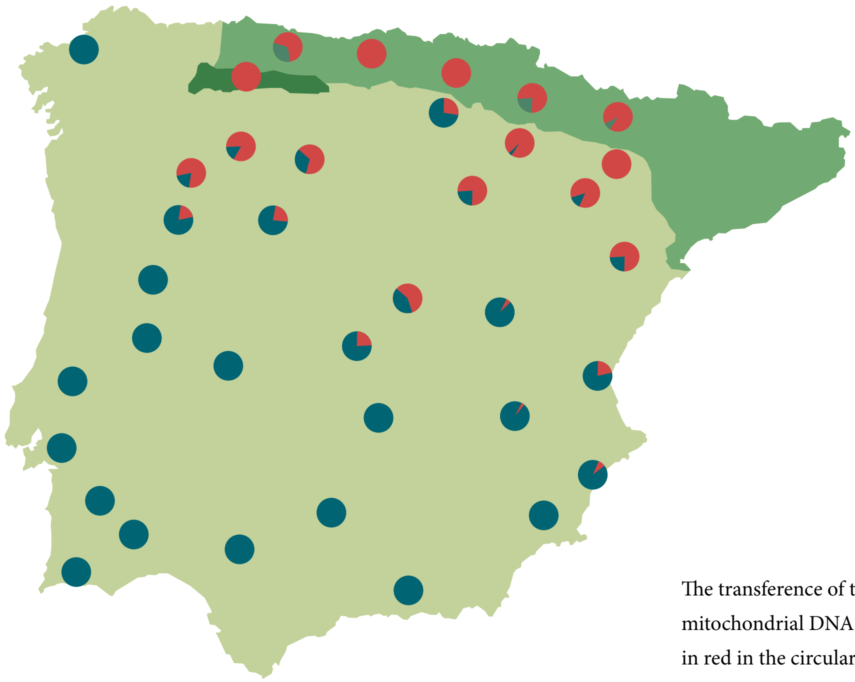
The mountain hare is well adapted to live in regions with low temperatures and long periods with snow cover. And about 21 thousand years ago, during the Quaternary glaciations, the Iberian Peninsula had those conditions. Today we can no longer found this species in Iberia but we can find its vestiges.

The fossil record show that during the Pleistocene the mountain hare lived in areas where it no longer exists, such as the Iberian Peninsula or the South of France. It also shows a very curious fact: there were shifts in the range of the areas where the mountain hare lived during the glacial cycles of the Pleistocene. Why is this result important for this story? Because those shifts allowed the mountain hares to contact with other hares and thus increase the probability of mating and consequent exchanging of genetic material between individuals from the different species!



The mountain hare is an arctic/boreal species that inhabits the North of Eurasia; some isolated populations occur in Scotland, Poland, the Alps and Japan.

The proof that the mountain hare did in fact mate with species that inhabit Iberia today is that even though there is no longer mountain hares in this region its DNA persisted. Analysing several individuals from the three Iberian species showed that all broom hare individuals and almost all European hares that live in Iberia have mitochondrial DNA from the mountain hare. This DNA is also very frequent in Iberian hare individuals from the Northern half of the Peninsula.



The transference of the arctic mitochondrial DNA - represented in red in the circular graphics - to the hares from Iberia was a large scale phenomenon that affected the three Iberian hare species, the Iberian hare (in light green), the broom hare (in dark green) and the European hare (in median green). However, the transference frequency was higher in the Northern part of the Peninsula.

HARES AND CLIMATIC CHANGES

The fast climatic changes faced by planet Earth due to human actions will primarily affect species with more specific ecological needs; that is, species that can only live in places with certain characteristics. When talking about hares these climatic changes may have a major impact in their long term survival. An example is our now familiar mountain hare. Climatic models had already showed that this species prefers cold places, with marked differences between day and night temperatures and very dry summers.

Species with a higher dependence for the maximum temperature will be the first to show higher sensibility to climatic changes because this thermal limit is highly conserved in nature. And what does this means? It means that species that inhabit regions where temperatures are close to their maximum tolerable temperatures are the ones that will have more difficulties to adapt to global warming.

In fact, man-driven global warming is already affecting the survival of the mountain hare in some places like the Alps or in the Southern limit of its distribution. In both cases the reduction of the suitable habitat for the mountain hare is accompanied by the arrival and slow substitution of this species by the European hare. Going back to the past, it is possible that the post-glacial natural warming of the planet may have affected the distribution of the mountain hare in the same way: reducing the areas where it could live and favouring the occupation of the habitat by the other species of hares that inhabited the Iberian Peninsula.

Why do we say Iberian hares have mountain hare mitochondrial DNA instead of saying there is mountain hare in Iberian Peninsula?

The answer is somewhat simple: the analysis of the nuclear DNA from the four species shows in general four distinct groups. This means that if we use nuclear DNA as genetic information to identify the species then we have four species and the mountain hare does not exist in Iberia. But if we use only the information from the mitochondrial DNA then the identification of the species becomes confusing because some Iberian and European hares will be identified as mountain hare and we will say that the mountain hare inhabits the Iberian Peninsula. And this is not true because we must use other information to classify and identify species besides genetic information. And in this case these species are easily distinguishable if we use information from morphology and ecology.

