Alma Mater Studiorum - Università di Bologna

Istituto Nazionale per la Fauna Selvatica

DOTTORATO DI RICERCA

Biodiversità ed Evoluzione

Ciclo XX

Settore scientifico disciplinare di afferenza: BIO/05 ZOOLOGIA

Assessing the patterns of genetic diversity in otter (*Lutra lutra*) populations in Europe

Presentata da: **Dott. NADIA MUCCI**

Coordinatore Dottorato:

Prof. GIOVANNI CRISTOFOLINI

Relatori:

Prof. FRANCESCO ZACCANTI

Prof. ETTORE RANDI

Esame finale anno 2008

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1 - INTRODUCTION TO THE SPECIES

1.1- Lutra lutra: ecology

The European Otter, *Lutra lutra*, is known as the Eurasian River Otter, the Common Otter and the Old Word Otter; it is a small member of the *Mustelidae* or weasel family.

These animals have a sleek, streamlined body with a thick tail that tapers to a point. They grow to a length of 1-1.3 meters. Males are heavier than females, weighting 7-9 Kg.

The ears are small and the nose is shaped like a hexagon. The limbs are short and the feet are webbed, the toes ending in sharp claws.

Long whiskers are located on each side of the snout and are very sensitive to touch. The sense of sight, smell and hearing is acute. The eyes are located at the top of the head.

Their fur is brownish-grey, their throats being a buff or cream colour. The guard hairs are stiff and coarse and covered in an oil than the European otter produces. This oil acts as a water repellent and is so effective than the skin never gets wet.

European otters have flaps over their ears and nose that close shut while the otter is swimming. The eyes remain open.

Although the otter is an exceptionally good swimmer and fish catcher, it can only hold its breath for 20 seconds as it dives for its prey.

Otters communicate using vocal expression as whistles, birdlike twittering and spitting. They also communicate using spraint. River otters have paired scent glands at the base of their tail which give off heavy, musky smell. Over 100 different scent components of otter dropping have been identified. About 17 of these are thought to contain information on sex, age, and even individual recognition, which can be used by other otters.

Spraints are deposited in prominent places along rivers or streams in an otter's range, such as on fallen tree trunks, tree roots, boulders or ledges under bridges. Spraints might be also found at several places along a well-used otter path and at the entrance to holts or resting places. If there are not prominent features the otter might make a sign heap out of mud, sand or twists of grass and leave a spraint on top of it. When regular sprainting occurs on grass, tufts are often greener and taller than surrounding grass.

Fresh spraints can be easily identified by their sweet musky odour. They are often greenish black and covered with mucus when fresh and usually contain fish bones and scales since small fish are otter's primary food source.

However fur, feathers and invertebrate remains may also be found when a spraint is dissected.

Dried out spraints crumble and lose their colour so that they appear like grey ash. Size and shape are variable, from compact cylinder shape spraint up to 6 cm long to a small piece or tarry smear.

Otters have a preference for rivers and lakes with clean transparent water, a high flow rate and well-vegetated steep banks. Otters may also live along the coast, in slat water, but require regular access to freshwater to clean their fur. Typical vegetation includes mature trees and woodland, particularly deciduous species, willow and alder; scrub and tall bank side vegetation such as hawthorn, blackthorn, bramble and dog rose; willow herb and reed and sedge beds (Bytchkov & Chanin, 1994).

The roots of mature trees, particularly ash, oak and sycamore, provide potential holt sites and reed/sedge beds are used to make 'couches'. Important feedings grounds are associated with gravel bottom and narrow stream or tributaries since these features are optimal for fish.

Permanent, well vegetated mid channel islands provide secure lying up and breeding sites. Additionally, ditches and ponds provide alternative food supplies such as amphibians, especially during the winter months and when rivers are in flood.

An otter's diet mainly consists of fish, but can also include birds, insects, frogs, clams, crustaceans worms, sometimes small mammals and a small amount of vegetation.

They use their *vibrissae* (whiskers) as sensing organs underwater to monitor the movements of fishes and other prey. River otters hunt and feed several times a day, consuming about 1kg of food daily (Grzimek, 1990, Heggberget, 1994).

The European Otter is an opportunistic both in terms of diet and habitat and forages in coastal and freshwater environment. It has a high metabolic rate and needs to consume about 10% of its body weight each day. To meet these energy demands, otters require a large territory in which to forage, typically a 16-20 km stretch of river.

River otters are most active at dusk and during the night.

Lutra lutra tends to live alone, except during mating and for a short time after the cubs are born. The young stay with the mother for approximately 13-15 months.

Cubs can be born at any time of the year, but we find there is a definite preference for spring and again in late autumn.

On average 2-3 cubs are born after a gestation period of 61-74 days weighing only 40 g, covered in a pale grey fur and with closed eyes. They develop slowly and the eyes will open in five weeks. At seven weeks they will start to run and take solid food. It is at this time that they will venture out of the holt to the toilet outside but they will not go much further until they are 10 weeks old and they are fully weaned at 14 weeks.

Young otters are not natural swimmers and the fluffy coat of the young makes it difficult, so they are often dragged into the water by the mother at 16 week old. They learn quickly and soon catch their own food. However they are still dependant on mother and will stay with her for over a year.

The juvenile cubs will start to disperse at 14 to 15 months and venture into new areas to find their own territories. Survival in the harsh environment on their own is very difficult as they tray to find a territory and good feeding grounds.

Mating takes place at anything from 17-20 months in males and 2 years in females and the male pick up the scent of the female in season and goes looking for her. The two otters chase each other along the shore, disappearing into the sea and diving, swimming and rolling together before mating take place. The male often stays close to the female about a week before venturing on to pastures new.

Otter will be lucky to reach the age of 4. However, there have been instances of otters living from 8-12 years, although possibly only one or two in a hundred will survive until this sort of age.

1.2 - The status of the Eurasian otter in Europe

The European otter is widely distributed in Europe; the species range includes North Africa and Asia. It has been described as having one of the widest distributions of all Palearctic mammals (Corbet, 1966).

The individual in Asia tend to have a lighter overall colour than those in Europe and it is found as far south as Indonesia (Foster-Turley & Santiapillai, 1990).

Its range originally extended from Portugal in the west to Japan in the east, and from Northern Europe and Asia, to the southern shores of the Mediterranean. Over the past 40 years there have been marked declines in the number of animals throughout much of the otter's range, particularly in Western Europe, and concern expressed for the survival of the species in several countries.

Details about otter distribution in the European states studied in this project are listed below.

1.2.1 - The British Isles

The otter was once widespread throughout the British Isles, and this is reflected by its inclusion in many early natural history books, the fact that bounties were paid on them and that hunts were organized as a sport and as a means of control, with in some areas dramatic effects.

Populations appear to have been still relatively healthy in the early 1950s (Stephens, 1957), but shortly afterwards the situation changed. There was a serious decline in numbers, which started suddenly about 1957/58, and occurred simultaneously throughout much of England, Wales and the

Scottish Borders. Chanin & Jefferies (1979) reviewed the situation and concluded that the factor most likely to have been responsible for these events was the introduction in 1956 of the organochlorine groups of insecticides (see also Jefferies & Hanson, 2001).

Nowdays population seems growing; a reintroduction project occurred in the East Anglia in the past century using captive animals bred in the Otter Trust Centre (UK).

1.2.2 - Western Europe

The otter is widespread and thriving throughout much of Portugal, with animals being found both on the coast and in freshwater habitats (Trindade, 1994; Santos-Reis et al., 1996).

The animals are most common in the north-east and south-west parts of the country and least common in the central area. Portugal could, therefore, hold one of the most important otter populations in Western Europe (Trindade *et al*., 1998). There is no evidence to suggest that the population is currently under threat, although Santos-Reis (1994) identified a new potential danger, periods without rain, resulting in many watercourses becoming dry in the summer.

The species has been fully protected since 1974, but still subject to illegal hunting.

It was thought that there had been a marked decline in otter numbers in Spain since the mid 1960s (Blas-Aritio, 1978). In the early 1980s, however, it was still widely distributed in the west, but by the end of that decade was considered threatened in the east, and restricted in the Central Region (Delibes, 1990).

Signs of otters were most frequent in the north and north-west of the country: Galicia, Asturias, and in West Central Spain on the borders of Portugal, where there is also a healthy population (Santos-Reis *et al*, 1996).

In 1993 individuals from Asturias, Galicias, Extremadura (Spain) and Portugal were released in the province of Girona (Catalonia- Spain), in order to restore the disappeared population.

At the end of the past century, marked increases were found in five regions, Catalonia, Aragón, Asturias, Galicia and Western Andalucía, but declines were reported in others, including Navarra, the Basque country, Rioja and to the north of Castilla-León (Ruiz-Olmo & Delibes, 1998). Increases have also been reported from both the coastal and subalpine areas of the Pyrennes (Ruiz-Olmo, 1994).

The otter was once found in Andorra (Ruiz-Olmo & Gosálbez, 1988), but is now thought to be extinct (Ruiz-Olmo *et al*., 2001).

At the beginning of the 20th century, the otter was found in every region of France except Corsica, and remained common throughout the country until about 1930 (Rosoux *et al*., 1996). Over the next two decades a decline occurred and since the 1950s the species has disappeared from

47 of the 95 French Departments. Otters are widespread in the area west of Brittany, south to the Pyrennes and in the Massif Central, but are absent from much of the north and east of the country (Rosoux *et al.*, 1996).

There has been a recolonisation, in the area around the Massif Central, which started around 1984.

Recent surveys also confirm an expansion in Brittany which is now thought to contain about 25% of the country's otters (Lafontaine, 1993).

In Germany, where the otter is fully protected by hunting law, the species is highly endangered in the old Federal Republic, with otters being rare or extinct in many of the federal states.

Otter is locally common in the former German Democratic Republic, but even here, the distribution is becoming more restricted, possibly because of changes in land-use practices and the rapid increase in the volume of traffic in the former German Democratic Republic following reunification in the late 1980s. The species is absent, for example, in the more lowland regions and along the Baltic coast (Stubbe, 1989; Macdonald & Mason, 1990,1994; Reuther, 1992; Stubbe et al., 1993). Stubbe & Stubbe (1994), however, reported that the species was now endangered and rare in the former German Democratic Republic, with both populations and distribution area declining.

In Lower Saxony the species appeared to occur in relatively large numbers and was distributed fairly uniformly throughout the Province.

By 1920, the species had become rare in the southern part of the province.

Recent surveys in Lower-Saxony suggest that a recovery has taken place there in the past decade.

The Austrian population is also expanding. In their account of the status of the otter in Austria, Macdonald & Mason (1990) reported that the species was threatened.

There are two main populations. The larger is found in the northern parts of Upper and Lower Austria and recent surveys have shown that this population is expanding southwards and has crossed the Danube (Kranz, 1994). The smaller population, in the south-east of the country, expanded between 1986 and 1993-94 (Sackl *et al.*, 1996).

Both populations continue to expand (Bodner, 1994; Gutleb, 1994) and there is some evidence that the two populations have made contact in the Northern Limestone Alps (Kranz, *pers. comm.*).

1.2.3 - Scandinavian Peninsula

The countries of Scandinavia have shown a slight increase in the range of the otter over the past few years.

In Denmark, game bag statistics showed that otters had been killed all over the country with no apparent effect on the population (Jensen, 1964).

The otter population at the end of the 1970s was thought to be between 200 and 500 individuals (Jensen, 1980).

The critical state of the population was evident from surveys in the early 1980s with the majority in Central and North-west Jutland.

Today the otter is still regarded as endangered, but Madsen (1996) concludes that after 10 years work, there are positive indications of a successful enhancement of the living conditions for otters in Denmark.

The Norwegian populations are fragmented in the south, but large and widespread in the north, where it is widely distributed along the coast and inland in lower densities (Heggberget, 1994).

A survey of otters in 1989-1990 (Christensen, 1995) indicated that the present distribution of otters in Norway is characterized by a metapopulation in the north, where 85% of coastal sites surveyed had evidence of otters.

The species had all but disappeared from along the south-eastern coasts.

In the western provinces, between the northern and south-eastern areas, otters signs were found at 22.1% the sites visited, there was an increase in the number of sites with evidence of otters from south to north. The northern region is considered to hold a viable otter population.

Based on two survey in the mid1960s and 1970s as well as information from game bags, it was concluded that that the otter population in Sweden was declining, a decline which probably began around 1950 (Erlinge, 1971; Erlinge & Nilsson, 1978; Erlinge, 1980). The decline, at least in part of the country continued, and by 1997 it was estimated that there were only 500-1500 otters in the country, which was less than the annual otter harvest for around 1950 (Erlinge & Nilsson, 1978).

Surveys in the 1980s showed only 5% of 2,000 sites visited in southern part of the country with evidence of otters (Olsson & Sandegren, 1986), while in Northern Sweden otters were evident in slightly more, 10% of the sites visited (Olsson in Macdonald & Mason, 1994).

More recent research, based on the otter reintroduction program in Central Sweden has shown an expansion of the otter population, (Sjöåsen & Sandegren, 1992; Sjöåsen, 1996). There is evidence that the reintroduced otters are now in contact with the northern population and signs of otters are being found in areas where there have been none reported for nearly 20 years (Sjöåsen, *pers. comm.*). The species is classified as vulnerable in the central and northern parts of Sweden and endangered in the south (Sjöåsen, *pers. comm.*).

Historically, otters in Finland were found throughout the country, including the coasts and on small offshore islands.

The population declined and, despite protection being reintroduced in 1975, and Macdonald & Mason (1990) reported that populations were becoming more fragmented in some areas and absent in others. Kauhala (1996) confirmed otter numbers declined in the 1970s, but increased again in the 1980s, with a marked increase in distribution between 1981 and 1991.

In Finland, otters are currently thought to be widespread, but with a patchy distribution (Skarén & Kumpulainen, 1986; Hagner-Wahlsten & Stjernberg, 1991), and while they are rare in the southern part of the country and in coastal areas, good populations are found inland in eastern and central parts of the country (Skarén, 1990).

The most recent surveys, using snow tracking suggest decreases in otter numbers throughout much of the country over the period 1989-1997 (Helle *et al.*, 1997).

The overall picture, therefore, appears confused, with increases in some areas, decreases in others and some marked fluctuations over the past ten years. The species is classified as "declining, in need of monitoring" (Skarén, *pers. comm.*).

1.2.4 - Eastern Mediterranean and Balkans

The Italian otter population is endangered and its survival depends upon the conservation of the populations living in the southern part of the country (Prigioni & Fumagalli, 1992). In the early 1970s the species range already appeared to be highly restricted (Cagnolaro *et al.*, 1975).

Some areas have been surveyed more recently with differing results. In the Sele-Calore river catchments, for example, the population appears to be stable, while some populations in Southern Tuscany and Northern Latium showed apparently dramatic decreases by late 1990, and are now extinct (Cassola, 1994). The persistence of otters in several water bodies in Campania, Basilicata and Calabria was confirmed in 1994, when 45% of 35 sites visited had evidence of otters; the local density and demographic trends of the species in these regions remain unknown (Reggiani *et al.*, 1997).

Otters were found on 50 water bodies, mainly in Southern Italy, during the period 1984 to 1994, with the population fragmented into five main groups (Prigioni, 1997).

The situation in old Yugoslavia was difficult to determine because of the conflicts. Macdonald & Mason (1990) reported that the species was found throughout much of the country, with the exception of the mountainous north-west area of the Adriatic coast. Inland, along the main rivers, the species was thought to be at a low density of extinct.

In Serbia and Montenegro Paunović & Milkenović (1994) concluded that the species was more widespread than previously reports. Animals were found in most areas except for the central

part of Serbia and West Central Montenegro. It is found along the coast (Paunović & Milkenović, 1998). The otter is currently protected (in Serbia since 1976 and Montenegro since 1982) as a 'natural rarity' under the hunting laws.

1.2.5 - Baltic Republics

In the Baltic Republics otters are widely distributed. Despite intensive hunting pressures, otters are widespread throughout Latvia, being found on most water courses (Ozolinš & Rantinš, 1992a), but with an uneven distribution. More dense populations are in the western and eastern parts of the country, with less dense populations in the north, north-east and on the coastal plain. Ornicans (1994) detailed the changing otter population in Latvia this century, from around 500 individuals in 1914, numbers dropped to an all time low of 255 in 1947. This decline was associated with the rapid development of agriculture and land reclamation and persecution by fish and crayfish breeders (Ozolinš & Pilãts, 1995).

Between 1980 and 1987, the otter was included in the Red Data Book of Latvia, but was subsequently removed when it became clear from hunting returns that the species was numerous, It is thought that the successful re-establishment of the beaver in Latvia has benefited the otter (Ozolinš & Rantinš, 1995), the latter making use of the beaver lodges and fish ponds.

1.2.6 - Eastern Europe

There have been extensive surveys of the otter in the former Czechoslovakia, and these have continued in the newly formed Czech and Slovak Republics.

Toman (1992) reported the results of the surveys using both snow tracking and spraints.

He estimated 300-350 animals in the Czech Republic in three isolated populations - a small one in the north extending to the German border, another, in the east, joining with Slovakia and a third, the main centre of otter activity, in the South Bohemian fish pond area, a population that extends into the Austrian Waldviertel.

In Slovakia, the status seems unclear, (Kadlečík, 1994) regarded the species to be seriously endangered, but the same author (Kadlečík, 1992) had earlier stated the species was still widely distributed, with the main population in the central and eastern parts of the country.

In the Czech Republic the species has been protected since 1949, but in 1996 was listed in the new hunting laws, with a year-long open season. Thus the otter is currently subject to two conflicting laws, although the former still ensures the animals' protection (Toman, *pers comm*.). The species is listed as "endangered" in the Red Data Book (Baruš, 1989). In Slovakia, the otter is strictly protected under the Act on Native and Landscape Conservation, and is listed as "vulnerable" in the Red Data Book (Štollmann, *et al*., 1997).

