

# Molecular Markers for Genetic Diversity Studies of European Hare (*Lepus europaeus* Pallas, 1778) Populations

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## Abstract

The purpose of this article is to give an overview of different molecular techniques which have been used in studies concerning population genetic issues of *Lepus* species and specifically of *L. europaeus*. The importance of these researches is ever-growing as the European populations of the brown hare have suffered several falloffs as a consequent upon both natural and anthropogenic effects. With developing tools and techniques molecular genetics have become the centrepiece of population genetics and conservation biology. Nucleic acid methods based on both bi- and uniparentally inherited DNA (allozymes, microsatellites, Y chromosome, mtDNA) are often used to study genetic structure, diversity and phylogeography of different species' populations due to their effectiveness in identifying genetic variability.

**Keywords:** brown hare, genetic methods, review

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## 1. Methods

### *Allozyme methods*

Developing the method of protein electrophoresis have provided a rather large set of marker genes thus making possible for researchers to identify homo- or heterozygosity at a particular nuclear DNA locus. At the beginning of the history of allozym surveys they were used to describe genetic variations in human and fruit-fly populations [1-3]. A considerable amount of these genetic variations have been described during times hereby there is rich literature available on allozyme data concerning for example populations' structures or broad scale variations across species' ranges [e.g. 4-6]. The method clearly has its advantages such as the samples can be processed in large quantities and there are many statistical procedures available for data

assessment so the routine requires less time and training as other DNA methods [7].

On the other hand there are disadvantages of using allozyme techniques as well. Endemic species and populations which have gone through genetic bottlenecks commonly lack polymorphic loci [8]. Furthermore it has been described that one can find to be monomorphic all or most of the allozyme loci even in species with large geographic range [9]. Allozymes in addition can have different metabolic functions [10, 11] and several studies have shown that selection can act on allozyme frequencies [12, 13]. Therefore it can be determined that noncoding DNA sequences may be better genetic markers than gene products directly exposed to natural selection.

Multiple studies have been carried out on *Lepus europaeus* populations of Central and South-Eastern Europe from Poland to Greece as well as Anatolian and British ones [14 – 21] to describe genetic diversity within and among them. Along with morphological characteristics and mitochondrial DNA markers Hartl et al. [16] studied allozymes, and this method turned out to

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be the most informative in that particular case. However they found that neither of the methods considered separately is representative for overall gene pool diversity within populations. They found that the average heterozygosity and polymorphism was significantly different among populations and higher than values reported by several other studies of the species (Poland, [15]; Austria and Bulgaria, [19]) and those of *Lepus timidus* populations of Europe [22]. They have not found correlation between age or gender and heterozygosity. Vapa [23] and his colleagues surveyed the allozyme variability in brown hare populations of the region of Vojvodina (Serbia). Genetic variability have been found within the range described for other Central European populations [14-15, 17] as well as for the Bulgarian and the Greek [21] populations.

#### *Microsatellite DNA (SSRs, STRs)*

Microsatellite DNA can effectively be used in population genetic studies because of the very high amount of alleles (30–50) on single loci [24]. These fragments of the DNA are composed of tandem-repeat units of a few bases. The number of alleles is the consequence of the balance between the mutation-driven formation of new alleles and the elimination of existing ones by natural selection or genetic drift [25]. The high heterozygosity of microsatellite alleles suggest a considerably high mutation pressure along with a low value of fitness differences between the alleles [25]. These markers are eligible for describe allele frequencies in population genetic studies [7]. They show high levels of gene diversity therefore are used in phylogeographic surveys [26]. Furthermore they are not any less easy to use than other PCR – gel electrophoresis techniques once the primers are identified. Finding the usable primers for new species can be expressly time-consuming, however there has been described that microsatellite regions are often flanked by highly conserved sequences at the priming sites [27-29]. This phenomenon provides an easement for beginning researches on species which have not been previously studied if there is information on their close relatives. Researches on *L. europeus* also have used microsatellite makers to describe genetic structure [e.g. 30, 31], diversity [e.g. 32, 33] or introgression by hybridization among Leporids of Europe [e. g. 34, 35].