SYSTEMATICS OF HARES

Systematics is the science that deals with the classification of the living beings considering their evolutionary history and relationships. In the case of the hares, their evolution has some interesting particularities. For example, many hare species evolved in approximately the same time – a type of evolution called a "fast radiation". This is the case with hares from Iberia that probably originated within the last 2 million years. One of the consequences of this type of rapid and almost simultaneous diversification is that different species conserve many of the characteristics from the ancestral species, sharing a fraction of its genetic patrimony and thus also many of its characteristics (such as morphology, physiology or ecology). This reason alone would be enough to complicate classifying hare species but when talking about hares from the Iberian Peninsula we have an additional complication: the existence of individuals with a "ghost" mitochondrial DNA, the mountain hare mitochondrial DNA!

Then what is the best way to classify living beings? For any case the best is to always use multiple types of data and critically evaluate the information we get from each.

Shall we try? We will need:
- cards with information from a mitochondrial DNA fragment from individuals of the 4 species that inhabited Iberia about 21 million years ago: the mountain hare, the Iberian hare, the European hare and the broom hare
- cards with information from a nuclear DNA fragment that encodes the albumin gene from individuals from the same 4 species
- cards with images of individuals from the same 4 species

The cards (attached) with each type of data - mitochondrial DNA, nuclear DNA and morphology - should be distributed to each player or team of players in turns. Each time a player gets a set of cards, they should group them using the similarity between information as criteria. For example, if playing with the cards with the mitochondrial DNA fragment, one should look for fragments with the least number of mutations - with the least number of differences - between each and group them.

After having all the cards grouped according to each type of information, confront the groups with the "real" identification of each individual and discuss the importance of using several types of data to classify biodiversity. We can also use the information given throughout these pages to make the discussion more complex, and for example debate the role of natural selection and the differences in information we get from the different genetic regions.

We also recommend a discussion about the nature of science: in the example of the hares, if in the early studies using genetic information to classify species we'd only used mitochondrial information what would be the result? There is mountain hare in Iberia! But science is a process, it never stop and is always questioning. Thus we look for more data and re-evaluate conclusions: we know that there is indeed mitochondrial DNA from the mountain hare in Iberia but it is like a ghost, a molecular fingerprint of a species that used to live in Iberia but is no longer there since the Last Maximum Glacial, approximately 21 million years ago. What we know today does not mean that the early scientists working on this would be wrong; it only means that our conclusions depend on data and that we should always keep an open but critical mind to incorporate new data and re-evaluate our conclusions.

There is yet another interesting result: the arctic mitochondrial DNA present in the hares from Iberia shows differences in relation to the arctic mitochondrial DNA present in the mountain hare. This is in agreement with the hypothesis that this mitochondrial DNA was transferred to the Iberian populations a long time ago - the 21 thousand years since the last maximum glacial! - and since then is evolving independently from the mitochondrial DNA from the extant mountain hare.

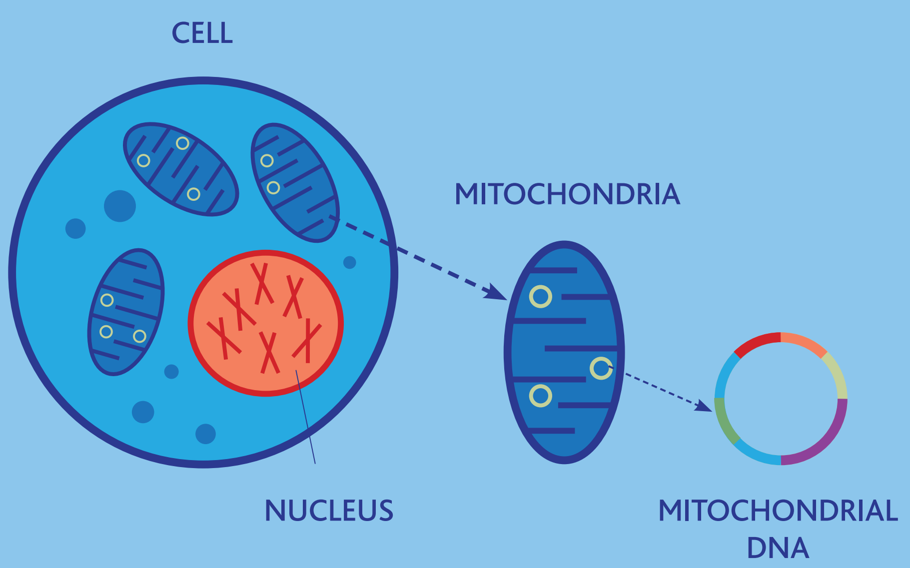
Lastly the data also suggest that there were two times to the transference of the mitochondrial DNA from the mountain hare to the other hares from Iberia: one during the Middle Pleistocene that affected the common ancestral of the broom and the Corsican hares (*Lepus corsicanus*; a species that exists only in the South of Italy, Sicily and Corsica) and another during the last maximum glacial that affected the three species that inhabited Iberia.