The Hungarian otter population is thought to be stable, but there has been a decline in the area east of the Danube (Nechay, 1980).

The population was reported as being stable, but growing. There was, however, concern about illegal killing (Egyetemes *et al*., 1997).

The species was given "strict" protection in 1978, but legal killing of individuals can be sanctioned after it has been established that they have been responsible for damage (Rakonczay, 1990; Lanski & Köromendi, 1996).

The otter in Belarus is widespread, and, since 1995 can only be hunted under license. Numbers were thought to have stabilized over the period 1984-1989, except for a slight decrease in numbers in the south-west of the country, and in areas of high human population (Sidorovich, 1991).

1.3 - Threats

There are many factors that contribute to making the otter's life very hazardous. These include habitat destruction, both on land i.e. the building of roads and the loss of previously undisturbed riverbank systems, and in the water (particularly in the sea, as otters may be caught in fishing nets where they can ultimately drown). Traffic injuries also pose considerable problems for otters, as new roads are built through their previously tranquil homesteads. Pollution from pesticides, PCBs, mercury and oil can all be severely damaging to the otter's health, when found in traditionally clean streams.

In the past, the European river otter was also mistakenly considered a pest for local fisheries. People later learned that the otters actually preferred slow-swimming fish species that were not used for human consumption. This misunderstanding did take a serious toll on their population, as did the fur trade, and their numbers declined rapidly.

1.4 - Legal protection

Today, the European river otter is protected by the European Protection of Wildlife and Living Habitats Agreement, which was enacted in 1979. This act strictly prohibits the hunting, capture, and trade of otters within their European range. The animals still face many threats from other human sources, and there are currently several studies looking into ways to clean up polluted habitats.The otter is listed on Appendix 1 of CITES, Appendix II of the Bern Convention and Annexes II and IV of the Habitats Directive. It is protected under Schedule 5 of the WCA 1981 and Schedule 2 of the Conservation (Natural Habitats, etc.) Regulations, 1994 (Regulation 38). The European sub-species is also listed as globally threatened on the IUCN/WCMC RDL.

2 - INTRODUCTION TO THE DEOXIRIBONUCLEIC ACID (DNA)

2.1 - DNA structure and function

DNA is organised in chromosomes that are contained in a cell nucleus (nuclear DNA), and in mitochondria, organelles present in the cell cytoplasm (mitochondrial DNA, mtDNA). DNA takes the form of double helix built by four nucleotides: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). The linear order in which these four nucleotides follow each other in the double helix is called nucleotide sequence. This simple structure is extremely stable and allows the DNA to act as a template for protein synthesis and replication.

The mechanism of DNA replication forms the basis of the hereditary transmission of genetic information. DNA is replicated before each division is completed. Each of the daughter cells receives a new complete set of chromosomes. Each of the two DNA strands (chromatids) is replicated when DNA is denatured and the double helix is opened. The enzyme that catalysed the replication, the DNA polymerase, bind itself to denatured area and starts to replicate, controlling the insertion of nucleotides.

The two new double helix are identical, each one formed by a parental chromatid and by a complementary chromatid.

In this way DNA sequences are faithfully copied and the genetic information coded in the sequences is preserved during cell duplication.

The process of replication is not perfect and some nucleotide mutations may be inserted by chance. Mutations modify DNA sequences and generate genetic variability.

The genome of vertebrates and many other living organism is largely made of non coding DNA sequences, that apparently have no function.

Genes, sequences present in single copy or in families made up of a small number of copies of the same gene, constitute the functional, non-repetitive DNA and codify for proteins. DNA sequences that make up the gene are organized in functional domains, have the role of regulating the transcription: the first part of the gene is made up of a promoter, a sequence of a few dozen nucleotides which is recognised by RNA polymerase. This is followed by coding sequences (exons) that normally alternate with tracts of sequences that are transcribed, but not translated (introns).

The gene ends with termination sequences, that interrupts RNA synthesis.

Non coding, tandem repeated DNA exists in the genome of every species (repetitive DNA). Tandem repetitive sequences, commonly known as “satellite DNAs” are classified into three major groups:

1 Satellite DNA: highly repetitive sequences with very long repeat lengths (up to 5000000 nucleotides) that are usually associated with centromeres. The satellite DNA is not used in population genetics or in forensic genetics.

2 Minisatellite DNA (Figure 1): are present in hundreds or thousands of loci in eukaryotic genomes. These tandem repeats often contain a repeat of more than 10 nucleotides and are present in multiple pairs that produce clusters of 500-30 000 nucleotides. Some minisatellites are hypervariable in array size and are widely used in forensic genetics to obtain DNA fingerprinting.

3 Microsatellite DNA (Figure 2): present in many thousand of loci in eukaryotic genomes. Microsatellites are made up of very short repeats (from 2 to 8 nucleotides) that are repeated only a few times and produce clusters of a few dozen or few hundred nucleotides at every locus. Microsatellites are used extensively in forensic genetics and are profiled through PCR.

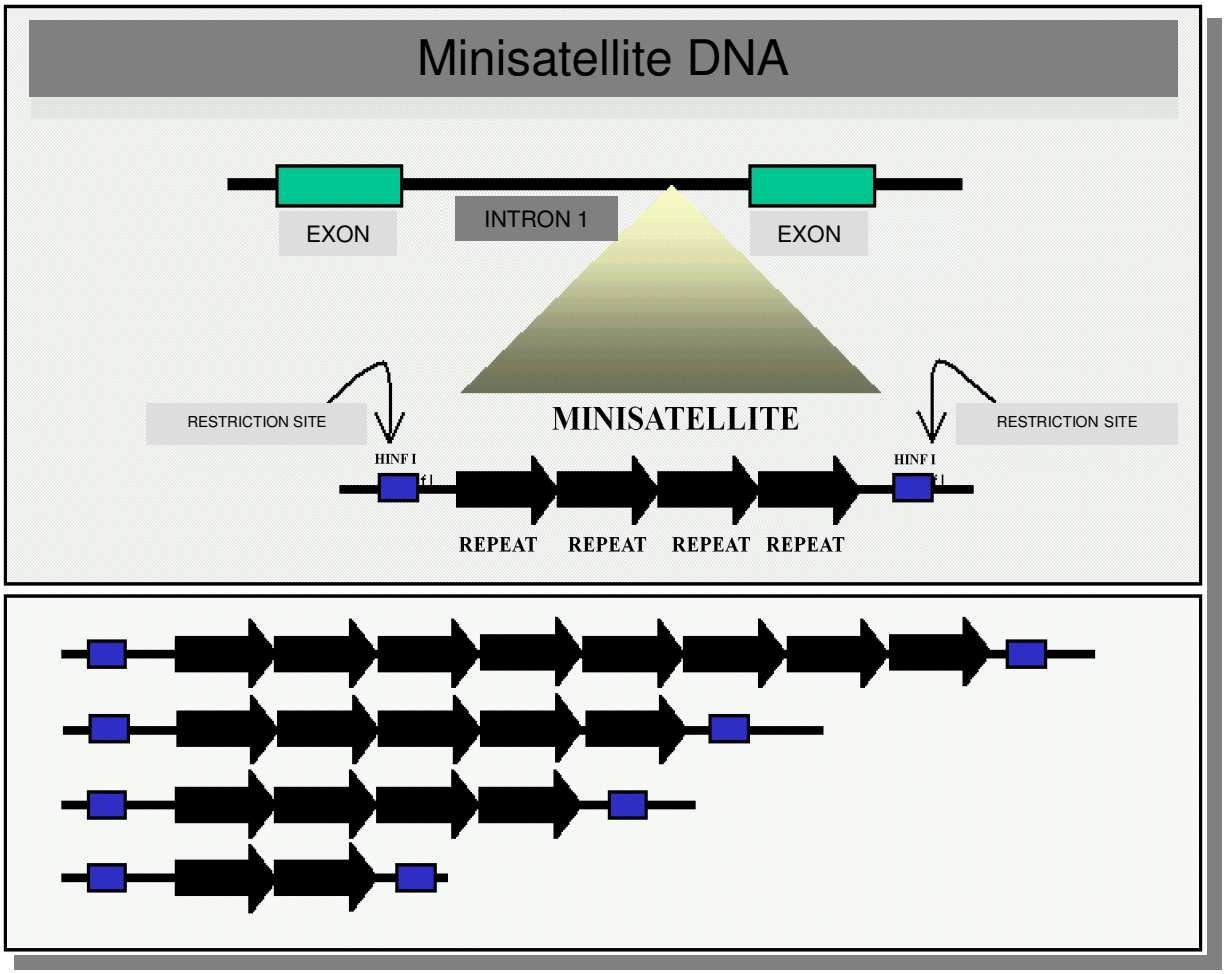


Figure 1 - Minisatellites are present in hundreds or thousand of loci in eukaryotic genomes.

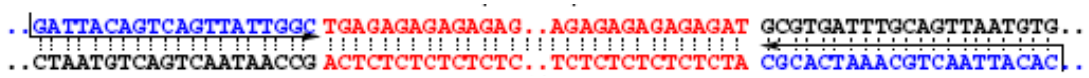


Figure 2 - Microsatellite DNA example

The different categories of functional or non- functional tandem repeated DNA evolve through different mutational processes that are associated with DNA structure and function.

A Nucleotide and amino acid substitution (Figure 3). The simplest type of mutation is the nucleotide substitution that is also called "point mutation". The mutations that do not change the amino acid substitution are non synonymous mutations.

B Insertion or deletion of a single nucleotide or a series of nucleotides. These mutation can modify the reading frame of the genetic code or inactivate the gene.

C Crossing over (symmetrical and asymmetrical) and recombination. Symmetrical crossing over produces exchanges of corresponding sequences between two chromosomes and produces genetic recombination. Asymmetrical crossing over occurs more frequently between sequences of satellite or minisatellite DNA, that is, between tandem repeated DNA that do not align themselves precisely. Asymmetrical crossing-over may occur between two chromatids of the same chromosome or between two different chromosomes.

D DNA slippage (Figure 4). Slippage occurs during replication when the nascent DNA separates and re-associates itself temporarily from the DNA template. During replication of non-repetitive sequences, the possible disassociation of the sister chromatid does not usually generate mutations, because the nascent DNA can re-associate only and exactly in the complementary point of the template. During the tandem repeated DNA replication, the single strand nascent DNA can pair in another point of the DNA template. When replication continues, the nascent DNA is found to be longer or shorter than the template.

E Gene conversion. Gene conversion produces the transfer of a DNA sequence from one allele to another.

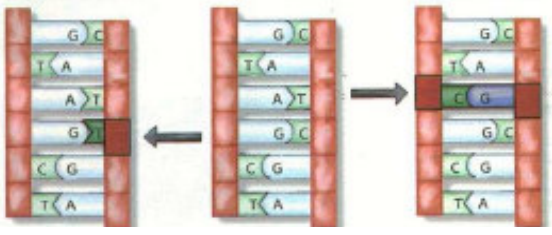


Figure 3 - DNA mutation

Figure 4 - DNA slippage

2.2 - Genetic mutations and polymorphisms

Mutations generate genetic variability in individuals and populations. A variable gene is defined as polymorphic. Polymorphism indicate the presence of two or more variants of a DNA sequence.

Gene coding polymorphism can generate protein polymorphism.

All these can be used as markers in the identification and individualisation of samples in forensic science.

The highly variable non coding DNA sequences, that apparently are not subject to strong pressures from natural selection and therefore evolve rapidly and neutrally, make up the most useful and reliable genetic markers in acquiring evidence in forensic genetics.

2.3 - Genetic Markers

A genetic marker is a known DNA sequence that can be identified by a simple assay.

It can be described as some sort of variation present can arise due to mutation or alteration in the genomic loci that can be observed.

A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism), or a long one, like microsatellites.

Genetic markers have to be easily identifiable, associated with a specific locus, and highly polymorphic, because homozygotes do not provide any information. Detection of the marker can be direct by DNA sequencing, or indirect using allozymes.

Some of the methods used to study the genome or phylogenetics are RFLP (Restriction Length Fragment Polimorphism), AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplification of Polimorphic DNA), SSR (Simple Sequence Repeats).

2.4 - Mitochondrial DNA (mtDNA)

The animal mitochondrial DNA is a circular molecule of 15-20 kb in length and in vertebrates it contains genes for 22 tRNAs, 2 rRNAs and 13 mRNAs coding for proteins involved in electron transport and oxidative phosphorylation. The only major non-coding area of the mtDNA is the control region, typically 1 kb, involved in the regulation and initiation of mtDNA replication and transcription. The use of mtDNA has become increasingly popular in phylogenetic

and population genetic studies, first with the developments in methodology for mtDNA isolation and use of restriction enzymes to detect nucleotide differences (Lansman *et al,.* 1981), and further with the development of PCR methodology and applicability of 'universal' primers (Kocher *et al.,* 1989) for amplification of mtDNA. Much of the interest is related to the fast rate of substitutions in mtDNA. The approximate mutation rate in mtDNA is 10^{-8}/site/year (Brown *et al.,* 1979, Ferris *et al,.* 1983, De Salle *et al,.* 1987) compared to 10^{-9}/site/year in nuclear genes. Most differences between mtDNA sequences are point mutations, with a strong bias for transitions over transversions (Brown *et al,.* 1982).

The mtDNA is haploid and uniparentally inherited (with some exceptions) and thus the variability is introduced by mutations alone. Compared to diploid nuclear autosomal genes with biparental transmission, the effective population size of mtDNA is one quarter of that for nuclear autosomal genes (Moore, 1995). Therefore, a mtDNA tree is more likely to be congruent with a species tree due to a high probability of coalescence even when speciation events have occurred within short time-periods.

Mitochondrial genes are inherited as one linkage group in the absence of recombination (Hayashi *et al.,* 1985, Hoech *et al.,* 1991). Recently, the clonal nature of mtDNA has been questioned and the possibility of recombination has been advocated based mainly on linkage disequilibrium in human and chimpanzee mtDNA, excess homoplasy in human control region and the existence of a globally rare transitional mutation found in more than one well-supported mtDNA clade present in one population (Awadalla *et al.,* 1999, Eyre-Walker *et al.,* 1999, Hagelberg *et al.,* 1999). Subsequently, the methodology used has been criticised (Ingman *et al.,* 2000, Kumar *et al.,* 2000) and alternative explanations have been preferred, such as the presence of hypervariable nucleotide positions or selection (Wallis, 1999). The necessary enzymatic machinery for recombination does exist in mammalian mitochondria (Thyagarajan *et al.,* 1996), and mtDNA recombination has so far been shown to occur in plants, fungi, protists (Gray, 1989) and phytonematodes (Lunt & Hyman, 1997). The prerequisite for recombination to create new variants is that different types of mitochondria are present in the same cell. Although individuals usually carry one type of mtDNA in their cells, heteroplasmy (existence of more than one extranuclear DNA sequence type in an organism) has also been reported. The most common cases are length variants in repetitive areas found within an individual (Berg *et al.,* 1995), but it has been suggested that these could be created *de novo* within a lineage (Lunt *et al.,* 1998). Contrary to common belief, the sperm mitochondria have been shown to enter the oocyte in most mammals (Ankel-Simons & Cummins, 1996) and paternal leakage or biparental inheritance of mtDNA have been reported (Kondo *et al.,* 1990, Gyllensten *et al.,* 1991, Zouros *et al.,* 1992). However, in mice

it has been shown that the paternal mitochondria are usually eliminated although the process of elimination does not work for interspecific crosses (Kaneda *et al.,* 1995). Then, if mtDNA recombination takes place, the hybrids of deeply diverged lineages could produce recombinant genomes e.g. in hybrid zones (Wallis, 1999), but as such this has yet to be reported.

2.5 - Mitochondrial control region

The control region is the main regulatory region and the only major non-coding area in animal mtDNA. It contains the heavy-strand origin of replication (Desjardins & Morais, 1990) and the promoters for heavy and light strand transcription (L'Abbé *et al.,* 1991). In mammals the length of the control region varies 880 to 1400 bp (Sbisà *et al.,* 1997). The variation in length has been attributed to variation in the tandem repeat number (Berg *et al*., 1995) and small insertions/deletions usually in the 5' and 3' ends of the control region.

Despite its functional importance, the control region is suggested to be the most variable part of the mtDNA. In the human control region, the estimates of the rate of substitution were found to range between 2.8 (Cann *et al.,* 1984) to 5 times (Aquadro & Greenberg, 1983) the rate of the rest of the mtDNA. Most of the studies in which control region sequences have been used have focused on intraspecific patterns of variability and phylogenetic relationships of closely related species, a prominent example being the study of human population history (Cavalli-Sforza *et al.,* 1994). A high mutation rate also means that the phylogenetic utility of the control region sequences diminishes in deep divergences due to saturation and ambiguities in homology determination.

Based on the distribution of the variable nucleotide positions and differential nucleotide frequencies in different parts of the control region, it is divided into three domains (Brown *et al*., 1986). Domains I and III are rich in L-strand adenine, whereas the central domain II is low in adenine. Most of the variability, both nucleotide substitutions and deletions/insertions, is concentrated in domains I and III whereas domain II is more conservative. Based on sequence similarity, tens of conserved sequence blocks with putative functional importance have been described (Southern *et al.,* 1988, Lee *et al.,* 1995, Sbisà *et al.,* 1997, Randi & Lucchini, 1998). The general structure of the control region and an overview of the sequence blocks are depicted in Figure 5.

