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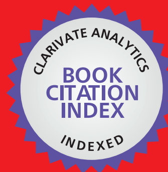

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Chapter 9

Isoenzyme Analyses Tools Used Long Time in Forest Science

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Additional information is available at the end of the chapter

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1. Introduction

1.1. What kind of tool are isoenzymes

A specific group of proteins – enzymes, characterized by similar chemical properties are isoenzymes (Hunter & Markert, 1957, Tanskley & Orton, 1983, Goncharenko, 1988, Mejnartowicz, 1990, Sułkowska, 1995). They are characterized by similar chemical, structural and catalytic properties almost identical within their functional group. They are characterized the same function in cell reactions, however they are products of different genes (described as allozymes).

Their origin is explained by gene duplication of polyploidisation mutation, which can create kinds of pseudogene – or nucleic acid hybrydisation. Different forms of an enzyme that are coded by variant alleles at the same locus are called allozymes, while isozymes are products coded by genes located at different loci. Even then, isozymes and allozymes are variants of the same genes and they possess the same catalytic functions they can be easily identified in biochemical way. First of all the substitutions of different amino acids are responsible for their variant electric charge and this can be the way how to identify them by electrophoresis process. This charge characteristics of isozymes and allozymes is the basis to use them as molecular markers.

An important feature of this group of proteins is that the presence of both alleles of a gene is disclosed in a manner independent of each other so cold co-dominant character, so it is possible to request a degree of heterozygosity within the study population characteristics (Rider & Taylor, 1980). In practice, this means that there is a simple way to distinguish heterozygotes from homozygotes. The isoenzyme molecules are proteins. It is important to define them as the first products of gene translation, so they possess information about their coding region of DNA. Their heredity of responsible genes is explained by Mendelian

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character. A large number of genes encoding enzymes shows a significant degree of polymorphism. The process of natural selection is still important factor, because genes are specialised to different functions.

Long time in forest sciences, the isoenzyme markers were the best tools to analyse genetic variation of populations despite of the different limits and restrictions of this method. Nowadays we have more informative tools based on DNA markers such as sequencing, microsatellites, PCR-RFLP and single genes analysis like SNPs. Most of this DNA based markers is still developed to make them more suitable in analysis of plant organisms. The level of complication of plant genome is higher comparing to animal. Average of size of *Pinus* genome is about 30 billion base pair (Grotkopp et al., 2004). We have up to now many possibilities to analyse the polymorphism of DNA fragments of plant material but not the particular genes. So, the isoenzyme markers represent still one of the best markers close to DNA level. It is possible to assess the variation of individuals at different level: within species, within population, and among populations within species. It is worth to add they are quick and cheap marker systems and good alternative to assay and identify level of genetic variation as pilot study of populations (Bakshi & Könnert, 2011) as well as conservation biology activities e.g. gene bank – enables choosing of proper sample for long time conservation (Bednorz et al., 2006) or as well as in quantifying mating system analysis (Mrazikowa & Paule, 1990).

The proteins of enzyme are consisted of polypeptides and are molecules characterised the specific conformation. The quaternary structure of particular enzyme forms may be different in relation to combination of peptides involved to build their molecules. We can observed (Figure 1) monomeric enzymes with two the same allozymes, dimeric forms with three allozymes and tetrameric proteins with fives allozymes. We can sometimes observe on gel after electrophoresis bands of some intermediate mobility reaction.

The isoenzyme analysis method enables the assessment of variability of isoenzymes in different types of tissues: young leaves, buds, pollen and seeds. There is possibilities to analyse diploid as well as haploid tissue. Especially it is important, when we study coniferous species genetic variation, then it is great tool to analyse mating system of trees, because of possibilities of using haploid tissue of mother trees and diploid tissue of embryos. The analysis requires very small amounts of plant material and is a very sensitive method. Simultaneously, it is possible to identify many samples/or individuals. The great advantage of this method is low cost of chemicals used to performed the studies.