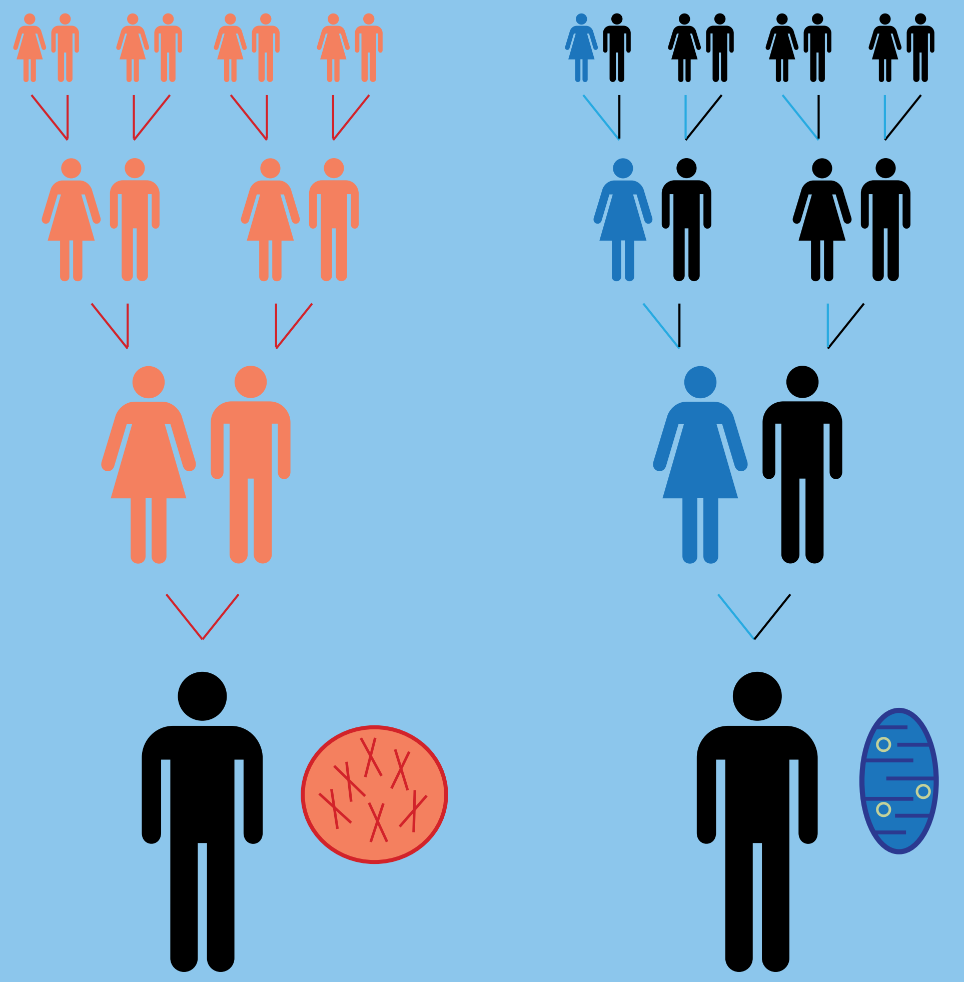
MITOCHONDRIA AND EVOLUTION

Mitochondria are cellular organelles found in a large number of cells of almost all eukaryotes. Its function is vital to the cells. Even though mitochondria are better known for their role in energy production, they also actively participate in several cellular processes such as cellular differentiation, cellular signalling or maintaining control of the cell cycle and cell growth. And there are even mitochondrial diseases caused by mitochondrial malfunctioning.

One of the most particular characteristics of this organelle is that it has its own genome. That genetic material is called mitochondrial DNA - or mtDNA. This DNA differs from the DNA from the nucleus not only because of its location but also because it has a circular configuration and, contrary to nuclear DNA, that is inherited from both parents and thus each one of our cells have two copies of each nuclear information, mitochondrial DNA is transmitted only by the mother.

These characteristics, together with the fact that its mean mutation rate is higher than the nuclear DNA and the fact that it was initially considered free from the influence of natural selection, made mitochondrial DNA a highly-used genetic marker especially in studies that aimed at reconstructing the evolutionary history of species. But the apparent neutrality of the mitochondrial DNA has been challenged: considerable evidence that this marker is also targeted by natural selection has started to show up and this includes the mitochondrial DNA from the mountain hare!





And so, to sum up, what do we know about the presence of the mountain hare and the history of hares in Iberia? We know that there is arctic hare mitochondrial DNA in hares that live in the Northern half of Iberian Peninsula, and that this DNA is also more diverse in these populations. There are also some results that suggest that the transference of the arctic mitochondrial DNA to the Iberian hare happened when temperatures started to increase after the last maximum glacial and when the Iberian hare started to occupy the space of the mountain hare. Since some Iberian hares from the Southern Iberian populations have nuclear DNA from the mountain hare it is probable that the Iberian hare survived colder periods in refugees located in the middle of the Iberian Peninsula and colonized the land they now occupy from there.

What we still don't know very well is if the transference of the mitochondrial DNA from the mountain hare to the other hares that inhabit Iberia was an adaptive process; or, in other words, if natural selection influenced this transference.

If there was no influence of natural selection then the transference occurred only because of geographic distribution and demography: during an expansion there are usually only a few leading individuals arriving to the new places. And these usually find many individuals from species that inhabit that place. In the case of hares, since they probably had many similarities, individuals from that arriving species were able to reproduce with individuals from the resident species. And since genetic drift is very effective in small populations it enables the mitochondria from the resident species to cross to the arriving species and thus to substitute its own mitochondria! This substitution is even more effective if females from the expanding species are philopatric, have a tendency to stay close to the home place, as in the case of hares.

