UDC 570.15; 630.18

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# METHODS OF MOLECULAR GENETICS IN ECOMONITORING OF CONIFEROUS FORESTS: APPROACHES AND PROSPECTS

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The article presents a review of the main molecular techniques applied to population genetic research. The merits and shortcomings of each method are considered in relation with its possible application for genetic ecomonitoring of coniferous forests. It is concluded that AFLPs and microsatellites are likely to be the most suitable molecular markers for these purposes.

*Key words: molecular markers, allozymes, RFLP, RAPD, AFLP, microsatellites, ecological-and-genetic monitoring.*

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МЕТОДИ МОЛЕКУЛЯРНОЇ ГЕНЕТИКИ В МОНІТОРИНГУ СТАНУ ХВОЙНИХ ЛІСІВ: ПІДХОДИ І ПЕРСПЕКТИВИ

Розглядаються переваги і недоліки основних методів молекулярної генетики, застосовуваних у популяційних дослідженнях. Обговорюються напрямки і перспективи використання цих методів в еколого-генетичному моніторингу хвойних лісів. На підставі літературних даних зроблений висновок, що найбільш перспективними маркерами для зазначених цілей є AFLP і мікросателіти.

*Ключові слова: молекулярні маркери, алозіми, RFLP, RAPD, AFLP, мікросателіти, еколого-генетичний моніторинг.*

Extensive anthropogenic environmental pollution has either an acute («digression») or a chronic effect on forests (Johnson et al., 1992, and others). Numerous studies have shown that this pollution causes genetic changes in the populations of woody species, for example, alterations of allele frequencies of allozyme loci, increase in mutation rate, etc.

Based on the available literature data, the effects of industrial pollution on forests may be classified as follows:

1) Pollution may either cause new mutations or simply favor selection against dominant genotypes. The level of new mutations may be particularly high in small areas under so-called «point» pollution (e. g., Chernobyl, industrial agglomerations, etc.) (Кальченко, Спирин, 1989; Тарабрин и др., 1990; Кальченко и др., 1991, 1993).

2) Chronic low-dose pollution may result in environmental changes, which organism is not able to adapt to (Johnson et al., 1992, Anonymous, 1995) and cause its death.

Forest-forming coniferous species are of great importance not only as an ecological factor (animal habitat, prevention of soil erosion, $CO_2$ utilization, conservation of coasts, etc.), but also as a source of raw wood.

During the last decades, rates of deforestation in the countries of the former Soviet Union have steadily increased. This process becomes more severe due to extensive industrial pollution. Thus, the total forest area of Ukraine, including artificially planted forests, is about 145,000 km$^2$ (Букштынов и др., 1981), and almost 40 % of these forests undergo digression due to industrial pollution, melioration, increasing proportion of artificially planted forests, and other anthropogenic factors.

Previously we considered the basic principles of using the main Eurasian forest species, Scots pine (*Pinus sylvestris* L.), as an object for phytomonitoring, as well as the advantages and disadvantages of morphometric and physiological-biochemical methods used for these purposes (Дворник, 1996). Currently, the methodology of phytomonitoring is used to develop more accurate and versatile methods, which allow to obtain reproducible results and to extrapolate the situation for the near and more distant future. From this point of view, molecular genetic methods are the most appropriate for the above purposes. In particular, their application makes it possible to approach the following problems:

1. The estimation of mutation rate at the level of protein (enzyme electrophoresis) and nucleotide sequences (DNA-based methods) in populations under ecological stress.

2. The estimation of genetic variability of populations and determination of the factors affecting this variability.

3. Prediction of changes in a population gene pool due to the effect of various natural or anthropogenic conditions.

4. The use of data about a population gene pool for conservation measures.

5. Mapping of the quantitative trait loci (*QTL*s) and the application of these data to breeding.

The present article considers two main groups of these methods.