1.2. Advantages of isoenzymes and week points of the utilisation

The using of isoenzyme electrophoresis method is useful tool especially in assessment of gene frequency of specific genes, determining of genetic similarities and genetic distances between the two objects e.g.:

- Good tools to support conservation and management of forest trees genetic resources
  - To characterize forest tree stands genetic structure

- To asses initial gene pole
- To present selection processes of forest stands and to maintain rich of natural diversity of stands

- Genetic characteristics of different forest tree species reproductive material:
  - Mother stands and progeny stands gene flow analysis
  - Seed orchards and progeny plantations mating system
  - The representation of populations selected for preservation in gene banks or in situ or ex situ measures
- Solving of problems from seed stands management point of view:
  - clonal/pedigree identification/selection process
  - pollen contamination especially important in management of artificial tree stands like forest seed plantations
  - patterns of gene flow and mating system in natural and artificial stands

Enzyme/Gene Type	Homozygote	Heterozygote	Homozygote
Monomeric			
Dimeric			
Tetrameric			

Figure 1. The graphic illustration of bands of proteins on the gel after electrophoresis process when we study various types of isoenzyme proteins.

1.3. Methodical problems

The isoenzyme studies are suitable only when their heritability is explained by Mendelian character – often the same enzyme systems are not suitable when we study different species. The enzyme loci are not randomly distributed over the genome, so they are not representative for total genome variation (Hubby & Lewontin, 1966). We have problems to compare analysed data with reference studies when the number of analysed enzyme system is different. At the first studies some enzyme systems were described by different numbers enzyme gene loci systems e.g. peroxidases or number of loci controlling analysed enzyme. For example GOT (transaminase glutamine oxylo-acetate) was assumed to be coded by 2 or

3 or 5 gene loci for Scots pine (Müller-Starck et al., 1992). Some alleles are not identify as the bands on the gel (null alleles). It is not possible to use particular enzyme markers when linkage disequilibrium is occurred for analysed loci, so it obvious to verify the genetic control system and inheritance of chosen enzyme markers.

Still seems to opened the question are allozyme neutral or adaptive?

Some researchers showed the results studies only for polymorphic enzyme systems without any information about monomorphic ones, so the genetic variation was described as higher than it was real.

The obtained information about isoenzyme variation is not a representative sample of the total variability of the analyzed species, which illustrates the genetic variability within the ranges of their occurrence, due to too small amount of tested samples. Evaluation of variation by this method applies only to a very small part of the genome.

2. Methods

2.1. The idea of isoenzyme electrophoresis

The charge characteristics of isoenzymes enables to use them as genetic markers, which can be distingished. First of all the substitutions of different amino acids are responsible for their variant electric charge and this can be the way how to identify them by electrophoresis process. The identification of proteins molecule characterised a specific electric charge can be dissolved in a buffering solution, after homogenisation of plant or animal tissue. The non-denatured proteins are separated on the carrier gel and they migrate under the influence of an applied electric field. The molecules of enzyme proteins can change conformation shape and it will be followed by modification of net charge. This changes of molecules charge can be detect using electrophoresis method.

The rate of migration of molecules in the gel and segregation of bands of enzymes in the gel is the result of interaction of: the applied electric field, pH of gel and electrode buffers (Concle et al., 1982). After completion of electrophoresis on gel plate, the proteins are stained by using the appropriate staining reactions.

2.2. Migration process of proteins in different media and their visualization possibilities

The carrier of proteins in electrophoresis may be different types of substances: polyacrylamide gels and potato starch gels. The second one were historically used, but the great advantage of them still is the possibility of cutting them to obtain slices, which can be analise to identify different protein markers using the same gel and tissue material, because all the proteins are active in the gel.

Proteins separated in the gel during electrophoresis can be detected by staining or UV analysis e.g. fluorescent esterase enzyme. Methods of detection of enzymes are relatively

well developed and reported in many publications (Concle et al., 1982, Goncharenko, 1988, Wang & Szmidt, 1989, Liengsiri et al., 1990). The visualisation of enzymes is possible by precipitation of soluble indicators like tetrazolium salts using cofactors of NAD or NADP to transfer them into reduced insoluble forms (Figure 2). It is important to put attention that the staining reactions require the active forms of enzymes and they simulate work of proteins in functional tissues of organisms. Most of reactions are performed in the temperature 37 °C. During relatively short time one day of laboratory work we have result and evaluation of the analysis for about 50 samples e.g. individuals using 10 or more enzyme systems represented by one or more loci each of them. It is important as well that the staining process does not requires complicated laboratory equipment. The resolving of proteins during electrophoresis can be performed in horizontal or vertical chambers, but the crucial is to protect the activity of enzymes by providing of cooling system. The power of electric field applied for migration of proteins is often up to 280V, so the heating of gel is observed and have to be reduced.