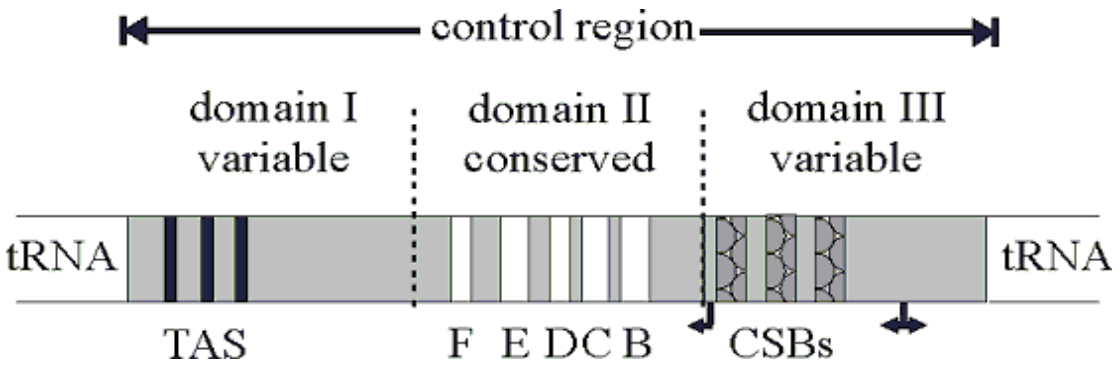


Figure 5 - General structure of the vertebrate mitochondrial control region. The arrows indicate the location of the H-strand replication origin and the bidirectional promoter for L- and H-strand transcription. TAS, termination associated sequence; F through B, conserved sequence boxes in the central domain; CSBs, conserved sequence blocks.

2.6 - Microsatellites

Microsatellites are simple sequence tandem repeats (SSTRs). The repeat units are generally di-, tri- tetra- or pentanucleotides. They tend to occur in non-coding regions of the DNA although a few human genetic disorders are caused by (trinucleotide) microsatellite regions in coding regions. On each side of the repeat unit are flanking regions that consist of "unordered" DNA. The flanking regions are critical because they allow us to develop locus-specific microsatellites; the probability of finding that particular stretch more than once in the genome becomes vanishingly small. In contrast, a given repeat unit may occur in thousands of places in the genome. We use this combination of widely occurring repeat units and locus-specific flanking regions as part of our strategy for finding and developing microsatellite primers.

Microsatellites are useful genetic markers because they tend to be highly polymorphic. Their variability is mainly due to mutations their mutations that occur in a fashion very different from that of "classical" point mutations. The mutation process in microsatellites occurs mainly through what is known as slippage strand mispairing.

Microsatellites are useful markers at a wide range of scales of analysis.

They have become the primary marker for DNA testing in forensics contexts, both for human and wildlife cases. The reason for this prevalence as a forensic marker is their high specificity. Match identities for microsatellite profiles can be very high.

In a biological/evolutionary context they are useful as markers for parentage analysis. They can also be used to address questions concerning degree of relatedness of individuals or groups. For captive or endangered species microsatellites can serve as tools to evaluate inbreeding levels (F_{IS}).

From there we can move up to the genetic structure of subpopulations and populations (using tools such as *F*-statistics and genetic distances). They can be used to assess demographic history

(e.g., to look for evidence of population bottlenecks), to assess effective population size (N_e) and to assess the magnitude and directionality of gene flow between populations. Microsatellites provide data suitable for phylogeographic studies that seek to explain the concordant biogeographic and genetic histories of the floras and faunas of large-scale regions. They are also useful for fine-scale phylogenies up to the level of closely related species.

The advantages of microsatellites as genetic markers are shown in Box n.1.

- **Locus-specific** (in contrast to multi-locus markers such as or RAPDs).
- **Codominant** (heterozygotes can be distinguished from homozygotes, in contrast to RAPDs and AFLPs which are dominant only).
- **PCR-based** (means we need only tiny amounts of tissue; works on highly degraded or "ancient" DNA).
- Highly **polymorphic** ("hypervariable") loci: provide considerable pattern.
- Useful at a **range of scales** from individual ID to fine-scale phylogenies.

Box n 1 - Advantage of the genetic markers

2.7 - Sex chromosomes

The karyotype of every individual includes a certain number of chromosomes pair that are similar each other (autosomes) and a single pair of chromosomes that have a clearly distinct form (heterochromosomes). Heterochromosomes are also called sex chromosomes because they contain the DNA sequence that determines the sex of the individual.

In mammals males have one X and one Y chromosome (XY is the heterogametic karyotype), while females have two X chromosomes (XX is homogametic caryotype).

Molecular sex identification is carried out in biological traces analyses.

2.7.1 - Genes linked to chromosome Y

Genes that determine the sex in males have recently been discovered. These genes map in the Y chromosome, even though the X chromosome may present structurally similar genes.

The zink-finger Y gene (ZFY) have modified counterparts in the X chromosome.

3 - METHODS OF ANALYSING DNA VARIABILITY

3.1 - Collection of biological samples

Molecular analyses techniques that are based on PCR require small amount of DNA, and therefore any type of biological sample can be utilised. Samples of animal origin that are most commonly used are the following:

- Blood samples from living animals that can be preserved in Longmire buffer.
- Samples of solid tissue taken from dead animals can be conserved in absolute ethanol.
- Hair samples from living animals can be conserved in absolute ethanol.
- Biological traces (biological fluids deposited on solid surface) can be conserved in absolute ethanol; these samples contain little DNA frequently degraded and contaminated from exogenous DNA.

3.2 - DNA extraction

Biological samples in ethanol or in buffer solution are always preserved in freezer upon reaching the laboratory of forensic genetics. There are different procedures that can be used to extract DNA from samples, but in all of them three main step are present.

The first treatment carried out in extracting DNA is the lyses of the cell membranes and proteins. This treatments disintegrate all the protein structures and make DNA free in the solution. Digestion buffer can contain Proteinase k (ProK) that digests proteins or thiocyanate guanidine (GUS) that produces the chemical disintegration of the protein structure. The activity of ProK and GUS is assisted from the presence of an anionic detergent that solubilizes the cell membranes and denatures the proteins.

DNA is separated from the residues of the proteins and cells digestion in order to obtain a DNA solution free from other biological substance.

DNA is re-suspended in a buffer solutions, that are based on Tris and EDTA activity and maintain a pH value that inhibits the activity of nuclease.

3.3 - The polymerase chain reaction (PCR

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It is used to exponentially amplify a fragment of DNA by *in vitro* enzymatic replication. PCR permits

amplification of a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece (Figure 6). PCR can be performed without restrictions on the form of DNA, and it can be extensively modified to perform a wide array of genetic manipulations.

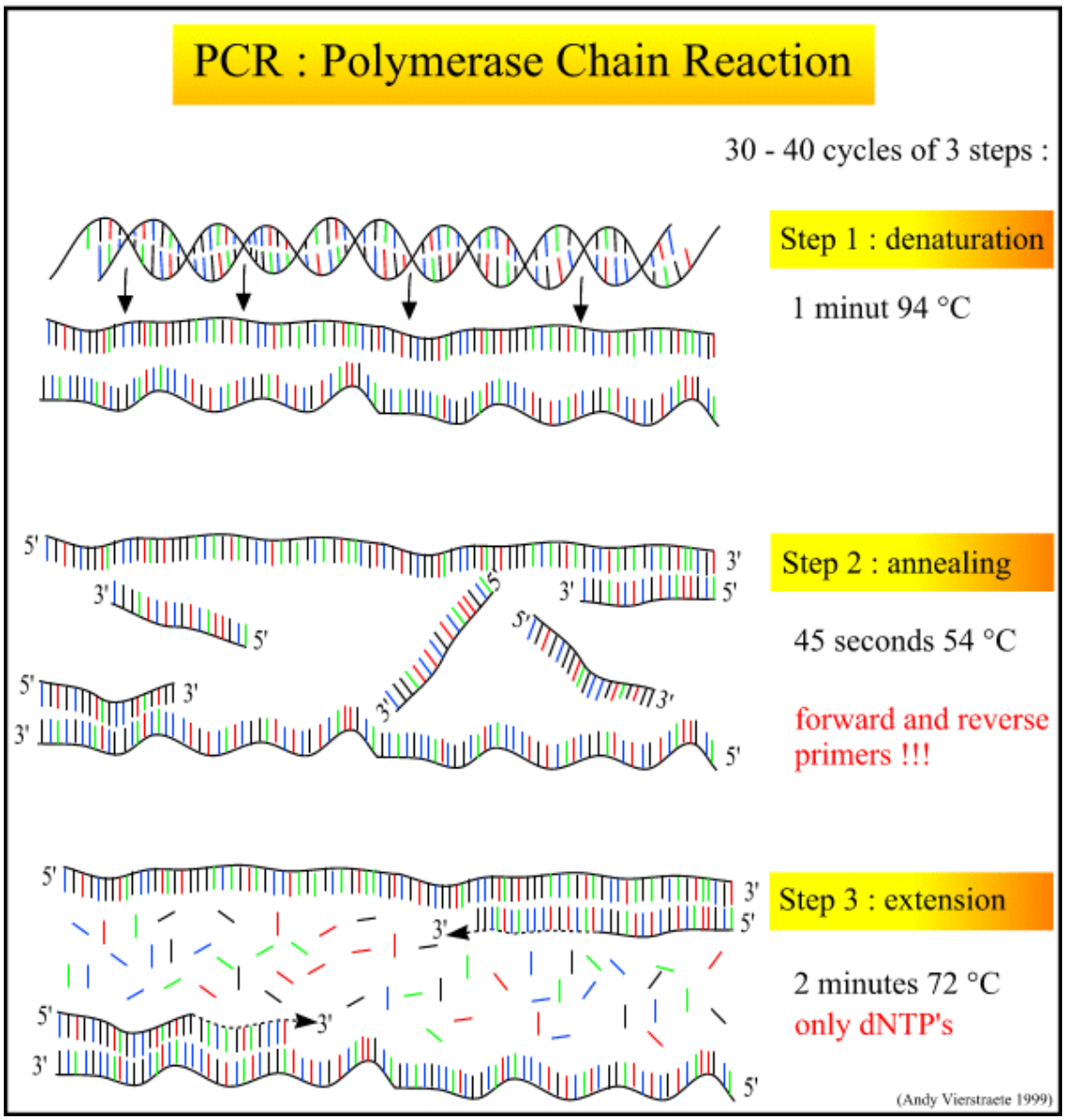


Figure 6 - Polymerase Chain Reaction

PCR was developed in 1983 by Kary Mullis and it is now a common technique used in medical and biological research labs for a variety of tasks.

PCR is used to amplify specific regions of a DNA strand. This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb).

A basic PCR set up requires several components and reagents. These components are listed in Box n.2.

- ***DNA template*** contains the DNA region to be amplified.
- One or more ***primers***, which are complementary to the DNA regions at the 5' (five prime) and 3' (three prime) ends of the DNA region.
- a **DNA polymerase** such as *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70°C.
- ***Deoxynucleotide triphosphates*** (dNTPs).
- ***Buffer solution***, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- ***Divalent cations***, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.
- ***Monovalent cation*** potassium ions.

Box n 2 - Polymerase chain reaction reagents

The PCR is commonly carried out in a reaction volume of 10-100 µl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Modern thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

3.4 - Sequencing

The term DNA sequencing encompasses biochemical methods for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide. The sequence of DNA constitutes the heritable genetic information in nuclei, plasmids, mitochondria, and chloroplasts that forms the basis for the developmental programs of all living organisms. Determining the DNA sequence is therefore useful in basic research studying fundamental

biological processes, as well as in applied fields such as diagnostic or forensic research. The advent of DNA sequencing has significantly accelerated biological research and discovery. The rapid speed of sequencing attainable with modern DNA sequencing technology has been instrumental in the large-scale sequencing of the human genome, in the Human Genome Project. Related projects, often by scientific collaboration across continents, have generated the complete DNA sequences of many animal, plant, and microbial genomes.

3.5 - Gilbert and Maxam method

Prior to the development of rapid DNA sequencing methods in 1975 by Sanger in England, a number of laborious methods were used. For instance, in 1973 Gilbert and Maxam reported the sequence of 24 basepairs using a method known as wandering-spot analysis.

It is noteworthy that RNA sequencing, which for technical reasons is easier to perform than DNA sequencing, could be considered one of the earliest forms of nucleotide sequencing.

3.6 - Chain-termination method

While the chemical sequencing method of Maxam and Gilbert, and the plus-minus method of Sanger and Coulson were orders of magnitude faster than previous methods, the chain-terminator method developed by Sanger was even more efficient, and rapidly became the method of choice. The Maxam-Gilbert technique requires the use of highly toxic chemicals, and large amounts of radiolabeled DNA, whereas the chain-terminator method uses fewer toxic chemicals and lower amounts of radioactivity. The key principle of the Sanger method was the use of dideoxynucleotides triphosphates (ddNTPs) as DNA chain terminators.

The classical chain-termination or Sanger method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP). These dideoxynucleotides are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand therefore terminates DNA strand extension, resulting in various

DNA fragments of varying length. The dideoxynucleotides are added at lower concentration than the standard deoxynucleotides to allow strand elongation sufficient for sequence analysis.

The new synthesized and labeled DNA fragments are heat denatured, and separated by size (with a resolution of just one nucleotide) by gel electrophoresis on a denaturing polyacrylamide-urea gel.

3.7 - Dye-terminator sequencing

An alternative to primer labelling is labelling of the chain terminators, a method commonly called 'dye-terminator sequencing' (Figure 7). The major advantage of this method is that the sequencing can be performed in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with a different fluorescent dye, each fluorescing at a different wavelength. This method is attractive because of its greater expediency and speed and is now the mainstay in automated sequencing with computer-controlled sequence analyzers. Its potential limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. This problem has largely been overcome with the introduction of new DNA polymerase enzyme systems and dyes that minimize incorporation variability, as well as methods for eliminating "dye blobs", caused by certain chemical characteristics of the dyes that can result in artifacts in DNA sequence traces. The dye-terminator sequencing method, along with automated high-throughput DNA sequence analyzers, is now being used for the vast majority of sequencing projects, as it is both easier to perform and lower in cost than most previous sequencing methods.

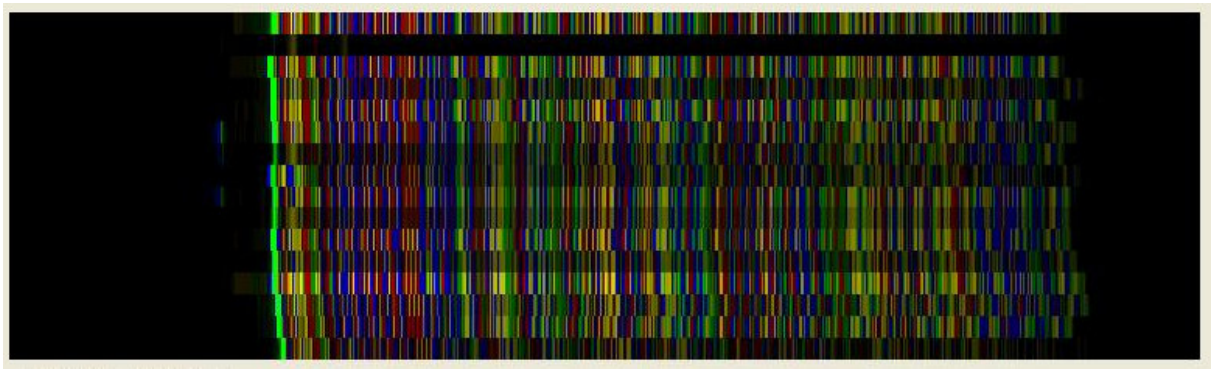


Figure 7 - Dye-terminator sequencing in ABI 3130XL

3.8 - Analysis fragment

Microsatellite analysis is done with an electrophoretic separation of the alleles present in a locus.

The differences in molecular weight dipend from the number of repetions. Not all microsatellite alleles are made up of a perfect repeat and sometimes the differences between two alleles are due to a single nucleotide.

Therefore the electrophoresic method can be able to detect fragments that differ in only one nucleotide, exactly as in sequencing. In automatic analyses of microsatellites, one of the two PCR primer is labelled with a fluorescent dye. The PCR product is separated electrofortically and the molecular weight of alleles is determinate with precision through the use of an internal standard.

3.9 - Automation and sample preparation

Modern automated DNA sequencing instruments (DNA sequencers) can sequence up to 384 fluorescently labelled samples in a single batch (run) and perform as many as 24 runs a day. However, automated DNA sequencers carry out only DNA size separation by capillary electrophoresis, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms.

4 - STATISTICS

4.1 - Genes in populations

The aim of population genetics is to describe the genetic composition of population and to understand the causes that determinate changes (evolutionary forces). Genetic variability in population is described through allele frequencies. Allele frequencies at each locus can vary in the course of generations due to mutations, natural selection, migration or genetic drift.

The different combinations of alleles present at each locus determine individual genotypes, whose frequency in populations can be calculated.

In an ideal population, in which population forces are not active, genotype frequencies remain constant from one generation, to the next.

Population genetics is based on an abstract, ideal population model, supported by a series of assumptions. The model must be simple, in such a way to render mathematic analyses possible, but consequently will not be very realistic.

The Hardy-Weiberg law defines the relationship that exists between allele and genotypes frequencies at each locus in a population. It states that in a Mendelian population, the proportion of genotypes remain constant from one generation to the next. In a locus with two alleles (a1 and a2), with frequencies p and q, with p+q=1, the genotype frequencies are obtained from the proportion: a_1a_1: $2a_1a_2$:a_2a_2=p^2:2pq:q^2.