But we must also consider the hypothesis of natural selection influencing this transfer. Because although early evolutionary biological studies considered the mitochondrial DNA free from the action of natural selection, we now have available several documented cases of the influence of this mechanism in the evolution of this genetic information. Looking to the strange case of the mitochondrial DNA of the hares from Iberia it is quite possible to think that maybe the mitochondrial DNA from the mountain hare had passed on so many times to the other hares because it is indeed advantageous. This means that this could be a case where mitochondrial DNA is under natural selection. And the truth is that recent data do suggest that! Another interesting aspect is that proteins that participate in the respiratory chain - the main process responsible for energy production in cells - have sub-units encoded by genes both in the mitochondrial and in the nuclear DNA. The interactions between the two genomes are indeed extensive and natural selection can influence simultaneously the interacting genes. This makes it possible that the passage of the arctic mitochondrial DNA to the hares from Iberia was accompanied by the interacting nuclear genes - a process of co-evolution! The more recent studies are also focusing on this aspect.

NATURAL SELECTION AND GENETIC DRIFT

Natural selection and genetic drift are two mechanisms that lead to changes in frequencies of genetic information in populations and thus influence their evolution. The major difference between both mechanisms is that while genetic drift is a purely neutral phenomenon and affects equally all portions of the DNA, mitochondrial or nuclear, natural selection acts specifically over certain information.

The two mechanisms are not mutually exclusive. On the contrary: we should assume that both act simultaneously, only different situations will make the effects of one or the other become more visible.

For example, men are continually producing spermatozoids, each with unique genetic information. However, only one in millions of spermatozoids fertilize the egg that will then grow to a new baby. And half of the baby' genetic information came from that single spermatozoid. Does that genetic information had any special characteristic? The simplest answer would be: no! That spermatozoid was able to fertilize the egg by chance. And that is genetic drift: chance. But at the same time it could happen that a portion of the genetic information in that spermatozoid confers some sort of advantage to the future baby. In this case, that information will be targeted by natural selection and the baby, then an adult, will have a higher probability to survive and pass on that information to his/her offspring thus contributing to increase the frequency of the information in the population.

In most cases genetic drift causes barely noticeable changes in the genetic frequencies of a population. Because in most cases populations are large enough to make it happen. But sometimes there are strong reductions in the size of populations that allow genetic drift to become effective in causing changes. In the case of our hares the expansion of the hares from Iberia and the colonization of the land occupied by the mountain hare would have happened with just a few individuals – a bit like a marathon: there are many runners near the starting line but when the whistle signals the start of the race only a few run away from the initial group. These individuals would have encountered mountain hare individuals with whom they mated. Since female hares are philopatric, meaning that they tend to stay close to their home place, mating would have happened between Iberian hare males and mountain hare females and thus the offspring of such reproductive events have the arctic mitochondrial DNA. The fact that there were more mountain hare individuals caused the number of individuals with the arctic mitochondrial DNA to increase in each generation.

But it can also happen that the arctic mitochondrial DNA has some characteristic that makes it especially advantageous, particularly in cold regions. In this case, the transmission of this genetic material from the mountain hare to the Iberian hare would not have happened by chance, as mentioned before, but instead due to natural selection.

Shall we see how the two mechanisms work?

Then we will need:
- two opaque boxes
- colourful beans
- colourful discs (6 different colours and one of the colours should be identical to one of the bean colours)

We fill one of the boxes with a mix of colourful beans and the other with beans of a single colour (for example, yellow). Beans should cover about 2/3 of the box. We separate 6 discs of each colour (don't forget to have yellow discs). Beans in the box simulate a given environment, discs simulate individuals of a hare species from the Iberian Peninsula – for example, the European hare – and colours simulate a type of mitochondrial DNA.

We place the discs we separated in the box with the colourful beans and mix well. One at a time, three participants have five seconds each to try to catch as many discs as they can. Then they should separate these discs - that were hunted and "died" – and take the remaining – the ones that "survived". Observe both groups and check for differences between them: Was there any colour that was most hunted? Do colours that "died" and that "survived" have any relation with the environment? If we repeat the game would we have groups with similar composition? The answer to this last question would be: no. Why? Because the evolutionary mechanism influencing the evolution of this disc population is genetic drift, chance. To continue playing we simulate the next generation giving 2 offspring to each "surviving" disc and taking these survivors out of the game. The offspring should have the same colour as the parent. Thus the second generation is composed only by the offspring of the first generation. Again, we hunt the discs, we observe the "dead" and "survived" groups and try to answer the same questions; now we will also be able to compare colours that were more and less hunted in the two generations: Were they the same? (probably not!)

To contrast evolution by genetic drift with evolution by natural selection re-start the game but this time placing the discs in the box with the single colour beans. Repeat the hunt, the observation of the two groups and the simulation of a second generation. Are there differences in the colours that were hunted in the previous game (with the colourful beans)? Probably the yellow discs were the least hunted. Why? Because contrary to the previous game where no characteristic was evidently advantageous in the colourful environment, in this game yellow seems to give an advantage allowing these discs to be less hunted and to have more opportunities to leave offspring that are also yellow - the frequency of yellow discs increases in each new generation while the frequency of the other colours decreases. This means that the evolutionary mechanism that is probably influencing the evolution of this disc population is natural selection.