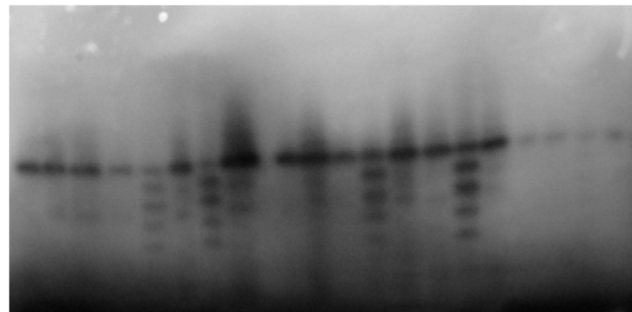

Figure 2. Menadione reductase electrophoretic pattern in European beech. We can observed The monomeric enzyme form with two the same allozymes (homozygote form), dimeric form with three allozymes (heterozygote form) and tetrameric proteins with fives allozymes (heterozygote form). Photo M. Sulkowska

2.3. Genetic differentiation evaluation process

2.3.1. Analysed parameters to describe population genetic variation and differentiation

The basic parameters evaluated to measure population genetic variation and differentiation are usually as it follows: number of alleles per locus, heterozygosity (*H*), percentage of polymorphic loci, genetic distance (G_{ST}). The terms of heterozygosity, diversity and differentiation are explained in different way by various researchers. Most of people used to work with genetic parameters elaborated by Nei (1973, 1978), when we consider heterozygosity observed or expected, what is estimated as well as a measure of genetic diversity heterozygosity observed (*Ho*), heterozygosity expected (*He* – or *D* – gene diversity). The value of this parameter is calculated as values from zero (no heterozygosity) up to nearly 1.0 (when we observe a large number of nearly equally frequent alleles). Instead of average number of alleles per locus more precise used to be measure of effective number of alleles per locus (n_e) – Crow & Kimura (1970).

$$He = 1 - \sum_{i=1}^{n} pi^2 \quad (1)$$

$$n_e = \frac{1}{\sum_{i=1}^{n} pi^2} \quad (2)$$

p_i – frequency of n allele occurrence in population

Heterozygosity is often one of the most important "parameters" when we describe the genetic data. Using this measure we explain the general trends in the structure of analysed populations – even is their history and future genetic structure is concerned. Low values of heterozygosity is influenced by small population size and processes of genetic drift e.g. bottlenecks effect. A lot of heterozygotes in population is equal high genetic variability and the opposite. When we compare the level of the observed and expected heterozygosity in balanced populations concerning random and open mating system it means under Hardy-Weinberg equilibrium and heterozygosity is higher than expected we can expect flow of alien pollen outside of population. If the observed heterozygosity is lower than expected we can assume that some inbreeding processes may occur in the population.

The interpopulational variation is described very often as G_{ST} (Nei, 1973) and it is used as equivalent of F_{ST} statistics (1969, 1978) and it enables to asses for each population the distance from other populations.

$$G_{ST} = \frac{H_T - H_S}{H_T} \quad (3)$$

H_T - heterozygosity interpopulational
H_S - heterozygosity intrapopulational

$$F_{IT} = (H_T - H_I)/H_T \quad (4)$$

F_{ST}, cold as fixation index is the measure of proportion of the total genetic variance within subpopulations in relative to the total genetic variance. The values of this parameter can range from 0 to 1. High F_{ST} implies a considerable degree of differentiation among populations.