It is possible to estimate the genotype frequencies of a population in Hardy-Weiberg Equilibrium (HWE) using the observed allele frequencies.

If a population is not in HWE an estimate of genotype frequencies, starting from the allele frequencies, may be wrong. Deviation from HWE may be caused from non-random mating, gene flow, founder effect, bottleneck and random drift.

The consequence of migration and reduction in population size could have important consequences, determining mixed population or population with high levels of inbreeding, that can be in Hardy-Weiberg Disequilibium (HWD).

Migration and the admixture of differentiate populations, causes stratified populations that are genetically heterogeneous. If a population is divided into subgroups, with random mating into subgroups, then the total population could be in HWD.

This phenomenon is known as the Wahlund Effect: the homozygosity observed in a population made up of subgroups that have recently admixed and are not panmitic, is significantly higher than the homozygosity estimated on the basis of HWE in the total population. Methods to

analyse admixed populations that identify the subgroups present and assign every individual to the subpopulation of origin are available (Pritchard et al., 2000). This methods use a statistical bayesian approach.

4.2 - Deviations from Hardy-Weinberg equilibrium

Violations of the Hardy-Weinberg assumptions can cause deviations from expectation. When random mating does not occur, the population will not have the Hardy-Weinberg proportions. Deviations from random mating are:

1 - Inbreeding, which causes an increase in homozygosity for all genes.

2 - Assortative mating, which causes an increase in homozygosity only for those genes involved in the trait that is assortatively mated.

3 - Small population size, which causes a random change in genotypic frequencies, particularly if the population is very small. This is due to a sampling effect, and is called genetic drift.

There are other assumptions affecting the allele frequencies, but they do not affect random mating. If a population violates one of these, the population will continue to have Hardy-Weinberg proportions each generation, but the allele frequencies will change with that force. The are:

A - Selection, that causes allele frequencies to change, often quite rapidly. While directional selection eventually leads to the loss of all alleles except the favored one, some forms of selection, such as balancing selection, lead to equilibrium without loss of alleles.

B - Mutation that will have a very subtle effect on allele frequencies. Mutation rates are of the order 10^{-4} to 10^{-8}, and the change in allele frequency will be, at most, the same order. Recurrent mutation will maintain alleles in the population, even if there is strong selection against them.

Migration genetically links two or more populations together. In general, allele frequencies will become more homogeneous among the populations. Some models for migration inherently include nonrandom mating (Wahlund effect, for example). For those models, the Hardy-Weinberg proportions will normally not be valid.

4.3 - F-statistics

4.3.1 - F-statistics

In population genetics, *F*-statistics (also known as fixation indices) describe the level of heterozygosity in a population; more specifically the degree of a reduction in homozygosity when

compared to Hardy-Weinberg expectation. Such changes can be caused by the Wahlund effect, inbreeding, natural selection or any combination of these.

The concept of *F*-statistics was developed during the 1920s by the American geneticist Sewall Wright, who was interested in inbreeding in cattle. However, because complete dominance causes the phenotypes of homozygote dominants and heterozygotes to be the same, it was not until the advent of molecular genetics from the 1960s onwards that heterozygosity in populations could be measured.

F-statistics measure the correlation between genes drawn at different levels of a subdivided population. This correlation is influenced by several evolutionary forces, such as mutation and migration, but it was originally designed to measure how far populations had gone in the process of fixation owing to genetic drift.

The measures F_{IS}, F_{st}, and F_{IT} are related to the amounts of heterozygosity at various levels of population structure. Together, they are called F-statistics, and are derived from *F*, the inbreeding coefficient. In a simple two-allele system with inbreeding, the genotypic frequencies are:

$$p^2 + Fpq \text{ for AA; } 2pq(1 - F) \text{ for Aa; and } q^2 + Fpq \text{ for aa.}$$

The value for F is found by solving the equation for F using heterozygotes in the above inbred population. This becomes one minus the observed number of heterozygotes in a population divided by its expected number of heterozygotes at Hardy-Weinberg equilibrium where the expected value at Hardy-Weinberg equilibrium is given by

$$p^2 + 2pq + q^{2=1}$$

where *p* and *q* are the allele frequencies of A and a, respectively. It is also the probability that at any locus, two alleles from the population are identical by descent.

The different F-statistics look at different levels of population structure. F_{IT} is the inbreeding coefficient of an individual (I) relative to the total (T) population, as above; F_{IS} is the inbreeding coefficient of an individual (I) relative to the subpopulation (S), using the above for subpopulations and averaging them; and F_{ST} is the effect of subpopulations (S) compared to the total population (T), and is calculated by solving the equation:

$$(1 - F_{IS})(1 - F_{ST}) = (1 - F_{IT}),$$

F_{IT} can be partitioned into F_{ST} due to the Wahlund effect and F_{IS} due to inbreeding.

Consider a population that has a population structure of two levels; one from the individual (I) to the subpopulation (S) and one from the subpopulation to the total (T). Then the total *F*, known here as F_{IT}, can be partitioned into F_{IS} (or f) and F_{ST} (or θ).

This may be further partitioned for population substructure, and it expands according to the rules of binomial expansion, so that for *I* partitions:

4.3.2 - Fst

A reformulation of the definition of F would be the ratio of the average number of differences between pairs of chromosomes sampled within diploid individuals with the average number obtained when sampling chromosomes randomly from the population (excluding the grouping per individual). One can modify this definition and consider a grouping per sub-population instead of per individual. Population geneticists have used that idea to measure the degree of structure in a population.

Unfortunately, there is a large number of definitions for Fst, causing some confusion in the scientific literature.

4.3.3 - Derivation and explanation of F-statistics

The extent of deviation in observed heterozigosity (H) can be used to quantify the level of genetic differentiation between the subpopulations. This quantification has been formalised (in the first instance by Wright (1978) in a series of hierarchical F-statistics.

INBREEDING COEFFICIENT = FIS = (HS - HI) / HS

- the mean reduction in H of an individual due to non-random mating within a subpopulation
- i.e., a measure of the extent of genetic inbreeding within subpopulations
- can range from -1.0 (all individuals heterozygous) to +1.0 (no observed heterozygotes)
- sometimes referred to simply as F rather than FIS

FIXATION INDEX = FST = (HT - HS) / HT

- the mean reduction in H of a subpopulation (relative to the total population) due to genetic drift among subpopulations
- i.e., a measure of the extent of genetic differentiation among subpopulations
- can range from 0.0 (no differentiation) to 1.0 (complete differentiation - subpopulations fixed for different alleles)

OVERALL FIXATION INDEX = FIT = (HT - HI)/HT

- the mean reduction in H of an individual relative to the total population

Note: FIT combines contributions from non-random mating within demes (FIS) and effects of random drift among demes (FST).

The relationship between the three F-statistics is:

$$(1 - FIT) = (1 - FIS)\ (1 - FST)$$

4.3.4 - Calculating probability and testing significance

A significance test has to be performed for each statistical analysis to verify if the results obtained are only due to the chance.

If differentiation among population has to be demonstrated, null hypothesis is that $F_{ST} = 0$, and alternative is that $F_{ST} > 0$. A simplest method of calculating the probability of an F_{ST} value is by using the simple equation $\chi^2 = 2N\ F_{ST}$, and comparing this value to the standard χ^2 distribution. A very useful non-parametric approach is to jackknife or bootstrap over loci, which provides approximate confidence intervals. A most flexible approach is to permute (for example) individuals among the subpopulations, calculate an F_{ST} value, and repeat this 100-1000 times to give a 'random' distribution of the statistic, against which the 'true' statistic is compared. The random probability of getting the F_{ST} (or greater) is then simply the proportion of the randomised values that are equal to or greater than the 'true' value.

One last, but very important, point about calculating probabilities in the context of subpop differentiation.

When a number of tests are performed at the same time, for example in a matrix of pairwise F_{ST} 's between subpopulations, then the probabilities actually should be adjusted for the fact that one of the many tests may be significant simply by chance. A Bonferroni-type adjustment should be applied to account for this (Rice, 1989).

F_{ST} has proved to be a very useful parameter in many respects, as described above. One major advantage is the possibility that it may tell us a lot about the processes leading to divergence between subpopulations or the maintenance of that divergence. When two subpopulations begin to diverge after, say, a vicariant event that separates them (e.g., a mountain range uplifts), then two processes begin acting, and in opposite directions. The first process is that, under the influence of genetic drift, the subpopulations start to diverge genetically.

Over time, F_{ST} will gradually increase, until it finally approaches 1.0, if there is no continued migration between the subpopulations.

4.3.5 - What FST may tell us about population divergence and gene flow

F_{ST} has proved to be a very useful parameter in many respects, as described above. One major advantage is the possibility that it may tell a lot about the processes leading to divergence between subpopulations or the maintenance of that divergence. When two subpopulations begin to diverge after a vicariant event that separates them (e.g., a mountain range uplifts), then two processes begin

acting, and in opposite directions. The first process is that, under the influence of genetic drift, the subpopulations start to diverge genetically. Over time, F_{ST} will gradually increase, until it finally approaches 1.0, if there is no continued migration between the subpopulations.

On the other hand, there is likely to be some level of continued migration between the two subpopulations, as they diverge. This migration will tend to limit the genetic divergence between the subpopulations Thus there are two opposing forces determining the divergence between subpopulations, and hence, F_{ST}. Genetic drift over time will allow them to diverge, while migration acts to keep them similar.

4.4 - Principle Components Analysis (PCA)

PCA is commonly used as a cluster analysis tool. It is designed to capture the variance in a dataset in terms of *principle components*. In effect, one is trying to reduce the dimensionality of the data to summarize the most important parts whilst simultaneously filtering out noise (Figure 8). Normalization, however, can sometimes remove this noise and make the data less variable, which could affect the ability of PCA to capture data structure (Yeung & Ruzzo, 2001).

***Definition*: Principle Components -** A set of variables that define a projection that encapsulates the maximum amount of variation in a dataset and is orthogonal (and therefore uncorrelated) to the previous principle component of the same dataset.

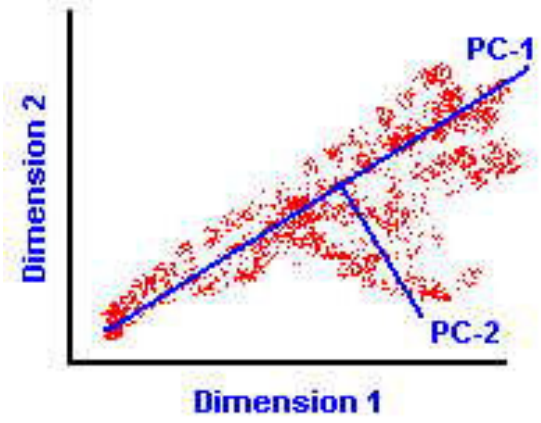


Figure 8. The blue lines represent 2 consecutive principle components. Note that they are orthogonal (at right angles) to each other.

4.4.1 - General Outline

Suppose you have a dataset composed of 1000 genes, each of which have an expression value over 10 experiments. The dimensionality of that dataset is therefore 10 (i.e. there are 10 axes).

The data, though clumped around several central points in that hyperspace, will generally tend towards one direction. If one were to draw a solid line that best describes that direction, then that line is the first principle component (PC).

Any variation that is not captured by that first PC is captured by subsequent orthogonal PCs.

The first 3 PCs could themselves act as Cartesian axes. The data they capture can therefore be plotted in terms of these axes. Hence there is a reduction of dimensionality.

When the data is plotted in this manner they are said to be plotted in *PC-space*.

4.5 - The analysis of molecular variance (AMOVA)

The analysis of molecular variance was initially introduced as an extension of the analysis of gene frequencies (Cockerham, 1973; Weir & Cockerham, 1984) for molecular haplotypes in an essentially in an essentially haploid system. The typical input for AMOVA consisted of a matrix of pairwise Euclidean distances between all multisite haplotypes and files containing the frequency of those haplotypes within each population.

Population structure by AMOVA is based on analysis of variance of gene frequencies, taking into account the number of mutation differences between molecular haplotypes or genotypes.

The AMOVA treatment provides a general framework for the analysis of population genetic structure, as the assumption on the evolution of a single polymorphism can be embedded within the definition of an Euclidean distance without affecting the essential structure of the AMOVA analyses.

The AMOVA approach was initially developed to estimate genetic population structure from molecular haplotype frequencies in haploid organism using an analysis of variance framework. The same framework can be used for diploid organism. The AMOVA treatment has already been applied to multilocus nuclear data in diploids by Peakall *et al*. (1995). These authors have also described the use of AMOVA to estimate intra-individual variance components and measures of inbreeding such as F_{IS} and $F_{IT.}$

4.6 - Bayesian statistics

The subjective theory of probability defines the probability *p* as an estimate of likelihood that the event *H* occurs.

We can have conviction (subjective) or information (objective, even though not exactly quantifiable) than an event may more or less occur frequently.

Circumstances, that do not correspond to the frequency determined experimentally, allow the probability of an event to be assigned. From this point of view, the probabilities assigned to an event are conditional and they are valid only in the presence of certain circumstances.

Prediction of the results from tossing a coin does not depend on a low of physics, but on the conditions associated with the toss.

In what way can we determinate the probability of un uncertain event in which its frequency cannot be experimented? That is, what is the Pr (*H*|*E*)= the probability that an event *H* occurs given the evidence *E*?

The factors that influence PrE can be many, for example: Pr(H|S, C, I), where S, C and I indicate the data (that is , the quantifiable observations) and the information (that is, data that is not exactly quantifiable) that are important in determining PrH. The data and information constitute the evidence. If we consider that all the probabilities can be influenced in some manner, that Pr (*H*|*E*)=Pr(*E*), and the two notations are equivalent.

4.7 - Haplotype networks

Haplotype networks are relationships among haplotypes (not individuals) often measured using a simple genetic distance, such as how many different mutation apart two haplotypes are.

In contrast to bifurcating trees, haplotype networks can have multifurcations and, in principle, cycles (biologists might call them loops).

Typically haplotype networks presented in papers show only some minimal representation of the network: for example a minimum spanning tree (Figure 9).

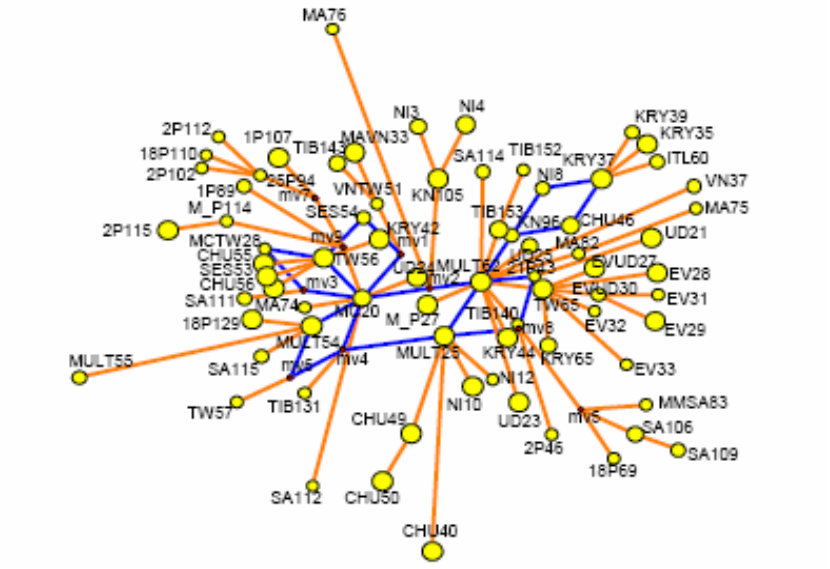


Figura 9 - Network example

5 - AIMS

Many species of large vertebrates are losing gentic diversity because of population decline and extinction (Avise and Hamrick, 1996).

It's well known that in the Paleartic region the population reacted to the Quaternary climate fluctuation with local extinction and shift in distribution in size (Hewitt, 1999). The complex dynamics during late Pleistocene/Holocene made it difficult to disentangle the genetic consequences of natural climatic and habitat change from the consequences of human habitat alteration and overexploitation of natural populations.

The otter (*Lutra lutra*) was widespread throughout suitable European wetland areas until the end of the XIX century (Mac Donald & Mason, 1994). In the past century, chemical pollution, habitat destruction/fragmentation and direct persecution lead the species to decline and become fragmented in the Central and Western Europe (Mac Donald & Mason, 1994).

Otters are now largely absent from most of the of the suitable areas in Central Europe where large gaps separate fragmented populations. Conservation projects aim to protect populations, to improve the habitat condition of corridors that could reconnect fragmented populations and support carefully planned local reintroduction projects. (Reuther, 1994).

Few information about population genetics and philogeographic structure in Europe are available: low level of polymorphism were retrieved in otters using mitochondrial DNA (Effenberg & Suchentruch, 1999, Mucci *et al*., 1999, Cassens *et al*., 2000) and microsatellite loci (Pertoldi *et al*., 2001, Hájková *et al*., 2007)

The aims of the project are the following:

1) to identify if the microsatellite loci panel used in the study is able to describe the genetic variability of otter populations;
2) to describe the main patterns of genetic diversity in the wild populations;
3) to describe and compare the genetic composition of wild, captive-reproduced and restocked otter populations;
4) to identify diagnostic markers and detect the consequences of translocations;
5) to develop a genetic database to be used for otter reintroduction plans in EU.

6 - MATHERIALS AND METHODS

6.1 - Sampling

We analyzed 700 samples collected in 19 European countries and in one Northern Israelian population.

Six additional specimens from captive stocks were also collected and analyzed (Table n.1).

Sampling distribution is shown in the map (Figure 10), in which green spots represent areas where reintroduction project occurred. Geographical coordinates were available for each sample.