In the story of our hares we can then discuss if the arctic mitochondrial DNA is the "yellow colour" in a "yellow" environment, that is, if in fact it confers any advantage to the individuals that carry and transmit it to their offspring.

Now what?

Between 2014 and 2015 a team of Portuguese and Spanish researchers tried to understand the meaning of this strange "ghost mitochondria" phenomenon. This work was a natural extension of previous work but giving priority to the biogeographic analysis of the involved species. The reasoning is that combining the genetic information of a species with its spatial distribution we are able to understand better the evolutionary history of that species. This combined analysis of genetics and geography is called "phylogeography".

BIOGEOGRAPHY AND EVOLUTION

The branch of science that studies and seeks to interpret the distribution - past, present and future - of species on Earth is called Biogeography. An important component of biogeographic studies is the analysis of the physical characteristics of the environment and the way these influence species and shape their distribution in space.

Alfred Russel Wallace, the naturalist that presented the theory of evolution by means of natural selection with Charles Darwin, was a pioneer using geographic and ecological data together with concepts from the theory of evolution to characterize patterns of species distribution. Contrary to the predominant idea that species are created according to the environment they inhabit, Wallace realized that geography plays an important role in species distribution, creating barriers that influence the evolution of different species in similar climatic regions. Wallace observations about the way climate, geography and ecology influence species evolution and spatial distribution are still recognized as valid.

Wallace, as Darwin, had the opportunity to travel across several regions of the Earth and was very meticulous in his observations. And that's how he realized that the Earth has six major ecologically distinct regions. For example, during his stay in Indonesia he found numerous differences between species that inhabited the Asian side and those that inhabited places closer to Australia. He then defined a line that divides these two regions. This line is known as the "Wallace line" and Wallace is recognized as the "father of biogeography".

The biogeographic regions described by Wallace grossly correspond to the six continents. And together with what we know about continental drift and plate tectonic were a major contribution to our understanding of the dynamics of species spatial occupation throughout time and their evolutionary relationship. And thus among other results we know that species from continents that separated more recently have also less genetic differences among them.

By studying the close relation between species and regions they occupy as well as the relation they establish with other species inhabiting the same space, biogeography is fundamental to our understanding of species' capacity to survive in the face of rapid climatic changes, as we face today, and to be able to define appropriate conservation measures to each species and each region.

To start this work the team asked two major questions:
1) Why is the transmission of an arctic hare mitochondrial DNA to temperate hare species so common?
2) Does this arctic DNA confer an adaptive advantage to the affected species in the Northern range of their distribution?

The first thing they found was that in fact Iberian hare populations with more individuals carrying the arctic mitochondria are in areas that would have had a good climate for the mountain hares while they inhabited Iberia. That is, it seems that places where we find today hares from Iberia with arctic mitochondrial DNA are almost the places where we would actually see the mountain hare if we had been there during the last maximum glacial! This is an interesting result because it suggests that maybe the transference of the arctic mitochondria to the hares from Iberia in these areas might be related with a local adaptation, meaning that it gives some clues that favour the hypothesis of natural selection acting on this transference. And it is also an important result for us to know better the relation between climate and hare evolution.

After having the suggestion about the places where the mountain hare might have existed while in Iberia we look carefully to the places where hares with the mountain hare mitochondrial DNA currently live. And we see that the number of individuals from Iberian hare species with the arctic DNA increases northwards and that there are signs that this increase is related with an expansion of the Iberian hare to zones inhabited by the mountain hare.

What probably happened is that during the last maximum glacial, when temperatures started to increase, Iberian hare individuals living in a central Iberia refuge invaded areas occupied by the mountain hare, outcompeted and replaced them. It is also possible that some of the Iberian hare individuals left the refuge to occupy other regions in Iberia where today we find the arctic hare DNA in lower frequencies.

We know from other studies that even during the last maximum glacial Iberia had regions in the North and in the South that would have been good for the Iberian hare, but that this species only survived in central refuges. Thus the most likely explanation for this geographic expansion of the Iberian hare happening only in the end of the last maximum glacial must have been related with the presence of the mountain hare throughout Iberia. On other words, when temperatures were very low the mountain hare would have been better adapted and impeded the Iberian hare to live in those places; when the temperature started to increase the Iberian hare was able

to invade and expel the mountain hare from Iberia - but while that happened some individuals from both species reproduced, allowing the transference of the mountain hare mitochondrial DNA to the Iberian hare! The same could have happened with the European hare that must have arrived in Iberian after the last glacial maximum and occupied the Northern part of Iberia, where it still lives today.

But what about the advantage, could that be that the hares carrying the arctic DNA have in fact some advantage in relation to the other? It seems so.

And how do we know that?

First we tried to understand if the arctic hare mitochondrial DNA is more frequent in a certain type of environment or if its distribution is just the result of geography. These analyses showed that Iberian hare individuals with the arctic mitochondrial DNA live in environments that are significantly different from the environment where individuals without this DNA live. This means that the distribution of the arctic hare mitochondrial DNA is concordant with some environmental variables which is a result supporting the probable adaptive advantage of the arctic mitochondrial DNA.