F_{IS} (inbreeding coefficient) is the proportion of the variance in the subpopulation contained in an individual. High F_{IS} implies a considerable degree of inbreeding. Values can range from -1 (outbred) to +1 (inbred).

$$F_{ST} = (H_T - H_S)/H_T \quad (5)$$

$$F_{IS} = (H_S - H_I)/H_S \quad (6)$$

H_T - heterozygosity total for population
H_S - heterozygosity within subpopulation
H_I - heterozygosity of individual

2.3.2. *Software*

One of the oldest programs enabling computing of isoenzyme analysis data is BIOSYS-1. This program was elaborated to help biochemical population geneticists to describe the analysis of electrophoretically detectable allelic variation. It can be utilised to study allele frequencies and genetic variability measures, to test for deviation of genotype frequencies from Hardy-Weinberg, expectations, to calculate F-statistics, to perform heterogeneity of chi-square analysis, to calculate a variety of similarity and distance coefficients, and to construct dendrograms using among others cluster analysis procedures. The program, documentation, and test data are available from the authors (Swofford & Selander, 1981).

Another one interesting software enabling as well analysing of DNA data markers is POPGENE (Yeh & Boyle, 1997). The current version of POPGENE is designed specifically for the analysis of co-dominant and dominant markers using haploid and diploid data. It performs most types of data analysis encountered in population genetics and related fields. It can be used to compute summary statistics, including: allele frequency: estimates gene frequencies at each locus from raw data, effective number of alleles per locus, percentage of all loci that are polymorphic, observed homozygosity, expected homozygosity, Shannon Index, gene diversity Nei's (1973), F-Statistics, Gene Flow: estimates gene flow from the estimate of G_{ST} or F_{ST} and many others parameters.

POPGENE is a good tool in analysing and simulations studies of population genetics, including: Hardy-Weinberg Equilibrium, multiple allele and loci inheritance, natural selection, genetic drift, migration, mutation and inbreeding.

Applied procedure of isoenzyme analysis is consisted of the following steps:

- Sample preparation (collecting of samples, homogenization and extraction of proteins from the tissue)
- Preparation of gels and running buffers requirements regarding analysed enzyme systems
- Development of isoenzyme electrophoresis
- Detection and staining of proteins
- Computing of the obtained data base with utilisation of proper software

3. Results

3.1. Genetic variation characteristics

The investigations of beech variation and differentiation in Europe showed (Gömöry et al., 2003, Sułkowska et al., 2012). The aim of this study was the assessment of genetic diversity

and differentiation patterns of European beech (*Fagus sylvatica* L.) populations within its natural range in Poland and to compare them to those in other neighbouring European countries including Slovakia, the Czech Republic, Ukraine, and even Romania, which was reported previously (Paule et al. 1995). These stands cover 5.2% of the forest area in Poland, and form the predominant forest tree communities throughout the Carpathians and Sudety Mountains, and the moraine landscape of the Pomeranian Lake District. Varying environmental conditions have resulted in a great number of ecotypes and populations which are characterised by various ecological requirements. Poland represents the northeastern limit of the beech's natural distribution. Genetic diversity and differentiation was assessed using allozyme gene markers employing 9 enzyme systems: glutamate-oxaloacetate transaminase (GOT - EC 2.6.1.1), leucine aminopeptidase (LAP- EC 3.4.11.1), isocitrate dehydrogenase (IDH - EC 1.1.1.42), malate dehydrogenase (MDH - EC 1.1.1.37), menadione reductase (MNR - EC 1.6.99.2), phosphoglucomutase (PGM - EC 2.7.5.1), phosphoglucose isomerase (PGI - EC 5.3.1.9), peroxydase (PX - EC 1.11.17) and shikimate dehydrogenase (SKDH - EC 1.1.1.25). The data revealed: high genetic diversity of beech, similar like in other neighboring European populations, slight decrease of average number of alleles per locus and level of differentiation towards the North of the natural range limit, which generally confirm the migration paths after glaciations but it is not the basis to distinguish geographic regions.

The population differentiation of beech provenances of selected seed stands and their progenies for chosen genetic parameters and on the basis of soil characteristics of the habitats were studied (Sulkowska et al., 2008, Sulkowska, 2010). Beech populations occurring toward the northeast of the natural range were characterised by a decreasing the average number of alleles per locus and percentage of polymorphic loci. The highest genetic differentiation was found in the East Carpathians.