#### *Mitochondrial DNA*

Mitochondrial DNA has often been used in gene flow studies over the past few decades. Its popularity is resulting from several attributes which make it easy to use, such as being strongly conserved, having no introns and very few duplications as well as short intergenic regions in the sequence. It is easy to be amplified due to the small size and the abundance in animal tissues of the molecule. The strict orthology of encoded genes make it a reliable phylogenetic marker [36]. Though it has been established that mtDNA is not by any means as perfect test subject as it was thought to be [37, 38] its usage in molecular ecology and conservation genetics has not been decreased due to the above-mentioned characteristics. The most frequently used mitochondrial markers are the control region [32, 39 – 41] along with the *cytB* region [41, 42]. Although there are exceptions [43, 44] mtDNA is typically inherited maternally in eukaryote species. Sperm-derived mitochondria do enter the oocyte but they degrade by autophagy almost immediately after fertilization in *Caenorhabditis elegans* [45], and it is believed that in mammals the method of avoiding heteroplasmy caused by paternal mtDNA inheritance could be the same [46]. Albeit information can be provided only in connection with the female germ line it is important that the molecule is transmitted consistently across generations. This nature of the transmission provides an important easement for describing the origin and kinship of a biological specimen since large amounts of reference samples of closely or distantly related individuals may be available for comparison [44]. Mitochondrial DNA regions show polymorphism in different species thus providing a valuable method for determining genetic identity or diversity among a species' populations [47] Based on main morphological parameters there are nine subspecies of the *Lepus europeus* [48] however genetic surveys do not confirm these taxonomic results, which probably have originated from the well-known intra- and interspecific morphological variability of the genus [e.g. 49]. MtDNA-based evolutionary hypotheses are inconsistent with those deduced from data of proteins or morphology hence practically representing the nuclear genome [21, 48, 50]. Transmission of the two genomes differ remarkably [51] This along with the sex specific

natal dispersal of these species [52, 53] possibly cause the incongruence.

Researches using only mtDNA markers have shown genetic divergence of some degree between European brown hare populations. Hartl et al. [16] found the haplotype diversity value to be  $h=0.158$  in Austria and Central-Europe. In Vojvodina region of Serbia and Montenegro the research of Djan et al. [47] showed an average value of  $h = 0.34$ . Mamuris et al. [54] described a high level of haplotype diversity ( $h=0.853$ ) and a large number of haplotypes in South-Eastern-Europe. This is expressly higher than the values described in Scandinavia (0.38%; [55]) and Italy [1.3%; 56] and three times lower than the average in the brown hare populations of the Iberian Peninsula (6.2%; 57).

Screening of single nucleotide polymorphisms (SNP) makes possible to use low quality template DNA in researches by not needing long molecule fragments, and could reduce the surveys' costs [58]. This can lead to the complete replacement of microsatellite techniques [35], thus they have recently been used as genetic markers in population genetics researches [59]. They can be used to identify alleles within the nuclear genome or haplotypes in mitochondrial DNA. The method is based on detecting polymorphic nucleotide positions in particular DNA sequences. Testing the scale of polymorphism and the prevalence of different alleles or haplotypes requires DNA sequence data and a reference population. Every position can provide four polymorphisms at the maximum (by the four nucleotides). Thulin et al. [35] identified single nucleotide polymorphisms in *Lepus europaeus* and *L. timidus* mtDNA researches.