Next, to have a clue about the possible advantage of the arctic DNA we determined a few parameters that help characterize the reproductive capacity and physical condition of hares with and without that DNA type. The analysis of almost 200 individuals from the Southern limit of the distribution of the arctic mitochondrial DNA showed that there is a strong association between the reproductive capacity and the presence of this DNA. Data also suggest that these individuals have a better diet quality which suggests that they have a better body condition. Using other words, the first results of an analysis that explicitly test for an association between having arctic mitochondrial DNA and having survival and reproductive advantage seem to indicate that this association does exists; that in fact natural selection seems to have influenced the arctic hare mitochondrial DNA evolution in the Iberian hare!

How can we confirm these first results?

Well, on one hand there are other fitness indicators such as leptin levels or parasite load that can help confirm the existence of a relation between having the arctic hare mitochondrial DNA and being fitter. The activity of the different mitochondria may also shed some light about the possible advantage of the arctic mitochondria.

On the other hand we know that population expansion and mating between the different species leave signatures on the genetic variation of extant Iberian hare populations. One such sign was the transference of the mitochondrial DNA from the mountain hare to individuals from the three Iberian species. But other portions of the nuclear genome – the information present in all genetic material found in the nucleus of the cell – might also been transferred.

If the transference of the artic species' genetic material to the species from Iberia resulted in higher fitness and survival and reproduction capacity then the DNA transference should preferentially affect genome regions involved in those functions and the mitochondrial DNA transference may have affected the transference of other genomic regions - because proteins that result from the information in the mitochondrial and the nuclear DNA work together in many essential cell functions such as energy production. These questions are being tackled using new DNA sequencing technologies that allow looking to the whole genomic information of an individual instead of looking at only a small part of it. And thus comparing genomes from hares from Iberia and the mountain hare will help to uncover instances of adaptation and co-evolution between nuclear and mitochondrial DNA!

SUGGESTION OF EDUCATIONAL RESOURCES

To know more about the project:
https://pelayoacevedo.wordpress.com/projects/ecomito/

For a more detailed description of the activities about genetic drift and natural selection:
Campos R, Sá-Pinto A (2013). Early evolution of evolutionary thinking: teaching evolutionary biology in elementary schools. Evolution: Education and Outreach 6:25.
[the paper is available at:
http://www.evolution-outreach.com/content/6/1/25]
Sá-Pinto X, Campos R (2012). As borboletas da floresta amarela. CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos. Porto, Portugal.
[the book is available at:
https://dl.dropboxusercontent.com/u/206969216/As_Borboletas_da_Floresta_Amarela.pdf]

For examples about the influence of genetic drift and natural selection on the evolution of human characteristics:
Campos et al. (2014). Somos mutantes!
[posters available at:
http://cibio.up.pt/upload/filemanager/somos-mutantes_cartazes.pdf]

For short "stories" about evolution and biodiversity and suggestions on how to correct some common misconceptions about evolution:
Campos et al. (2013). Um livro sobre evolução. CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos. Porto, Portugal.
[the book is available at:
https://www.dropbox.com/s/t2iw0czeobtid5g/Um%20livro%20sobre%20evolucao.pdf

Acevedo P, Melo-Ferreira J, Farelo L, Beltran-Beck B, Real R, Campos R, Alves PC (2015). Range dynamics driven by Quaternary climate oscillations explain the distribution of introgressed mtDNA of *Lepus timidus* origin in hares from the Iberian Peninsula. Journal of Biogeography 42:1727-1735.

Acevedo P, Melo-Ferreira J, Real R, Alves PC (2012) Past, present and future distributions of an Iberian endemic, *Lepus granatensis*: ecological and evolutionary clues from species distribution models. PLoS ONE 7(12): e51529

Acevedo P, Jiménez-Valverde A, Melo-Ferreira J, Real R, Alves PC (2012) Parapatric species and the implications for climate change studies: a case study on hares in Europe. Global Change Biology 18:1509-1519

Altuna J (1970). Hallazgo de una liebre artica (*Lepus timidus*) en el yacimiento prehistorico de Urtiga (Guipuzcoa). Munibe 22:165-168.

Alves PC, Ferrand N, Suchentrunk F, Harris DJ (2003). Ancient introgression of *Lepus timidus* mtDNA into *L. granatensis* and *L. europaeus* in the Iberian Peninsula. Molecular Phylogenetics and Evolution 27:70-80.

Alves PC, Harris DJ, Melo-Ferreira J, Branco M, Suchentrunk F, Boursot P, Ferrand N (2006) Hares on thin ice: introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. Molecular Phylogenetics and Evolution 40:640-641.

Alves PC, Melo-Ferreira J, Freitas H, Boursot P (2008). The ubiquitous mountain hare mitochondria: multiple introgressive hybridization in hares, genus *Lepus*. Philosophical Transactions of the Royal Society B 363:2831-2839.

Alves PC, Hackländer K (2008). Lagomorph species: geographical distribution and conservation status. In Lagomorph Biology: Evolution, Ecology, and Conservation (Alves PC, Ferrand N, Hackländer K, eds.), Springer-Verlag. Pp 395-405.