3.2. Ecotype variation characteristics

3.2.1. Geographic trends with an example of coniferous species - Scots Pine (Pinus sylvestris L.)

Allozyme differentiation in chosen European populations of Scots Pine (*Pinus sylvestris* L.) were studied in 17 populations from North and East-Central Europe (Prus-Głowacki et al., 1993). The samples were collected from provenance trial in Lubień (Poland). The provenances from Scandinavia, northern Poland, Netherlands and Belgium were more heterozygotic, more polymorphic and characterised higher number of alleles per locus. The source of seeds used to establish the provenance trial was unknown as far their autochthonous or introduced origin is concerned, but the results indicate a degree of coincident agreement with geographical distribution of stands. This effect seems to be blubbered by human activity (uncontrolled seed transfer).

The studies of 11 enzyme systems concerned on the genetic variation of *Pinus sylvestris* from Spain in relation to other European populations revealed genetic dissimilarity of populations from this region. The differences were observed as far as slightly higher

number of alleles per locus, but lower heterozygosity level in populations from Spain (Prus-Głowacki & Stephan, 1994).

In Poland, the isoenzyme studies of 5 systems variability of 8 populations revealed existence of two groups of populations – North and South groups (Krzakowa, 1979). There was a high variability within all analysed loci. The importance of existence of this groups was undertaken as well by field studies and low regulations (Dz.U. 04, nr 67, poz. 621, 2004, Matras, 2005).

3.2.2. Site plasticity with an example of deciduous species – European beech (Fagus sylvatica L.

Present genetic structure of beech population in Europe was formed many different factors not only environmental and genetic but also anthropogenic. Different environmental condition resulted in great number of ecotypes and populations, that characterized various ecological requirements (Dzwonko, 1990, Giertych, 1990). Very important factors affected the gene pool were glacial epoch and the location of beech refugia, for postglacial migration paths of species (Szafer, 1935, Huntley & Birks, 1983, Ralska-Jasiewiczowa, 1983, Hazler et al., 1997). One of the first studies on genetic variability of isoenzymes of European beech - *Fagus sylvatica* L. were conducted in France. The study of genes encoding peroxidase system. There was a relationship between the frequencies of particular alleles encoding these enzymes, and analyzed population, geographical and environmental factors (Cugen et al., 1985). Genetic variation of beech - *Fagus sylvatica* L., was also analyzed on a wider scale within the six enzyme loci, 130 population for southern and western Europe. Observed correlation between the frequency of alleles of genes encoding peroxidase and climate (Comps, et al., 1990).

Some genotypes are eliminated during natural selection process, when they are not efficient in the environment, it is shown using isoenzyme markers (Müller-Starck, 1985). In most case populations characterised higher level of genetic variation seems to be more tolerant to harmful environmental factors (Starke et al., 1996).

In a continuous process of verifying the adaptation of individuals, which occurs in nature, it is contributed to both human and natural selection. This was proven among other by differences in the genetic structure of two provenances of beech German and Romanian, grown in a greenhouse and natural conditions (Kim, 1985). It was found that beech seedlings with *Lap-A2* allele always were characterised a higher survival. Homozygous for the allele *Lap-A2* survived better in a greenhouse, while heterozygotes were characterized by higher vitality in the natural environment.

3.3. Using of isoenzyme markers in coniferous seed orchard research

Isoenzyme markers are very important tool concerning genetic parental or progeny identity (Wheeler & Jech, 1992). The estimation of gene flow in mating system of seed orchards is crucial for proper use of seeds to asses and if possible avoid pollen contamination outside of

the stands and seed orchard genetic efficiency. It plays important role for gene conservation of the stands and the improvement in forest tree selection (Concle, 1972, Rudin & Lingren, 1977).

The investigations of 122 trees in seed stand in Sweden were conducted for adult trees, embryos of seeds and progeny of the stand (Yazdani et al., 1985). The analyses revealed significant variation among different groups of studied objects at 5 enzyme loci. Genetic frequencies of alleles were close to Hardy-Weinberg equilibrium, but the deviations were found for embryos.

In Poland, also featured on the study of inheritance of a certain enzyme - GOT (transaminase glutamino-oxylo-acetate) for selected two homozygous plus trees, under the terms of this gene, and the pollination of these trees by surrounding neighbors. The study provided evidence of contamination at least 40% of the seeds with pollen outside the stands, which indicates a high out-crossing rate for pine (Krzakowa, 1980).

In the seed orchard in Slovakia, the mating system of trees was analysed on the basis of five enzymes studies inheritance (Mrazikowa & Paule, 1990). The study was conducted simultaneously for macrogametophytes of embryos and seeds. The degree of foreign seed pollination with pollen from outside the plantation was shown.