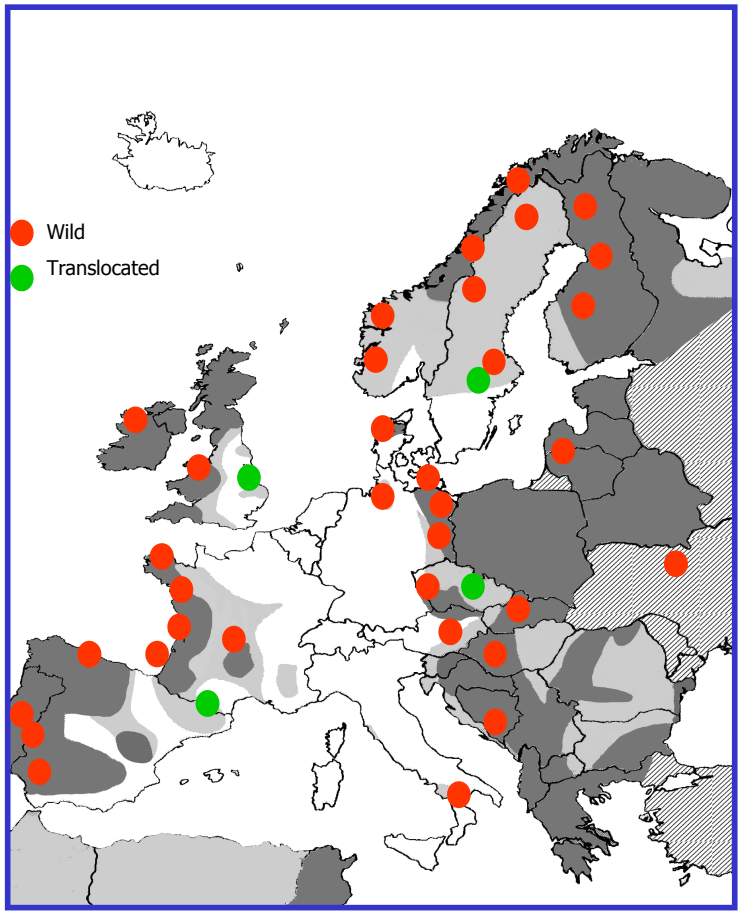


Figure 10 - European sampling map

Region	***n***
Portugal	30
Spain	40
France (>Atlantic)	42
England	5
UK (East Anglia)	47
Ireland	14
Denmark	15
Germany	171
Austria	18
Czech Rep.	32
Slovak Rep.	19
Hungary	6
Serbia	8
Latvia-Belarus	7
Finland	74
Sweden	43
Norway	69
Italy (Calabria Mainly NGS)	34
Israel	15
Captive (OT B line)	6

Table 1 - Sampling in Europe, Israel and in the captive stocks. *n* indicates the number of samples analyzed in the

6.2 - DNA extraction

DNA was extracted using the thiocyanate guanidine and silica method (Gerloff *et al*., 1995) and eluited in a TE buffer solution (Box n 3).

Preparation of the solutions :

GUS, stock solution (7,05M thiocyanate guandine):

500 gr of GUS dissolved in 200 ml of ddH_2O

GUS, lysis buffer (0,05M TRIS-HCl pH7; 0,025M EDTA pH8; 1,25% Triton X100; 4,23M GUS):

1M TRIS-HCl, pH 7,0: 12,5 ml

0,5M EDTA, pH 8,0: 12,5 ml

TRITON X100: 3,125 ml

ddH2O: up to 100 ml

GUS stock solution: 150 ml (final volume: 250 ml)

GUS, binding solution (0,05M TRIS-HCl pH7; 0,025M EDTA pH8; 4,23M GUS; diatomee 1%)

1M TRIS-HCl, pH 7,0: 12,5 ml

0,5M EDTA, pH 8,0: 12,5 ml

ddH2O: up to 100 ml

GUS stock solution: 150 ml

Diatoms (Sigma): 2,5 gr

final volume: 250 ml

GUS, washing solution (0,05M TRIS-HCl pH7,0; 4,23M GUS)

1M TRIS-HCl, pH 7.0: 25 ml

ddH2O: up to 200 ml

GUS stock solution: 300 ml

final volume: 500 ml

TE (TRIS-HCl 10mM pH 8,0; EDTA 0,1 mM pH 8,0):

1M TRIS-HCl, pH 8,0: 1 ml

0,5 M EDTA, pH 8,0: 0,02 ml

ddH_2O: up to 100 ml of final volume

Box n 3 - GUS solutions

6.3 - Mitochondrial DNA (mtDNA) analysis

A mitochondrial region 2000 bp long, including the last part of the *cytochrome b*, the entire *control region* and the first part of the *ribosomal subunit 12S*, was amplified using the forward primer CybL996 (5' CCT TAC CCT AAC CTG AAT CGG) and the reverse primer 12SH91 (5' CTA GAG GGA TGT AAA GCA CCG), that anneal respectively on the light and on the heavy strand of mtDNA.

Amplifications were performed in a 9700 ABI Thermocycler (Appliedbiosystems) using the following protocol:

Initial denaturation	94° X 2 ‘
40 cycles	94 X 30” *denaturation*
	55 X 30” *annealing*
	72 X 30” *extension*
Final extension	72 X 10’

Positive amplifications were detected on a 2% agarose gel and binding DNA with an UV fluorescent reagent (Gel Red; Società Italiani Chimici); PCR products were purified using 1 µl of a mixture of Exonuclease I and Shrimp Alkaline Phosphatase (GE Healthcare) that remove respectively unincorporated primers and dNTP.

Purified samples were sequenced with BigDye terminators v.1 (Appliedbiosystems) using the external PCR primers and the internal primers: the forward primer OTTD3 (ACAACATTTACTGTGCCTGCCC), the forward primer LLUdL225 (5’ CCCAAGACTCAAGGAAGAGGC),the forward primer OTTD4L (5’ CATCTGGTTCTTACTTCAGGGCC) and the reverse primer OTTD5H 5’ ACAAGTGGTGGGAGAGAGAAGCG).

Primer positions are highlighted in the reference sequence:

```
Reference sequence
AACATCCATTCATCACCATTGGCCAACTAGCCTCAATCCTTTACTTCACACTCCTCTTAATTCTCATACCAATCGCGAGC
ATTATCGAGAACAACCTATTAAAATGAAGAGTCTTTGTAGTATATTAATTACCTTGGTCTTGTAAACCAAAAATGGAGAA
TCCCATCTCCCCAAGACTCAAGGAAGAGgCAAAAaGCCCCACCATCAGCACCCAAAGCTGACATTCTAACTAAACTATTC
CCTGATTCTCTCACCCCACATTTCAATTCATATATTCAACAACATTTACTGTGCCTGCCCAGTATGTATTCCGCGCACCG
CCCCCTATGTATATCGTGCATTAATGGTTTGCCCCATGCATATAAGCATGTACATACTATGGTTGATTTTACATGTATCC
ACCTCACCTAGATCACGAGCTTGATCACCATGCCTCGAGAAACCATCAATCCTTGCGCGATGTGTACCTCTTCTCGCTCC
GGGCCCATCACATGTGGGGGTTTCTACCGTGAAACTATACCTGGCATCTGGTTCTTACTTCAGGGCCATAACAATCCTCA
ATCCAATCCTACTAACCTCTCAAATGGGACATCTCGATGGACTAGTGACTAATCAGCCCATGATCACACATAACTGTGGT
GTCATGCATTTGGTATCTTTAATTTTTGGGGGGGGAGAAATTGGTATCACTCAACTATGGCCAGGTGTGGCCTCGTAGCA
GTCAAATAACTTGTAGCTGGACTTATCCTTCATCATTTATCCCCGCCGCGTAGCTCTAAGGTGCTATTCAGTCAATGGTC
ACAGGACATACACACATAGATCCACCCCCGTGCACGTACGTATACACGCACACTCACGTACGtATACACGCACACCcCACG
TACGcATACACGCACACCcCACGTACGCATACACGCACACCCACGTACGnATACACGCACACCCACGTACGTATACACGCA
CACCCACGTACGCATACACGCACACCCACGTACGCATACACGCACACGCACGTACGCATACACGCACACGCACGTACGCA
TACACGCACACGCACGTATTCAACAGATATGAAACTAGCTTAATCAAACCCCCCTTACCCCCCCGTAACTTCAAAAGTATA
CAAATACCTATATTGTCCTGCCAAACCCCAAAAACAGnGCTAGGCACATGCAACGTATATGAGAAGTCACTTACACTGGC
GCCACGCATGCTAATCTCATTCACTGATTCATTAAAATAATTCATTAGAAATTCCTATCCAAAAGAAGCTATCTATAGAT
GTTATTTATATCTCCTACTACCCCCGTCAAAAACGCTTCTCTCTCCCACCACTTGTTAATGTAGCTTATTAAATAAAGCA
AGGCACTGAAAATGCCTAGAAGAGTCACAAGACTCCATAAACACAAAGGTTTGGTCCTGGCCTTCCTATTAGTTATTAAC
AGGATTACACATGCAAGTCTCCACATCCCGGTGAAAATGCCCTCTAAATCACCATGTGATTAAAAGGAGCTGGTATCAAG
CACACTTATAAGTGGCTCATAACGCCTTGCTCAACCACACCCCCACGGGATACAGCAGTGATAAAAATTAAGCTATGAAC
GAAAGTTCGACTAAGCCATGTTAGCACCAAGAGTTGGTAAATTTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAAT
TAATAGGCCCACGGCGTAAAACGTGTTAAGAACTACAACAATACTAAAGTTAAAATTTAACAAGGCCGTAAAAAGCTACT
GTTAATACAAAATATGCTACGAAAGTGACTTTACTATATCCGACAACACGATAGCTGAGGCCCAAACTGGGATTAGATAC
CCCACTATGCTCAGCCCTAAACATAGATAGCTTACATAACAAAACTATCTGCCAGAGAACTACTAGCAACAGCTTAAAAC
TCAAAGGACTTGGCGGTGCTTTACATCCCTCTAGAGGAGCCTGTTCTATAATCGATAAACCCCGATATACCTCACCACTT
```

CTAGCTGAATCAGTCTATATACCGCCATCTTCAGCAAACCCTCAAAAAAGGAAGAAAAGTAAGCACAATAATAATACATA
AAAAAGTTAGGTCAAGGTGTAACCCATGAAGTGGGAAGAAATGGGCTACATTTTCTAACCAAGAATACACTCACGAAAGT
TTTTATGAAAACTAAAAACTAAAGGTGGATTTAGTAGTAAATTAAGAATAGAGAGCTTAATTGAATGGGGCCATGAAGCA
CGCACACACCGCCCGTCACCCTCCTCAAGCAATATACCTAAACACTACATAATTTATTAGACAGACTAAAGCAAGAGGAG
ATAAGTCGTAACAAGGTAAGCATACTGGAAAGTGTGCTTGGATAAATCAAAGTGTAGCTTAACCAAAGCACCTGGCTTAC
ACCCAGGAGATTTCACACATTGATGACCACTTTGAACCAAACCTAGCCCAACCAATTACCAATTTAATTATCACGACAAT
ACCAATCAAAACATTTAATCACACCATTACAGTATAGGAGATAGAAATCTTATTTGGAGCTATAGAGAAAGTACCGCA

Sequencing protocol is described below.

25 cycles 94 X 10" *denaturation*
55 X 5" *annealing*
60 X 4' *extension*

final extension

Fragments were separated on a 3130 XL ABI automatic sequencer and results were analyzed and corrected using the software Sequencing Analysis v.5.3 and Seqscape v.2.0.

6.4 - Microsatellite loci analysis

Microsatellite loci were analyzed using the markers listed below.

Lut 453 HEX (Dallas & Piertney, 1998)
Lut 435 6-FAM (Dallas & Piertney, 1998)
Lut 604 6-FAM (Dallas & Piertney, 1998)
Lut 701 6-FAM (Dallas & Piertney, 1998)
Lut 715 6-FAM (Dallas & Piertney, 1998)
Lut 733 HEX (Dallas & Piertney, 1998)
Lut 782 HEX (Dallas & Piertney, 1998)
Lut 818 6-FAM (Dallas & Piertney, 1998)
Lut 832 HEX(Dallas & Piertney, 1998)
Lut 833 6-FAM (Dallas & Piertney, 1998)
Lut 902 6-FAM (Dallas et.al, 1999)

Amplifications were performed in a 9700 ABI Thermocycler (Appliedbiosystems) using the following protocol:

Initial denaturation 94° X 2 '
40 cycles 94 X 30" *denaturation*
55 X 30" *annealing*
Final extension 72 X 2'

Fragments were separated on a 3130 XL ABI automatic sequencer and results were analyzed and correlated using the software Genescan v.3.7, Genotyper v.3.7 and Genemapper 4.0.

6.5 - Statistic analyses

6.5.1 - Analyses of the mtDNA sequences

Phylogenetic trees were reconstructed using MEGA 3.1 (Kumar *et al*. 2001; http://www.megasoftware.net/), with the neighbour-joining procedure (Saitou & Nei 1987) and Tamura and Nei's TN93 genetic distance model (Tamura & Nei 1993), which is appropriate to describe the evolution of control region sequences.

Haplotype diversity (*h*), average pairwise nucleotide substitutions (*k*), nucleotide diversity statistics, were computed using DNASP 3.9 (Rozas *et al*. 2003).

Networks are better suited than phylogenetic methods to infer haplotype genealogies at the population level because they explicitly allow for extant ancestral sequences and alternative connections (Bandelt *et al*. 1999). We used the complete alignment with the median-joining network procedure (Bandelt *et al*. 1999), implemented in network 4.4.1 (http://www.fluxustechnology. com/).

6.5.2 - Analyses of the microsatellite loci diversity

Commonly used summary population genetic statistics (allelic frequencies, heterozygosity and deviations from Hardy-Weinberg equilibrium) were computed for each locus and population, and patterns of differentiation were visualized by a Factorial Correspondence Analysis (FCA) of individual multilocus scores using GENETIX 4.03 (Belkir et al., 2001).The partition of microsatellite genetic diversity withing and among populations was analysed by AMOVA (Excoffier et al., 1992) using Φ analogues of Wrigth's (1965) F-statistics with the software GeneAlEx 6.0 (Peakall & Smouse 2006).

Biparental multilocus genotypes were analyzed using a Bayesian clustering procedure implemented in Structure v.2 (Pritchard et al., 2000; Falush et al., 2003), which was designed to identify the *K* (unknown) populations of origin of the sampled individuals, and assign the individuals to the populations. Population clusters are constructed by minimizing the departures from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE), which could result from recent admixtures, migration or hybridisation. The samples are subdivided into a number of different sub-populations (clusters) and, simultaneously, individuals are assigned probabilistically to one (the population of origin) or more than one cluster (the parental populations) if their genotypes are admixed. Structure does not need perfect genetic equilibrium to cluster individuals, but attempts to minimize departures from HWE and LE within the inferred clusters. Genotypes were assigned with a percentage (*q* value) to one or more than

one clusters; the Credibility Interval was calculated for each sample; large and small intervals might respectively confirm or not the q value obtained. Predictably, the threshold values will strongly affect both efficiency and accuracy of groups identifications (Vähä & Primmer, 2006).

6.5.3 - Analyses of genotypes distribution in Europe

Although populations refer often to genetic structure only, it is often realistic to assume that populations are spatially organized. Therefore it makes sense not only to estimate population membership of each individual of a dataset but also to try to delineate spatial domains of each such population. In consequence, geo-referenced individual multilocus genetic data were the processed using Geneland 2.0.10 with the aim to detect population structure, i.e sub-populations. Toward this aim, Geneland makes use of both spatial and genetic information to estimate the number of populations in a dataset and delineate their spatial organization.

7 - RESULTS

7.1 - Mitochondrial DNA sequences

The mtDNA alignment (134 individuals, 1582 nucleotides), showed 25 haplotypes, defined by 23 polymorphic sites including 16 singleton polymorphic sites and 7 parsimony informative polymorphic sites (23 transitions, 1 transversions and two insertions/deletions). Mitochondrial DNA diversity was high in otter, which showed on average one distinct haplotype over 5.3 individuals (134/25 = 5.3). Haplotype diversity was high (h = 0.997 ± 0.012, standard deviation), but nucleotide diversity (π = 0.00166 ± 0.00021) and average number of pairwise differences (k = 2.613 ± 1.79) were small, suggesting that otter populations had historically large effective size (*Ne*), but that extant mtDNA lineages originated recently.

7.2 - Network analyses and geographical distribution of the mtDNA haplotypes

Network computed using the complete data set is a star like network, with no geographical structure (Figure 11).