### Some genetic peculiarities of conifers

Conifers have some specific genetic peculiarities, which should be taken into account when conducting population genetic research. Conifers are predominantly outcrossing species and have large effective population size (Muona, 1990). Their haploid megagametophyte (endosperm) makes them particularly suitable for genetic analysis of populations, because it helps to avoid problems of data analysis associated with allele dominance and provides direct haplotype data.

The chloroplast DNA of conifers is inherited paternally (i. e. through pollen), whereas mitohondrial DNA is transmitted maternally (through endosperm) (Wagner, 1992). This fact is of particular importance for population genetic analysis.

Moreover, conifers have exceptionally large genomes (20–30 pg) with a large amount of repetitive DNA (Wakamiya et al., 1993). Many genes in genomes of conifers form large gene families, which may have a few to hundreds of members (Kinlaw, Neale, 1997). To estimate homology between these members is a fairly difficult task. In addition, conifers are an evolutionarily old and, hence, a very genetically diverse group, which makes it difficult to apply the same methods for different species even within the same genus.

## ENZYME ELECTROPHORESIS

Since 70's, enzyme electrophoresis has been widely used for estimation of genetic variability and mutation rates in conifer populations (e. g., Muona, Harju, 1989; Hamrick, Godt, 1990; Parker, Hamrick, 1996; Berg, Hamrick, 1997 и др.), because conifers have a haploid megagametophyte that allows to detect mutations at early stages of ontogenesis (Алтухов и др., 1983; Духарев и др., 1984) and to trace recessive alleles, thus identifying the alleles which cannot be raced in diploid tissues.

Using enzyme electrophoresis in starch and polyacrylamide gels, researchers have found that populations of conifers under environmental pollution differ in genetic structure from the populations under normal conditions (Eckert, 1986, 1989; Prus-Glowacki, Godzik, 1991; Prus-Glowacki, Nowak-Bzowy, 1992; Bakhtiyarova et al., 1995). Certain allozymes were identified as markers of genetic changes in pine populations under industrial pollution (Духарев, 1983, 1985; Духарев и др., 1984; Mejnartowicz, 1986, 1989; Prus-Glowacki, Godzik, 1991), including esterases, which may be promising as genetic markers for tracing the effects of relatively mild ecological changes (Дворник и др., 1998).

Although enzyme electrophoresis is attractive due to its simplicity, it has serious limitations. First of all, it has low resolution. Using diploid embryos for analysis (see, e. g., Mejnartowicz et al., 1978; Prus-Giowacki, Nowak-Bzowy, 1992) makes the resolution even lower, due to failure to detect the rare alleles. Secondly, although electrophoresis itself is a highly standardized and fairly simple procedure, the set of loci used for analysis may vary greatly. Therefore, results obtained in different laboratories are often difficult to compare, especially if the set has fewer than 15 loci, which is very common for many studies (e. g., Gullberg et al., 1985; Muona et al., 1988; Wang et al., 1991; Prus-Giowacki, Bernard, 1994; Siedlewska, Prus-Giowacki, 1994; Zhelev et al., 1994). This is because, according to the extensive experimental studies (see Алтухов, 1989, for review), at least 20 loci are required to obtain estimates of a population's gene pool parameter, which are close to reality. Otherwise, the estimates of population variability obtained with olnlya few loci may be discussed only in terms of variability within this particular set of loci. In such case, the results are comparable only if the same loci are used (Дворник, 1997*a*,*b*).

Pine populations have relatively high genetic variability, but a low level of interpopulation differentiation, as estimated by average heterozygosity and $F_{ST}$ (e. g, Wang et al., 1991; Гончаренко и др., 1993; Goncharenko et al., 1995; Шигапов и др., 1995; Dvornyk, 2001). Differences

between populations are often observed as variations in frequencies of particular alleles. In such case, rare alleles are of particular importance as the markers for this differentiation. Their frequencies may differ significantly even in neighboring populations, which have been evolving in different ecotopes for a long time (Дворник и др., 1998). Likewise, changes in rare alleles frequencies may be caused by industrial pollution (Prus-Glowacki, Godzik, 1991; Prus-Glowacki, Nowak-Bzowy, 1992). Therefore, in order to determine whether such changes result from an anthropogenic influence or are due to natural genetic diversity, methods with higher resolution than enzyme electrophoresis should be applied.