4. Discussion

The isoenzyme markers are important tool in when we assay gene variability of forest trees. The complicity of genome of this organism makes it impossible in most cases to obtain information about particular genes. Nowadays, molecular markers are powerful tools, which enables to study genetic variation of the organism, but we are usually able to work with specific fragments DNA, not to assay the genes. The methods biochemical and molecular should be taken into account in case of genetic variability analyses of forest trees as complementary tools. It can be revealed by studies of migration paths of European beech using both types of markers (Magri et. al., 2006), where existence of one common refugium of the species was proved for most part of Central and West Europe.

Single genes are responsible to control proteins of particular features – genetic traits (Bergmann, 1991). They are not responsible to control the complex of morphological traits, physiological and adaptability of individuals as reaction of needs of environment, that are crucial for surviving of rooted in one site plants over their whole life.

Ecotype variation of forest tree species can be classified as relation to their geographic range (Müller-Starck et al., 1992):

- Species characterised large geographic range like Scots pine, Norway spruce and European beech and little genetic differentiation among populations within regions derived from the same refugia, but greater comparing various refugia
- Species with large geographic ranges, but with many subspecies like Pine species – *Pinus nigra, Pinus halepensis* with small interpopulational variation within subspecies, but differentiation among subspecies

- Species characterised small geographically ranges like fir but with great interpopulational differentiation and medium level of intrapopulational genetic variation – endemic species
- Species with extremely small geographic range like Siberian dwarf pine characterised relatively high interpopulational differentiation – relic species.

Using of isoenzyme studies as one of DNA responsible marker to solve the problems of reproductive and economic value of seed orchard stands is very crucial (Wheeler & Jech, 1992, Krzakowa, 1980).

A especially interesting seems to be using of isoenzyme analysis in estimation of gene flow in natural and artificial populations of forest trees, when the genetic values of artificial management stands is taking into account (Savolainen & Yazdani, 1991, Skrøpa, 1994). The reported by authors genetic diversities as well the level of outcrossing rates estimated on the basis of allozymes differentiation were comparable in natural and artificial stands. However, that was underlined the importance of possible changes in the important quantitative traits not revealed by neutral enzyme markers. It was undertaken (Skrøpa, 1994) the meaning of many aspects important for proper quality production of forest reproductive material e.g.: seed collection procedure, seedlings sylviculture management, progeny testing.

Low costs of the analyses are the reason why isoenzyme markers are good tools in pilot studies of gene pool, as well as conservation biology activities e.g. gene bank – enables choosing of proper sample for long time conservation. Using of isoenzyme analysis was the step to assess the genetic variation for *Sorbus torminalis* L. Krantz. natural populations in Poland (Bednorz et al. 2006), what was the basis to establish in the progeny stands in next step, as ex-situ measures for the species.

The present selection processes of forest stands should to maintain richness of natural diversity and do not allowed to use the trees with high economic as in of case natural populations of *Pinus wallichiana* A.B. Jacks (Blue Pine) in India (Bakshi & Könnert 2011).

5. Conclusions

The isoenzyme molecules are proteins, which are defined as first product of the first products coding region of DNA activity. Their heredity is known, when it is explained by Mendelian character of segregation it is possible to utilise as genetic markers.

Application of isoenzyme electrophoresis method is useful tool in forest trees genetic diversity assessment, in spite of their long history of their utilization, elaboration of DNA analysis markers as well as known limits of their possibilities to apply.

Especially the possibilities to use them on wild scale, because of low costs of analysis makes them important tools in:

- Genetic characteristics of different forest reproductive material of natural and artificial stands e.g.

  - Mother stands and progeny stands gene flow
  - Seed orchards gene flow
  - Gene banks representation of populations assessment
- Solving of particular problems in case of:
  - clonal/pedigree identification/selection process
  - pollen contamination
  - mating system
  - patterns of gene flow
- Good tools to support conservation and management of forest genetic resources e.g. to support following activities:
  - identification of migration path of species from postglacial refugia
  - selection and protection of ecotypes
  - to asses initial gene pole for needs of effective gene conservation measures
  - to present selection processes of forest stands to maintain rich natural diversity.

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