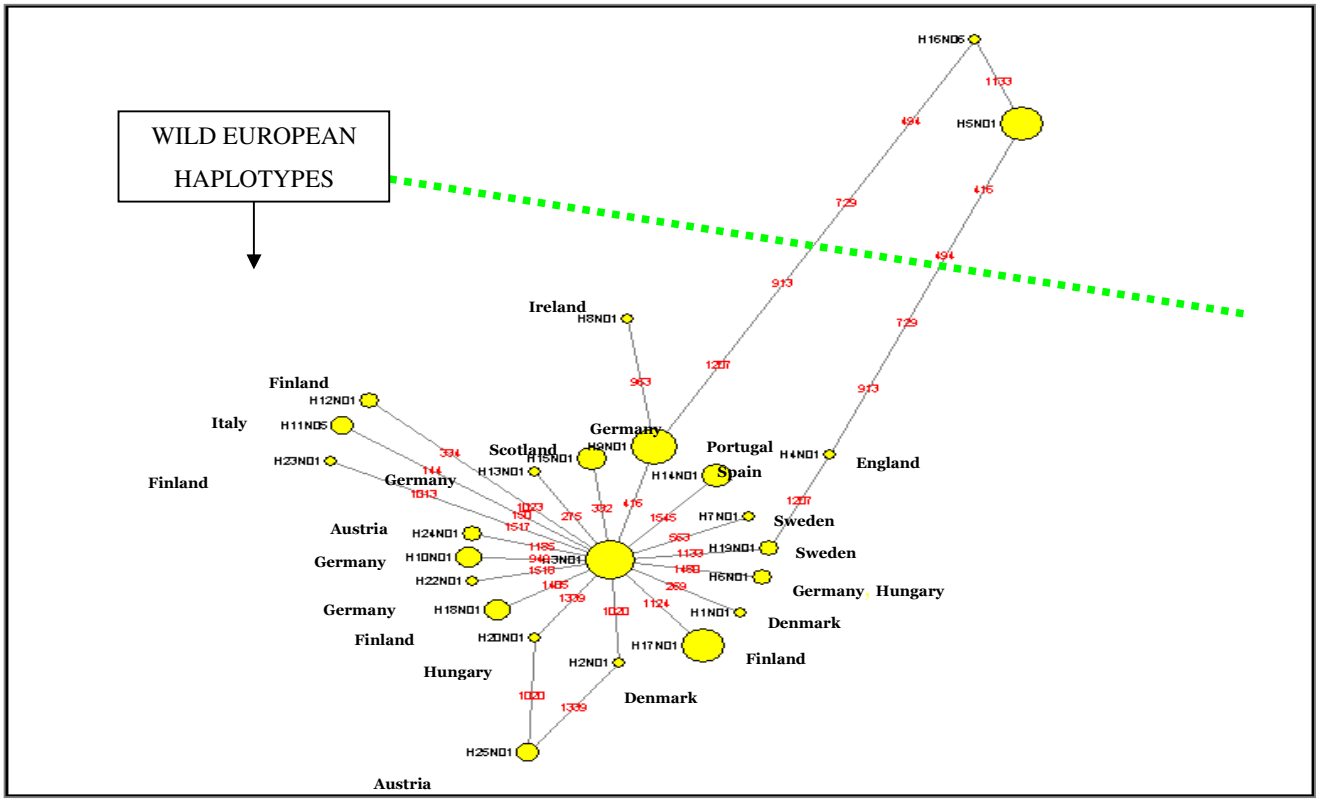


Figure 11 - Mitochondrial DNA haplotypes netwok.

Central haplotype (H3) is widespread in all the European countries sampled excepted in the population of Southern Italy.

All the European hapolotypes differ form the main widespread in one or two mutations.

The two main divergent haplotypes (H5 and H16) were retrieved in the animals from the captive stocks or in wild, but only in the areas in which individuals from captive breeding were released.

7.3 - Microsatellite loci results

7.3.1 - Genetic diversity among wild and European populations

The eleven microsatellite loci showed 99.13% polimorhic loci and an average of 4.52 alleles. Higher allele numbers were retrieved in northern populations (Germany 6,27; Finland 6.73, Sweden 6.63, Norway 6.81). In Italy and Denmark allele number was the lowest (2.54 and 2.90 respectively). Allele number with frequency higher than 5% showed a lower value (3.58 on average). Considering only wild European populations allele number values (all alleles and alleles with frequency >5%) increased (4.65 and 3.72).

Twenty-nine private alleles in a total of 121 alleles (23.9% private alleles) over all the populations were detected. The average private allele frequency was 0.052. Private alleles with frequency higher than 5% were found in Spain, England (not East Anglia), Ireland and Norway.

Observed and expected heterozigosity was similar in the populations (0.61 ± 0.042) except in Denmark and Italy in which was lower (Ho= 0.37 and 0.37 respectively).

Results obtained from the analysis of 700 samples is shown in Figure 12a.

The patterns of genetic differentiation by a multivariate PCA (Principal Component Analysis) is shown in the plot: wild European population tended to group at the bottom in the left side and show low geographical differentiation.

Divergences between Israelian and European population are evident.

In the right side of the plot are shown the genotypes of captive samples and the genotypes of wild animals collected in East Anglia (UK), where captive individuals were released.

In the plot, two individuals collected in Central Czech Republic out lie from the European grouping (Figure12b).

The Germany and the Italian population are differentiated (green and white on the left side of the plot).

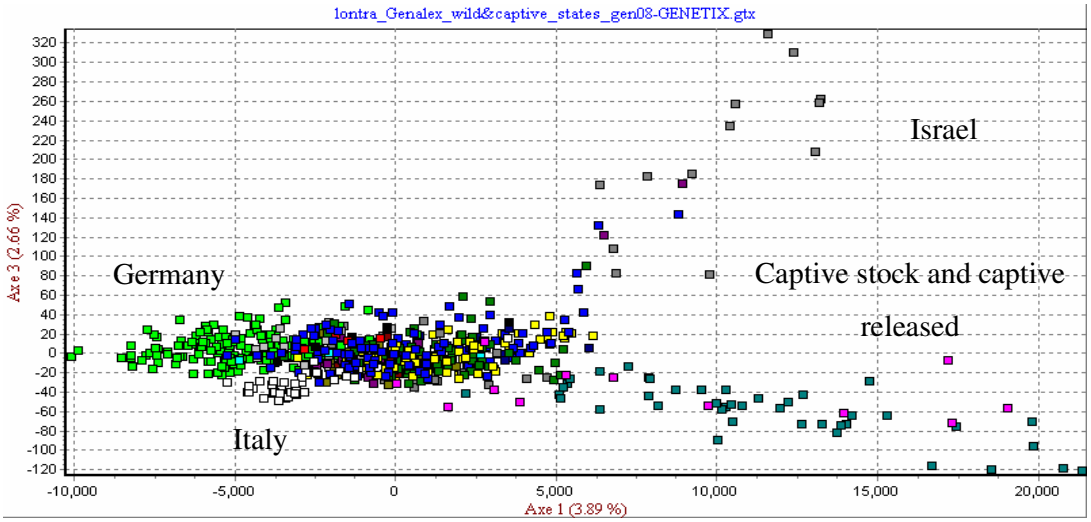


Figure 12_a - Principal Component Analysis. Axe 1-3

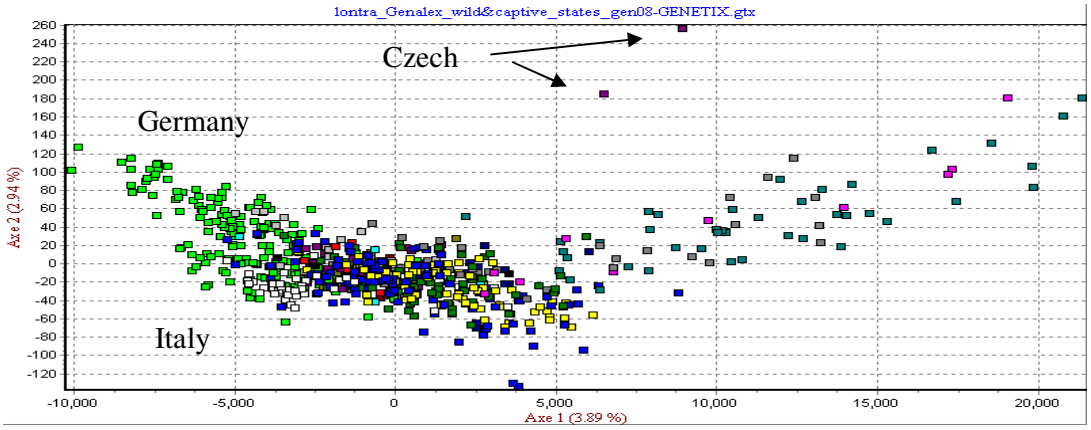


Figure 12_b - Principal Component Analyses. Axe 1-2

Variability is described from the factorial components: the first axe describe the 3.89% of the variability, the second 2,94%, the third 2.66% and the fourth 2.5%.

Higher significant average Fst values(P>0.005) were found in England, Denmark, in Italy and Israel versus other populations.

Genotypes from captive individuals, outliers (the two Czech samples), the individuals reintroduced in Catalonia (Spain) and the Israelian population were removed and 17 wild populations (Table 2) were analyzed separately.

Allele diversity was 4.65; alleles with frequency >5% showed a value of 3.72.

Twenty-eight private alleles in a total of 111 alleles (25.2% private alleles with an average frequency value of 0.044) over all the populations were detected.

The patterns of genetic differentiation of 616 individuals by a multivariate PCA plot is shown in Figure 13.

1.	Portugal	30	10.	Slovakia	15
2.	Spain	40	11.	Hungary	6
3.	France	42	12.	Serbia-Montenegro	8
4.	England	5	13.	Latvia Belarus	6
5.	Ireland	14	14.	Finland	74
6.	Denmark	15	15.	Sweden	43
7.	Germany	170	16.	Norway	69
8.	Austria	18	17.	Italy	34
9.	Czech Republic	27			

Table 2 - wild European populations

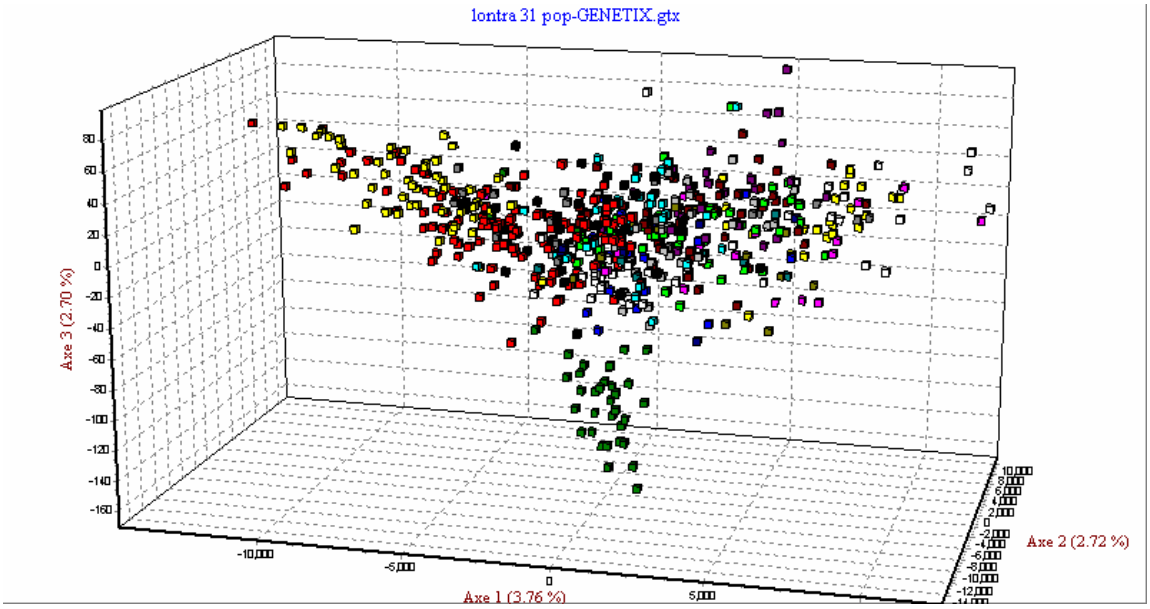


Figure 13 - Principal Component Analysis3D plot of European populations

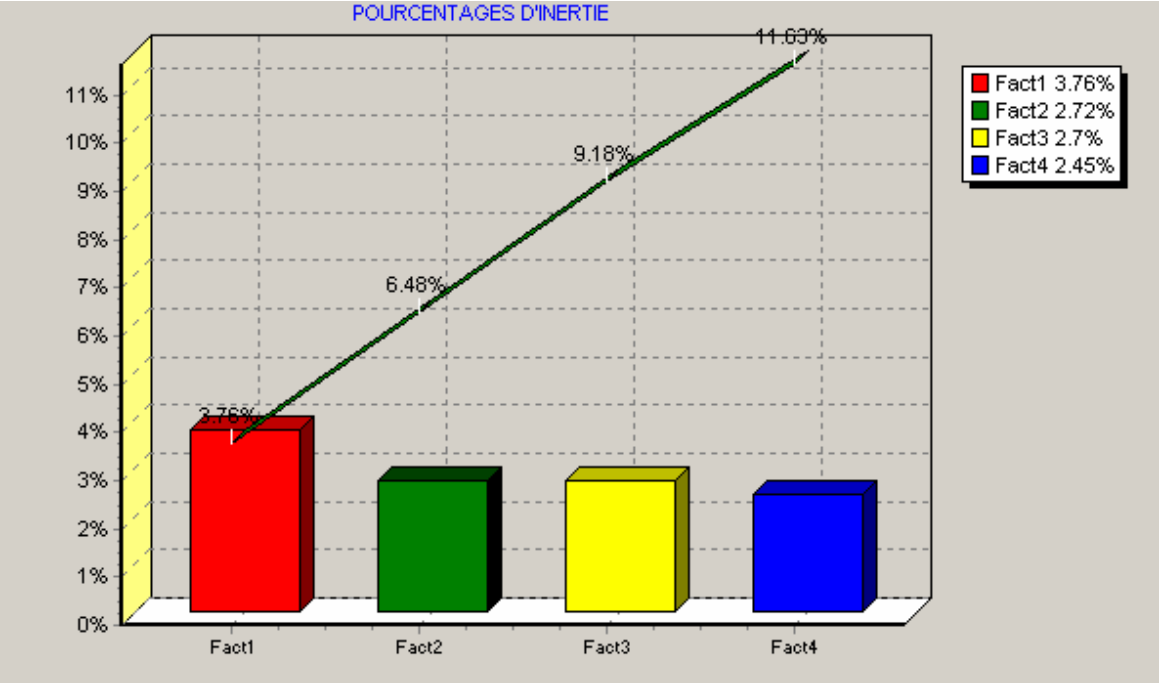
Variability described from the factorial components is lower if compared with variability described when captive animals are included.

European population are confirmed to be low differentiated; in the plot only the Iberian population (Spain and Portugal) and a part of the Germany population are separated on the left and the right side respectively.

At the bottom, the Italian population is well separated from the other European populations.

An AMOVA was performed; values of Fst analogous (**PhiPT**) = 0.21 were higher than Rst values = 0.17 (Figura 14).

Fst was not significant among Hungary, Serbia, Latvia, between Czech Republic and Austria, between Hungary and Austria and between Norway and Latvia.



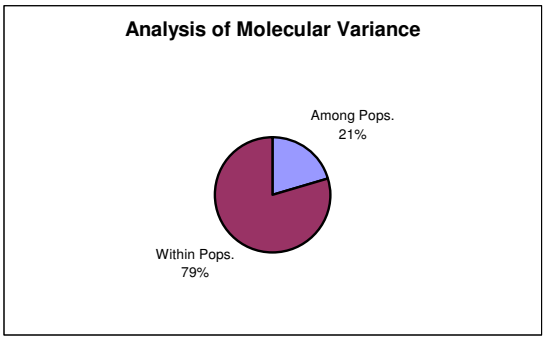


Figure 14 - AMOVA among 17 populations

Deviation from HWE was significant in 10 populations (Portugal, France, Denmark, Germany, Austria, Hungary, Finland, Sweden, Norway).

In England, Ireland, Czech Republic, Slovak, Serbia-Montenegro, Latvia, Belarus, Italy deviations from HWE were not significant (p>0,01%).

An additional internal subdivision in the geographical states was identified (Table 3). Our aim was to detect internal genetic subpopulations.

Values of Fst analogous (**PhiPT)** = 0.25 were higher than Rst values = 0.19.

Deviations from HWE were found in populations from North Portugal, North-West France, Germany Brandenburg, Austria, Hungary, South Finland, Centre-Sweden, North and Centre Norway.

AMOVA (Figure 15) and Hardy Weinberg deviation suggested the presence of a cryptic substructure or the presence of high level of admixture between the original populations.

1. Portugal		30	8. Austria		18
	i. S.Portugal	27	9. Czech Republic		27
	ii. N.Portugal	3	10. Slovakia		15
2. Spain		40	11 Hungary		6
	i. S_Spain	23	12. Serbia-Montenegro		8
	ii. C_Spain	13	13. Latvia belarus		6
	iii. N_Spain	4	14. Finland		
3. France		42		i. Finland_S	53
	i. SW France	6		ii. Finland_C	21
	ii. CS France	6	15. Sweden		43
	iii. NW France	30		i. Sweden_N	7
4. England		5		ii. Sweden_C	12
5. Ireland		14		iii. Sweden_S	24
6. Denmark		15	16. Norway		69
7. Germany		170		i. Norway_N	25
	i. Germany_LS	2		ii. Norway_C	24
	ii. Germany_MV	13		iii. Norway_W	20
	iii. Germany_BB	101	17. Italy		34
	iv. Germany_SA	3			
	v. Germany_SS	51			

Table 3 - European geographical subdivision

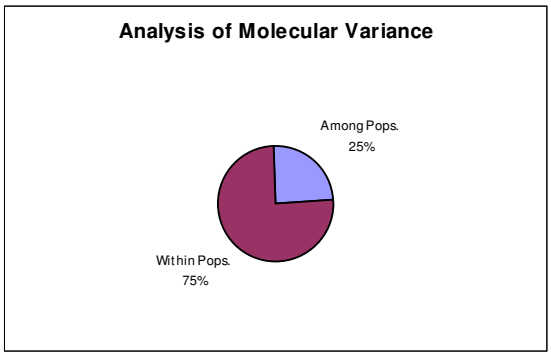


Figure 15 - AMOVA among 31 populations

7.3.2 - Bayesian assignment of genetic subdivision

Structure was run with all the samples (wild and captive animals) using POPFLAG = 0, iterations = 200000, length of the burning period = 20000, and K= 1-10.