Araújo MB, Ferri-Yáñez F, Bozinovic F, Marquet PA, Valladares F, Chown SL (2013). Heat freezes niche evolution. Ecology Letters 16(9):1206–1219.

Avise JC (2000). Phylogeography: the history and formation of species. Harvard University Press.

Chapman JA, Flux JEC (2008). Introduction to the Lagomorpha. In Lagomorph Biology: Evolution, Ecology, and Conservation (Alves PC, Ferrand N, Hackländer K, eds.), Springer-Verlag. Pp 1-9.

Lopez-Martinez N (1980). Les lagomorphs (Mammalia) du pléistocène supérieur de Jaurens. Nouvelles Archives du Museum d´Histoire Naturelle de Lyon 18:5-16.

Melo-Ferreira J, Boursot P, Suchentrunk F, Ferrand N, Alves PC (2005). Invasion from the cold past: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. Molecular Ecology 14:2459-2464.

Melo-Ferreira J, Boursot P, Randi E, Kryukov A, Suchentrunk F, Ferrand N, Alves PC (2007). The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. Molecular Ecology 16:605-618.

Melo-Ferreira J, Alves PC, Freitas H, Ferrand N, Boursot P. (2009). The genomic legacy from the extinct *Lepus timidus* to the three hare species of Iberia: contrast between mtDNA, sex chromosomes and autosomes. Molecular Ecology 18(12): 2643-2658.

Melo-Ferreira J, Alves PC, Rocha J, Ferrand N, Boursot P (2011). Interspecific X-chromosome and mitochondrial DNA introgression in the Iberian hare: selection or allele surfing? Evolution 65:1956-1968.

Melo-Ferreira J, Boursot P, Carneiro M, Esteves PJ, Farelo L, Alves PC (2012). Recurrent introgression of mitochondrial DNA among hares (*Lepus* spp.) revealed by species-tree inference and coalescent simulations. Systematic Biology 61: 367-381.

Melo-Ferreira J, Farelo L, Freitas H, Suchentrunk F, Boursot P, Alves PC (2014). Home-loving boreal hare mitochondria survived several invasions in Iberia: the relative roles of recurrent hybridisation and allele surfing. Heredity 112: 265-273.

Melo-Ferreira J, Vilela J, Fonseca MM, da Fonseca R, Boursot P, Alves PC (2014). The elusive nature of adaptive mitochondrial DNA evolution of an arctic lineage prone to frequent introgression. Genome Biology and Evolution 6(4): 886-896.

Rand DM (2001). The units of selection on mitochondrial DNA. Annual Review in Ecology and Systematics 32: 415-448.

Smith AT, Johnston CH (2008). *Lepus timidus*. In*: IUCN 2013. IUCN Red List of Threatened Species*. Version 2013.2. <www.iucnredlist.org>. Consulted on April 2015.

Thulin CG (2003). The distribution of mountain hares *Lepus timidus* in Europe: a challenge from brown hares *L. europaeus*? Mammal Review 33: 29-42.

Annexes

CLASSIFYING HARE SPECIES - INFORMATION FROM THE NUCLEAR DNA, FRAGMENT FROM THE ALBUMIN GENE (INTRON)

The information given in the cards is a 15 base pair sequence composed of nucleotides taken from the published complete sequence. The elimination of nucleotides from the complete sequence is only done to facilitate comparisons.

The complete information, sequences with 611 nucleotides, is published in the paper Melo-Ferreira J, Alves PC, Freitas H, Ferrand N, Boursot P. (2009). The genomic legacy from the extinct *Lepus timidus* to the three hare species of Iberia: contrast between mtDNA, sex chromosomes and autosomes. Molecular Ecology 18(12): 2643-2658.
That information can be used to, for example, discuss the existence of ambiguities related to the fact that this is a nuclear gene - if an individual receives different copies from each parent for a given position in the sequence then both information will be registered according to the code defined by the IUPAC – or the existence of other type of information – the complete sequences of broom hare individuals have less 4 nucleotides than those from the other species, suggesting that a deletion of 4 base pairs have occurred in that position in that species.

The identification of individuals on the cards corresponds to the following species: L1 to L8 - Iberian hare; L9 to L16 – European hare; L17 to L23 – Broom hare; L24 to L29 – Mountain hare.

L1 - TAAACCTAATGTGCT	L2 - TAAACCTAATGTGCT	L3 - TAAACCTAATGTGCT
L4 - TAAACCTAATGTGCT	L5 - TAAACCTAATGTGCT	L6 - TAAACCTAATGTGCT
L7 - TAAACCTAATGTGCT	L8 - TAAACCTAATGTGCT	L9 - TCGATCTAGTGTGTT
L10 - TCGATCTAGTGTGTT	L11 - TCGATCTAGTGTGTT	L12 - TCGATCTAGTGTGTT
L13 - TCGATCTAGTGTGTT	L14 - TCGATCTAGTGTGTT	L15 - TCGATCTAGTGTGTT
L16 - TCGATCTAGTGTGTT	L17 - TCGATCAAATGTGTC	L18 - TCGATCAAATGTGTC

L19 - TCGATCAAATGGGTC	L20 - TCGATCAAATGTGTC	L21 - TCGATCAAATGTGTC
L22 - TCGATCAAATGTGTC	L23 - TCGATCAAATGTGTC	L24 - TCGATCTAATGTGTT
L25 - TCGATCTAATGTGTT	L26 - TCGATCTAATGTGTT	L27 - TCGATCTAATGTGTT
L28 - TCGATCTAATGTGTT	L29 - TCGATCTAATGTGTT	

CLASSIFYING HARE SPECIES – INFORMATION FROM THE MITOCHONDRIAL DNA, FRAGMENT FROM THE CYTOCHROME B GENE

The information given in the cards is a 15 base pair sequence composed of nucleotides taken from the published complete sequence. The elimination of nucleotides from the complete sequence is only done to facilitate comparisons.