#### *Y chromosome*

As mentioned above most of times mtDNA surveys have been used in population genetics and conservation biology researches [e.g. 39; 60]. Although plenty of very important data have been provided by those, one can say, that neither of the methods are enough for getting an adequate panorama on the subject since no information about the male lineage is added to the results [61, 62]. This cannot be satisfactory in relation to species with females characteristically philopatric and among them the European brown hare [31, 63, 64]. This is the reason why researchers tend to use biparentally inherited genetic markers such as

microsatellites [65 - 67]. These methods seem to resolve the problem of lacking paternal data but the recombining loci and the mostly uncharted mutation model [68] present obstacles to the comprehensive analysis. Though the stepwise mutation model might explain the allele size distribution in satellites with short repeat units [69]. A viable solution to the problem of getting adequately synthetic image on the population genetics of species like the *Lepus europaeus* is using mtDNA and Y chromosome markers in comparison.

The Y chromosome in mammalian species is inherited strictly paternally, is characterized by a slow mutation rate in proportion to the mtDNA [70] and is almost entirely, approximately in 95%, nonrecombining (NRY). However, it has been described that the NRY can form palindromes by self-recombination and gene conversion [71, 72] at least in primates. This discovery has changed the terminology from NRY to MSY (male-specific region on Y). Mammalian Y chromosomes have lost most of their genes (in humans, more than 95%; [73]) and for this reason have become far smaller than their allosomal counterparts. They are believed to evolve by gene loss by certain theories [74, 75] and eventually settle in stasis. The sexdetermining region of Y (SRY) whose expression is the basis of the male sex development [76, 77], is the most conspicuous locus on the MSY. It has probably evolved by the truncation of the SOX3 gene on X [78]. About 5% of the Y chromosome's sequence recombines with the X chromosome. These recombining regions are termed as pseudo-autosomal due to their essentially diploidic nature. They code genes like the zink finger protein region (ZFY) or the amelogenin gene [79, 80]. Likewise autosomes MSY contains microsatellites [81], but there is little known regarding their evolution.

Y connected markers have been used by several studies in population genetics with the aim of shedding light on issues like male-driven evolution [e.g. 82, 83], demographic history of certain populations [84, 85] or the origin of male lineages [86, 87].

Hughes et al. [88, 89] have carried out a research to compare the conservation of Y-linked genes in humans and chimpanzees which revealed that there is excessive divergence between the two species' sequence structure. As a result of this study the MSY of the chimpanzee is now

sequenced as accurately as that of the human. However there is relatively few information on the Y chromosome of other mammalian species. On species of Lagomorpha a few studies have been carried out. There have been mapped Y chromosomal markers for *Lepus europaeus* including the complete coding sequence of the LeSRY locus, microsatellite loci (LeMS-Y) and introns of the zinc finger protein (ZFY) [62, 90]. Information on the Y chromosome sequence of *Oryctolagus cuniculus* have been published as well [91, 92].

## 2. Summary

This study have been given forth with the aim of making an attempt at providing an overview of what we have known of the nucleic acid markers used in brown hare (*Lepus europaeus* Pallas, 1778) studies concerning distribution, phylogeography, population structure and taxonomic status to this day. This Leporid is an important game species with an extended geographic range from Western-Europe to Mongolia. Being a species of economic value it has been introduced to various countries such as Argentina, Australia, Canada and Sweden [49]. These circumstances have motivated several researchers in carrying out studies on brown hare populations all over the European continent. Data have been provided on population structure, hybridization and introgression among species, however there are unclarified questions about the taxonomic status or the phylogeography of the brown hare. The molecular genetic techniques and large amount of markers identified so far could lead to a rapid progress in gaining population genetic data during conservation biology surveys. In studies concerning the populations of *L. europaeus* mtDNA markers have been used most frequently [e.g. 16, 56]. There are however valuable results of microsatellite and Y chromosome [63, 91] studies as well as provided by allozyme [e.g. 19, 23] researches. Though all of the above mentioned methods are useful and necessary they all have their disadvantages. For this reason one must consider carefully which technique is the best option to answer their particular questions having regard to both financial and scientific aspects.

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