The admixture model was applied; both independent and correlated frequencies models were run.

Each run was 5 time independently replicated.

Genetic variability was described with k=10, it has not the best likelihood, but permit to identify divergences among main European groups.

Population from Southern Iberia, England (only few samples analyzed), Ireland, Italy, Saxony (Germany), Mecklenburg (Germany), Czech Republic, Northern Norway and Western Norway were assigned to a single cluster with an high average percentage. Only Italian and Western Norway genotypes were associated to high Credibility Intervals.

Other populations were assigned to two or more clusters.

Captive individuals, samples from East Anglia and from Israel were associated to a unique cluster.

Individuals from the reintroduced area in Catalonia resulted partially associated to the Southern and Central Iberian population and partially associated with the French samples.

Italy, Saxony, Mecklenburg populations, captive reared stocks and Israel population revealed the higher allele- frequencies divergence among population (calculated with the Kullback-Leibler distance).

Structure was then run with samples collected in three separate areas:

- Iberia and France;
- Central Europe (Denmark, Germany, Austria, Slovak, Czech Republic, Hungary, Serbia-Montenegro);
- Northern Eastern Europe (Norway, Sweden, Finland, Latvia, Belarus).

7.3.3 - Genetic diversity and bayesian assignment of genetic subdivision in populations from Iberia and France

Allelic diversity was not different among Portugal, Spain and France (5.12 alleles, from 4.7 to 5.8); average values of expected and observed heterozigosity was respectively *He* = 0,61 (from 0.59 in France to 0.64 in Spain) and *Ho* = 0,54 (from 0.48 in France to 0.58 in Spain).

Two and three private alleles were found respectively in France and Spain with an average frequency of 0.015 and 0.075; no private alleles were found in Portugal.

Structure was run with K= 1-6, popflag =0, simulations = 500000, length of the burning period = 50000.

Optical genetic division was obtained with 4 populations (Figure 16) and 75% of samples were associated to a single population with a *q* value >80%.

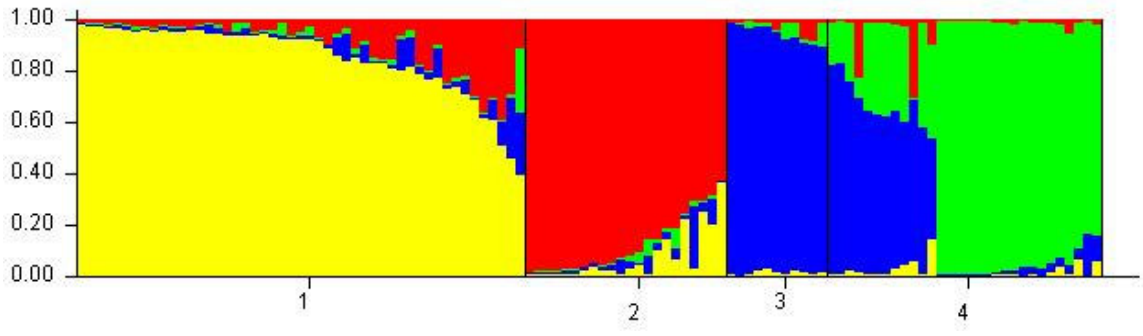


Figure 16 - Structure results in the populations from Portugal, Spain and France

Samples from Southern Portugal and Spain (Alentejo, Extremadura and Andalucia regions) were mainly assigned to the first population.

Samples from Northern Portugal and Central Spain were associated mainly to the second population.

French samples are divided into two principal groups (Northern and Southern France).

Considering a *q* value >80% and *IC* value >80% only 19% of the samples resulted correctly assigned to a single population.

Six samples from South Iberia (Portugal and Spain) were assigned to the first population, six samples from Central Western Iberia to the second population, only two samples from the middle west France to the third population and eight samples from Brittany to the last population.

7.3.4 - Genetic diversity and bayesian assignment of genetic subdivision in populations from Central Europe (Denmark, Germany, Austria, Slovak, Czech Republic, Hungary, Serbia, Montenegro)

Population from Central Europe showed different allelic diversity values: 2.9 in Denmark, 3.90 in Hungary and Czech Republic, 4.36 in Slovak and Serbia-Montenegro, 4.63 in Austria, and 6.2 in Germany. Private alleles were found in Denmark (one with frequency value= 0.036), in Germany (four private alleles with an average frequency value = 0.019), in the Czech Republic (2 private alleles with frequency = 0.019) and in Serbia.Montenegro (one private alleles with frequency = 0.063)

Expected and observed heterozigosity were also different: the lowest value (*He* = 0.38 and *Ho* = 0.34) was found in Denmark; other values were higher : *He* = 0.50 and *Ho* = 0.49 in the Czech Republic; *He* = 0.57 and *Ho* = 0,57 in Slovak; *He* = 0.57 and *Ho* =0.44 in Austria, *He* = 0.64 and *Ho* = 0.53 in Hungary; *He* = 0.66 and *Ho* = 0.66 in Serbia-Montenegro. The highest values were found in Germany (*He* = 0.65 and *Ho* = 0,58).

Structure was run with K = 1-9, popflag = 0, simulations = 500000, length of the burning period = 50000. Each simulation was 5 time replicated.

Optical genetic division was obtained with 7 populations (Figure 17) and 76.8% of genotypes were associated with a *q* value >80%.

Samples from Denmark were entirely associated to an unique population.

Genotypes from Meklenburg-Vorpommern and from the upper Brandenburg were associated to the second population.

Samples assigned to the third population were retrieved in Brandenburg.

To the fourth population were assigned genotypes retrieved in the Brandenburg, Saxony Anhalt and Saxony.

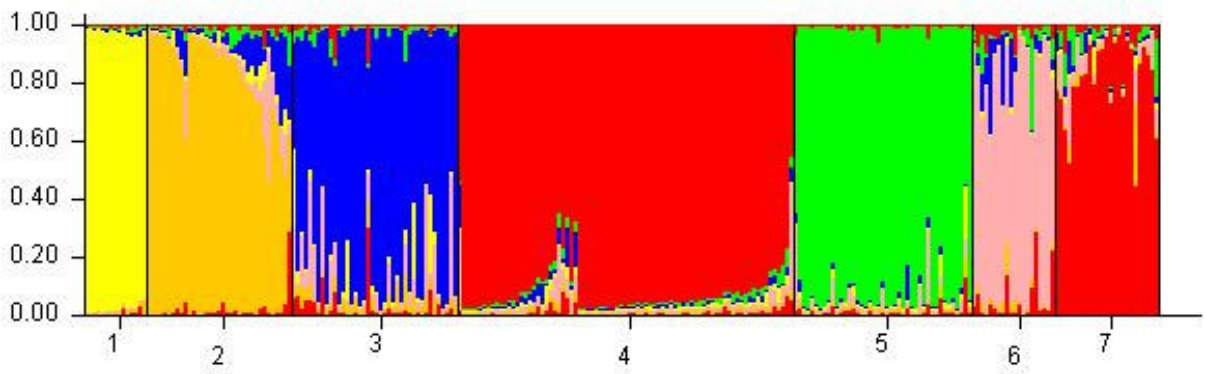


Figure 17 - Bayesian clustering of the populations in Central Europe

In the fifth population were retrieved samples collected in the Czech Republic and in Austria.

Samples from Serbia resulted partially associated with some Austrian and Germany genotypes in the sixth population and partially with samples from Hungary and Slovak in the last group.

Considering *q* and *IC* values >80%, only 16.6% of the genotypes was correctly associated to a single population.

Eight Danish samples resulted associated to the first population, four samples from Mecklenburg were assigned to the second population, only one sample from Brandenburg was associated to the third population, eight samples from Brandenburg and 12 samples from Saxony were associated to the fourth population, three Austrian individuals and seven Czech genotypes were associated to the fifth population; no one sample was assigned with *q* and *IC* value > 80% to the sixth and seventh population.

7.3.5 - Genetic diversity and bayesian assignment of genetic subdivision in populations from Northern Eastern Europe (Latvia, Belarus, Finland, Sweden, Norway)

Samples from Latvia and Belarus were considered as unique population because the geographical position and the low number of individuals. Allelic diversity was high: 4.27 in Latvia-Belarus, 6.54 in Finland and in Sweden, 6.81 in Norway.

Eleven private alleles were detected: one in Finland with a frequency = 0.020, four in Sweden with an average frequency value = 0.018 and six in Norway with an average frequency value = 0.041.

Average of expected and observed heterozigosity was high (*He* = 0.67 and *Ho* = 0.62) and not significantly differences among the populations.

Structure was run in 192 samples with K = 1-9, popflag = 0, simulations = 500000, length of the burning period = 50000. Each simulation was 5 time replicated.

Optical genetic division was obtained with 4 populations (Figure 18); an high level of admixing is present.

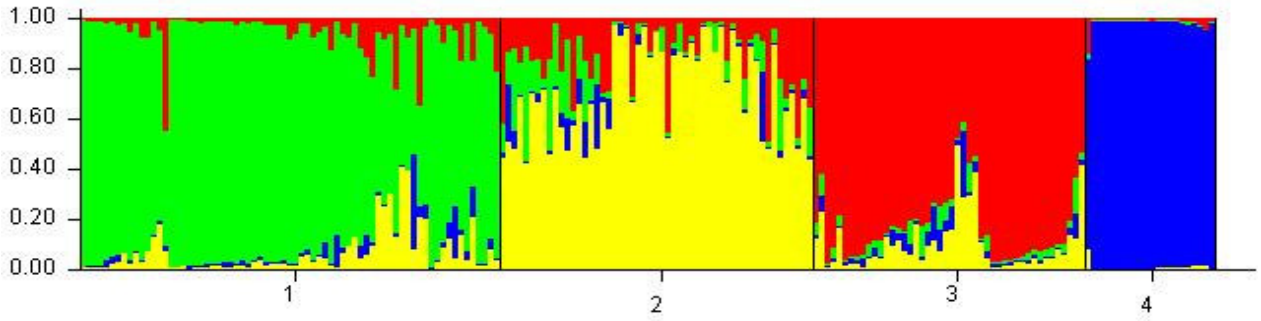


Figure 18 - Bayesian clustering of the populations in Northern Eastern Europe

Samples assigned to population number one were collected in Central and Southern Finland; few samples entirely assigned were collected in Central and Southern Sweden. The population from North Norway and few samples from North-Centre Sweden were assigned to the second population.

Genotypes assigned to the third population were collected mainly in Central Norway, few samples belong to the Central and Southern Sweden population.

Western Norway population was entirely assigned to the fourth population with high values.

Considering only samples associated with *q* and *IC* values >80%, only 21% of the samples was correctly associated to a single population.

Only eleven samples from Central and Southern Finland and one sample from Central Sweden were associated to the first population; six genotypes from Northern Norway were assigned to the second population; only one from Latvia, one from Central Norway and three from Southern Sweden were associated to the third population.

All samples except two from West Norway were assigned to the fourth population with a percentage = 90.4%. Removing Western Norway population, only 11.6% of the samples from Scandinavia were associated to a single population.

7.4 - Geographical distribution of the genotypes

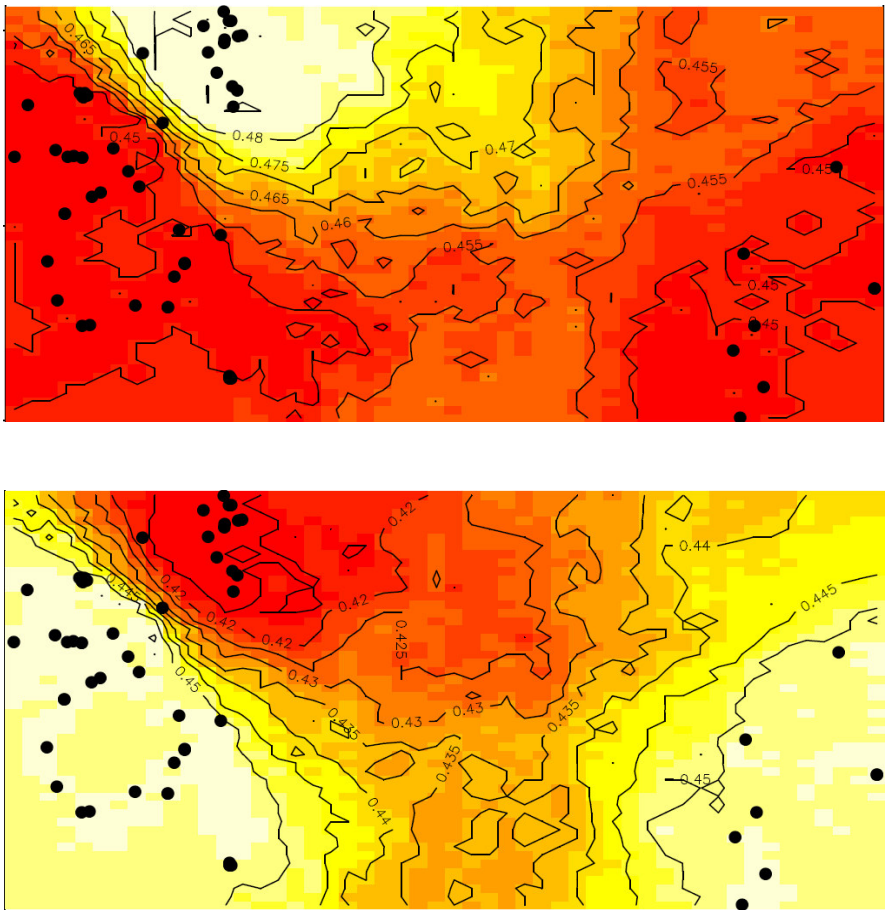
Geneland was run using 500000 iteration and a 100 length tinning period. Different values of uncertainty on coordinates was used, because the animals movements and mainly because the geographical locations were often approximate.

Data were processed separately in consequence of the samples lack in many geographical areas.

We performed the analysis in the Central and Southern Iberia, in the Western France, in Germany and in the Scandinavian Peninsula.

Spain Portugal

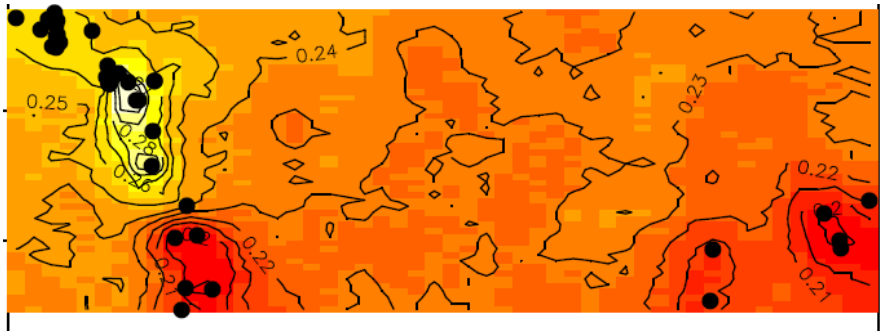
Two main areas were identified in the Iberian peninsula; populations living in the southern and in the central area of Iberia resulted genetically differentiated. Gradient between the two distribution indicate gene flow and a possible admixed area. Many samples collected in this admixed area were associated to more than one population in Structure.

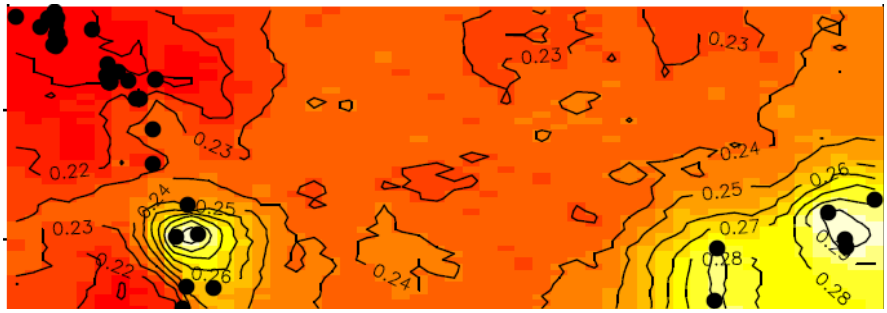


France

In France the software identified two main areas, in the North and in the South; two similar subdivisions were obtained also after the assignment test in Structure.

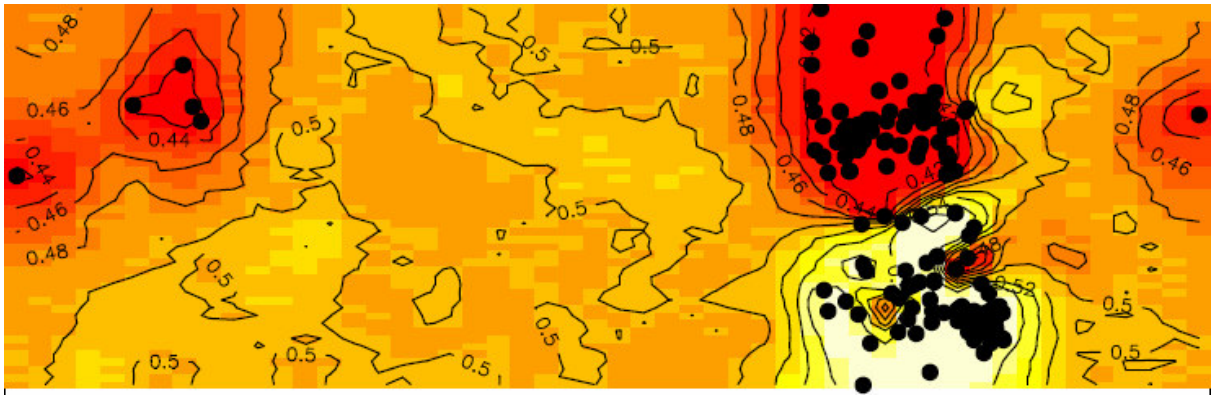
The gradient could indicate gene flow and admixed area.