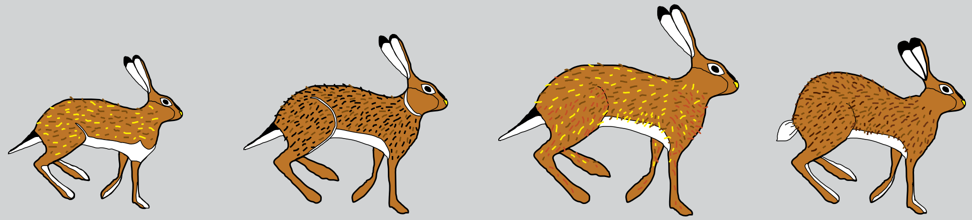
The complete information, sequences with 616 nucleotides, is published in the paper Melo-Ferreira J, Boursot P, Randi E. Kryukov A, Suchentrunk F, Ferrand N, Alves PC. (2007). The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. Molecular Ecology 16(3): 605-618.
That information can be used to, for example, discuss the existence of different types of artic mitochondrial DNA in Iberian hares, suggesting that there were contacts between the mountain hare and the Iberian species in different times - the most parsimonious hypothesis tells that the more alike are the mitochondrial DNA found in the mountain and in the Iberian species then the more recent was that contact and the introgression (that is, the mitochondrial DNA transference).

The identification of individuals on the cards corresponds to the following species: L1 to L6 - Mountain hare; L7 to L13 - Broom hare; L14 to L21 - European hare; L22 to L29 - Iberian hare. Thus, according to the sequences in the cards, the individuals L8, L20, L21, L22 and L26 have the artic mitochondrial DNA.

L1 - AAGTCGAATCCCGTT	L2 - AAGTCGAATCCCGTT	L3 - AAGTCGAATCCCGTT
L4 - AAGTCGAATCCCGTC	L5 - AAGTCGAATCCCGTT	L6 - AAGTCGAATCCCGTT
L7 - AAGTCAAACTCCGTT	L8 - AAGTCGAATCCCGTT	L9 - AAGTCAAACTCCGTT
L10 - AAGTCAAACTCCGTT	L11 - AAGTCAAACTCCGTT	L12 - AAGTCAAACTTCGTT
L13 - AAGTCAAACTTCGTT	L14 - AGGCTAAACCCTATT	L15 - AGGCTAAACCCTATT
L16 - AGGCTAAACCCTATT	L17 - AGGCTAAACCCTACT	L18 - AGGCTAAACCCTATT

L19 - AGGCTAAACCCTATT	L20 - AAGTCGAATCCCGTT	L21 - AAGTCGAATCCCGTT
L22 - AAGTCGAATCCCGTT	L23 - TAGCCGAGCCCCATT	L24 - TAACCGAGCCCCATT
L25 - TAGCCGAGCCCCATT	L26 - AAGTCGGATCCCGTT	L27 - TAGCCGAGCCCCATT
L28 - TAGCCGAGCCCCATT	L29 - TAGCCGAGCCCCATT	

CLASSIFYING HARE SPECIES – INFORMATION FROM MORPHOLOGY



Iberian hare *Lepus granatensis*	**Broom hare** *Lepus castroviejoi*	**European hare** *Lepus europaeus*	**Mountain hare** *Lepus timidus*
Small size	Big size but smaller the European	Big size	Big size but smaller than the European and with a rounded body
Fur yellow-brown	Fur brown-black	Fur yellow-brown and rufous in the neck and the upper side of legs	Fur with various tones of brown (Summer coat; most individuals change for a white coat in the Winter)
Upper side of the legs with white fur	Upper side of the legs without white fur	Upper side of the legs without white fur	Upper side of the legs with white fur
Without facial band	With white-gray facial band	Without facial band	Without facial band
Extended white ventral fur, reaching the upper side of the front legs, and with a sharp con-trast between ventral and dorsal fur	Less extended white ventral fur and with a sharp contrast between ventral and dorsal fur	Slightly extended white ventral fur and with a transition zone between ventral and dorsal fur	Slightly extended white ventral fur and with a transition zone between ventral and dorsal fur
Tail is black on the upper side and white on the under side	Tail is black on the upper side and white on the under side	Tail is black on the upper side and white on the under side	White tail
Hears with black tips	Hears with black tips	Hears with black tips	Hears with black tips and white edges

The identification of individuals on the cards corresponds to the following species: L1 and L5 – Iberian hare; L2 and L6 – Broom hare; L3 and L7 – European hare; L4 and L8 – Mountain hare.