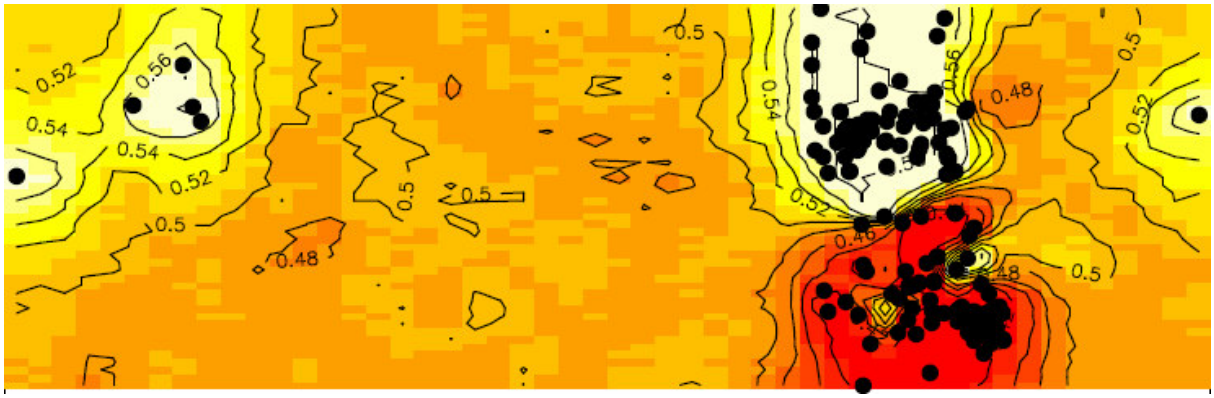




Germany

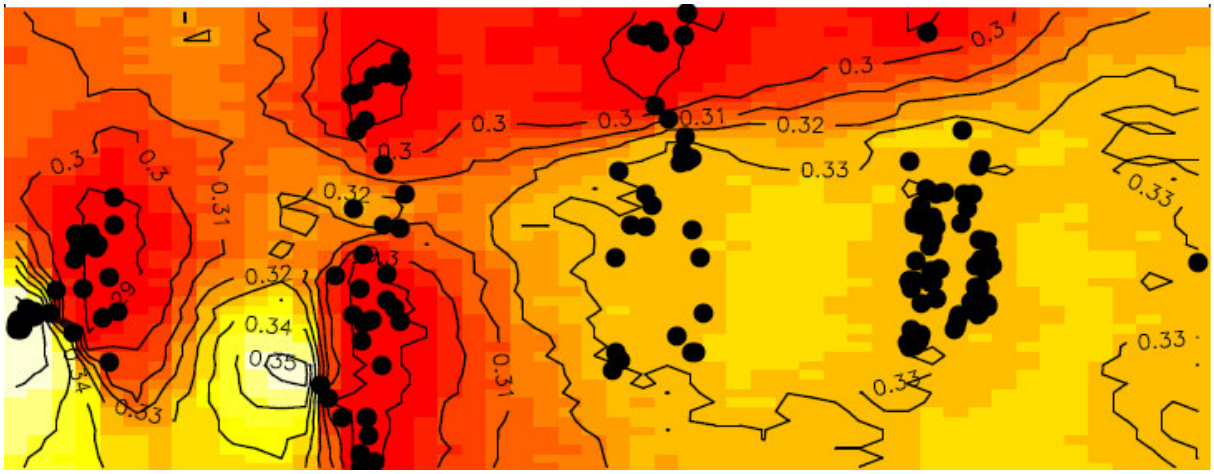
In Germany the software individuated two main groups: the first population located in the Mecklemburg and in the upper Brandenburg and the second population localized in the lower Brandenburg and in Saxony. This distribution and subdivision confirmed the data obtained with Structure.

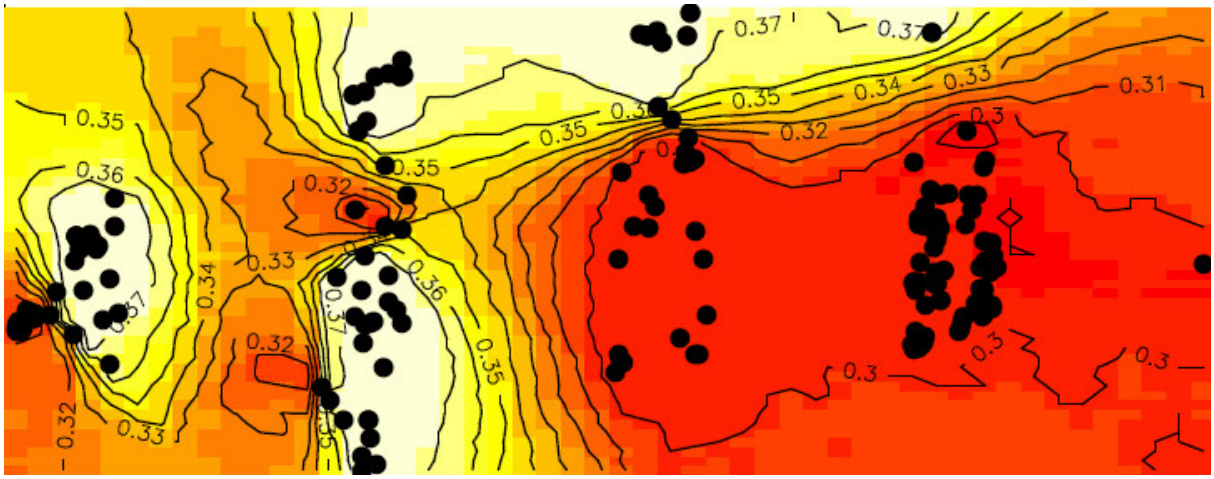


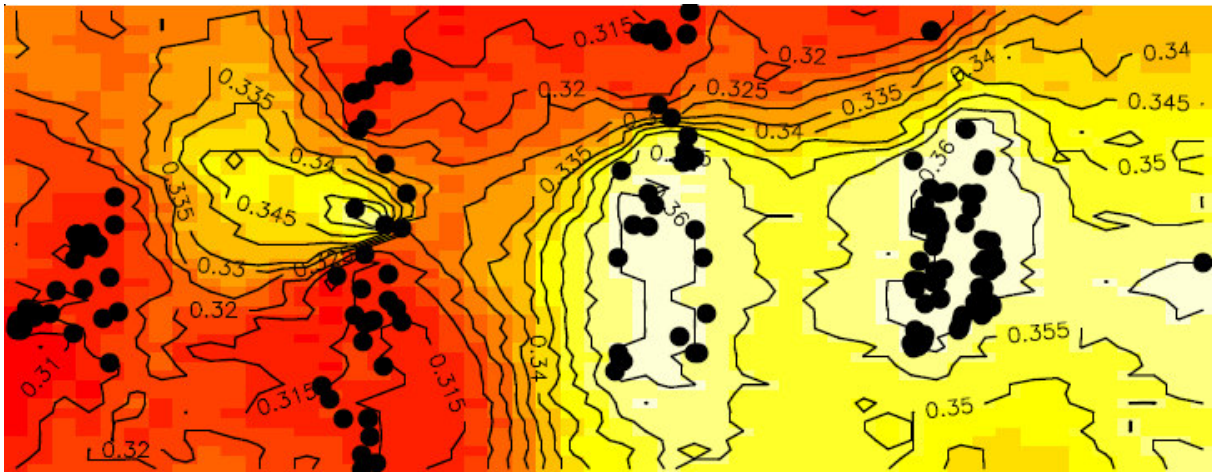


Scandinavia

In Scandinavia Geneland retrieved three main areas and groups: a Western Norway population, a group including the Central Norway and the Southern Sweden population and the last group including the Northern Sweden and the Finland. Gradient showed large values except between Western Norway and Sweden and between North Norway and Finland.



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8 - DISCUSSION

8.1 - Can microsatellite loci differentiate populations?

Low genetic variability was described among European otter populations (Pertoldi *et al*., 2001), Randi *et al*.,2003, Haikova *et al*., 2006); these results could be explained or assuming poor differentiation among populations or assuming that microsatellite loci chosen are not enough variable.

In the present work markers used has revealed able to describe variability.

As resulted from the PCA plot (Figure 12), variability described from the factorial components increased when Israelian populations and captive animals were included.

These data confirm that variability can be described and that low differentiation is due to poor differentiation among European populations.

8.2 - Genetic diversity within and among wild European populations

The otter (*Lutra lutra*) was widespread throughout suitable wetland areas in Europe until the end of 1800s. During the 20th century, habitat destruction, chemical pollution and direct persecution lead otter populations in Central and Western Europe to decline and become fragmented (Macdonald & Mason, 1994). Conservation projects aim to protect the existing core populations, and support planned local reintroduction project using both wild and captive stock animals (Reuther, 1994).

Recent findings (Randi *et al*., 2002, Pertoldi *et al*., 2001) suggested that current genetic structure in otters in Europe may not be explained by the most recent population decline, but is the likely consequence of more ancient historical processes.

Northern Europe was glaciated and almost the entire Central Europe was covered by permafrost until ca. 18,000 years ago. Afterward, almost all Europe experienced two extremely dry climate cycles, respectively at 13,000 and 10,000 years ago (The Younger Dryas, Starkel, 1991).

During those periods permafrost and dry steppe habitat conditions could have been adverse to the persistence of continuous otter populations throughout Northern and Central Europe. Post glacial founder events and re-colonization of northern Europe after the last glacial maximum, or more recent population declines during the Early Holocene in Central Europe, might have involved populations and genetic bottleneck.

Pertoldi *et a*l. (2001) suggested that population decline of otter in Denmark might have begun 2,000-3,000 years ago and have been due to human disturbance.

The low value of *He* of the Danish otter population could reflect the effect of drift, whose effects were probably stronger after gene flow from the neighbouring German population ceased (Mucci *et al*., 1999)

The data presented in this study indicate that the European otter populations are not genetically depauperate: estimated values of allelic diversity and heterozigosity were moderate to high (Goldstein & Pollok, 1997). Otters from Germany, Finland, Norway and Sweden showed higher level of allelic diversity

Mitochondrial DNA analysis showed low variability and the absence of a phylogeographic structure.

A widespread haplotype (H3) was found in all the European countries sampled, except in Southern Italy.

Although the low genetic variation makes the mitochondrial DNA an unsuitable marker to resolve the phylogenetic relationships among the European populations, it could provide information about history.

During Pleistocene, populations might have been restricted to a single glacial refugium where the main widespread haplotype might have been fixed.

During post glacial re-colonization otters have spread throughout Europe and the new haplotypes originated locally by one or two single mutation.

Star like network with a central abundant ancestral haplotype and derived locally types is typical for a population expansion after a bottleneck (Stanley *et al*., 1996).

Star like network cannot identify the origin of the ancestral population.

An increasing sampling in Southern Italy and the analysis of the museum samples collected in the Northern and Central Italy could give more information about haplotype presence in the past.

Results of AMOVA and Weir & Cockerham test (1984) indicated that most otter population were significantly differentiated. Different *Fst* and *Rst* values are probability due to the fragmentation of the European otter populations; in consequence Fst value might be more precise.

Results of multivariate distance and Bayesian cluster analysis were consistent and suggested that otters from Germany, Iberian Peninsula and Italy contributed most to population divergence.

Significant deviation from HWE in some populations could be due to the Wahlund effect (Hartl & Clark, 1989) or to recent admixing due to the recent expansion descript in most the European populations (Macdonald & Mason, 1994; Conroy *et al.,* 2000).

Deviation from Hardy-Weinberg Equilibrium observed in most of the area sampled suggest that, even within this region, the otter populations may not be homogenous and that there may be some sort of population structure at an even finer geographical scale. This could be also the situation in some countries (England, Ireland, Latvia, Belarus, Serbia-Montenegro), where the sample size may have been too small for detecting deviation from Hardy Weinberg Equilibrium.

Most of the populations did not joined into distinct clusters in the PCA plotting and in the Bayesian clustering, supporting the hypothesis of small-scale population distinction.

Assignment test showed a very low percentage of samples assigned to a single population and an high level of admixture; high admixture levels were retrieved in all the areas sampled except in the Western Norway, in Italy and in Denmark, in which it seems that an isolation process occurred.

Low variability in Italy and in Denmark could be explained either by post glacial founder event or a more recent population decline.

In Iberia, France, Denmark, Austria and Germany higher *He* values respect to *Ho* values could reflect a bottleneck occurrence; in Slovak lower *He* values respect to *Ho* values could indicate the presence of the Wahlund effect.

Landscape genetic analysis used genotypes and coordinates and identified some areas in which living otter populations have an high probability to belong to a distinct genetic population.

In the Iberian peninsula two main areas (Central and South Iberia) were identified; due to the poor sampling we lack information about northern populations. Distribution corresponds to the areas in which otters never suffered a reduction in size. In France two main groups were identified with high level of admixture. Northern population never suffered of reduction sin size, Southern population suffered a reduction from the middle to the end of the past century and the beginning of the present century surveys described an expansion of these populations.

An increasing sampling in the Southern France and in Northern Iberia could verify the existence of corridors between France and Spain. Costal area between the two countries that might connect the two populations until 1950, is actually an anthrophic area in which otter has disappeared.

In Germany two main areas identified correspond to the upper Brandenburg and Saxony; two different mitochondrial DNA lineages were also found by Cassens *et al.*, 2000. Genetic flow between upper and lower Brandenburg showed that the dense highway net did not prevent the genetic flow.

In the Scandinavian peninsula three main subdivisions were found; Western Norway was separated from the other regions and the data confirm the results obtained in the assignment test.

Sweden was divided into two areas: the Centre and the South associated to Central Norway and the North associated to Finland.

High levels of admixture confirm the presence of genetic flow and the absence of geographical barriers. Absence of genetic flow seems to exist between West Norway and Central and North Norway. The gap between the fragmented populations and mainly the presence of Joutunheimen, a mountain range covering an area of roughly 3500 Km2 in the Southern Norway explain the absence of gene flow.

8.3 - Genetic composition of captive otter

Reintroduction plans occurred in many European countries (Netherlands, Spain, Sweden, United Kingdom) using captive stock individuals or wild traslocated animals.

In this study we identified the genetic composition of the captive individuals raised in the Otter Trust Centre (UK) in which two different blood lines are bred: the Otter Trust *a* line (known origin of the animals) and *b* line (unknown origin of the individuals).

Animal released in United Kingdom descend from the Otter Trust *b* line; mitochondrial and microsatellite data suggest a probable non-European origin f these animals.

Genetic analysis of the samples collected in the French, in the Italian Breeding and in the Germany Centres (Hunawihr, Hanhensbuttel Centre, Parco Faunistico La Torbiera) confirmed that these animals originated from Otter *b* line.

Czech samples that lie outside the European genotypes were collected in an area in which cubs of a captive female born in Pavlov Otter Station (Czech Republic) and two males from south Bohemian population were released (Haikova *et al*., 2007). Based on records in Otter Studbook of European Breeding Programme, captive born female originates from B-line captive otters from Norfolk Wildlife Park (United Kingdom), with origin of paternal and part of maternal lines from wild otter population in England, however, the origin of two ancestors in maternal line is not known (A. Melissen *pers. com.*).

Only these two samples were collected in the restocked area that is located between the Czech and the Slovak viable populations; an additional sampling might describe the amount of genetic pollution.

Captive samples analyzed suggested avoiding the reintroduction or the restocking of otter from mixed captive-reared stocks of unknown geographical and genetic origin.

Low diversity among European populations suggested to prefer the translocation of wild animals from viable close populations.

The low population differentiation across Europe, as revealed in this study, suggests that risks of outbreeding depression in artificially admixed stocks are minimal (Marshall & Spalton, 2000).

In the province of Girona (Catalonia) a reintroduction plan have occurred in 1993, after the disappearance of the species at the middle of the past century; local human population was mainly in favor of the reintroduction.

The reintroduction project established the capture and the releasing of individuals from Extremadura, Galicia, Asturias and Portugal where population are healthy and increasing in number (Trinidade *et al.*, 1998; Ruiz-Olmo & Delibes, 1998).

Genetic analyses individuated a genetic similarity of these samples with the Central and Southern Iberian populations permitting to identify a probable origin of the translocated and resampled individuals. A genetic similarity was also found with French samples; these data might suggest a possible similarity of the Northern Iberian population with the Southern French population and consequently the existence of a corridor (maybe in the past) between the two countries; some information (Ainhoa Ferrando, *personal communication*) suggested that some individuals from Pyrenees were also released and that genotypes analyzed might be originated in this area.

However these data confirm that genetic information about Northern Spain are necessary.

8.4 - Conclusions

In this study, genetic methods were utilized to generate data on the threatened Eurasian otter, that may be directly applied to the ongoing conservation and management of the species.

Despite population decline, otters are still widespread in Europe and locally viable.

Genetic results from this study confirmed the data showed in the recent otter proceedings (Conroy *et al.*, 2000) and identified an increasing in number and an expansion of most of the European populations.

In order to avoid the problems originated by unwise reintroduction plans (Storfer, 1999) the best option, when possible, would be to sustain the ongoing trends of natural re-colonization through habitat naturalization and restoration of corridors (Reuther, 1994).

An increasing connectivity among scattered populations would predictably increase the effective population size and reduce the impacts of demographic and genetic stochasticity.

Habitat conservation and efforts to improve the quality of the water are to be considered more effective than r-introduction, more economical and they will provide benefit also for the whole ecosystem.

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