The similar methodological problems occur when a mutation rate, as estimated by mutant allele frequencies, is used as an indicator of industrial pollution effect. This is because about 90 % of mutations are repaired during the ontogenesis, so that mutation rate determined by the enzyme electrophoresis differs greatly from its actual value (Ryabokon et al., 1995).

Enzyme electrophoresis has one more serious disadvantage, which greatly reduces its resolution. It detects only the mutations, which alter the amino acid sequence of an enzyme and thus change electrophoretic mobility of the protein molecule (i. e., nonsynonymous mutations). However, such mutations are fairly rare. Most mutations are synonymous and therefore do not result in amino acid changes. Moreover, allozyme analysis cannot detect mutations in the DNA regions which are non-coding but functionally important. All these problems may be solved with the aid of the methods based on DNA analysis (DNA-based methods).

## DNA-BASED METHODS OF GENETIC ANALYSIS

These methods are based on the analysis of DNA rather than the products of gene expression. Despite their variety, DNA-based methods make it possible to reliably estimate genetic characteristics of forest tree populations and to compare the results (see, e. g., Neale et al., 1989; Bobola et al., 1992, 1996; Grattapaglia, Sederoff, 1994; Grattapaglia et al., 1996). These methods are widely used in forest genetics (Karvonen, Savolainen, 1993; Karvonen et al., 1994; Dumolin et al., 1995), including genetics of conifers (Kostia et al., 1995; Karhu et al., 1996; Dvornyk et al., 2002).

This article does not aim to give a detailed description of the DNA-based methods, which is available from a number of comprehensive manuals (e. g., Avise, 1994), but rather to evaluate their merits and shortcomings in terms of their application to population genetics of Scots pine and some other economically important conifers.

### RFLP (Restriction Fragment Length Polymorphism)

This method is based on digestion of genomic (mitochondrial or chloroplast) DNA with restriction enzymes and comparison of lengths of the obtained fragments. The fragments from genomes of different individuals (or populations) are separated on polyacrylamide gel, visualized and compared. An advantage of the method is that the RFLP markers designed for one species can usually be successfully applied to closely related species. However, large genomes with many repetitive DNA regions (e. g. gene families) may yield RFLP markers which are difficult to interpret (Karhu et al., 1996). RFLPs may be rather polymorphic (*Table*). The data on interpopulation variability obtained with RFLPs are generally in agreement with those of allozyme analysis (Karhu et al., 1996; Butcher et al., 1998).

A disadvantage of this method is the requirement for a relatively large amount of DNA (about 10 μg per analysis). This limitation can be overcome by using polymerase chain reaction (PCR) to amplify the DNA region (e. g. gene) of interest. The resolution of the method may be further increased by using more sensitive electrophoretic procedures, in particular single strand conformational polymorphism (*SSCP*) and denaturing gradient gel electrophoresis (*DGGE*).

Population genetics studies of conifers with RFLP, chloroplast or mitochondrial DNA is commonly used due to its uniparental inheritance (Vicario et al., 1995; Karhu et al., 1996; Sinclair et al., 1998; Zheng, Ennos, 1999).

### RAPD (Randomly Amplified Polymorphic DNA)

This technique is based on PCR. The method employs short (12–15 bases) *random* primers which anneal to the DNA regions with complement sequences. It results in multiple-

band patterns. RAPD markers are extremely polymorphic, because random annealing of the primers results in amplification of not only conserved (i. e., coding and regulatory), but also non-coding DNA regions (e. g., introns, etc.).

This method is simple and relatively inexpensive (as compared to the other DNA-based methods), because it requires only regular PCR and agarose electrophoresis. RAPD markers are dominant. However, it is impossible to determine whether both alleles or only one are amplified. The other disadvantage of this method is a lower reproducibility of banding patterns in different laboratories (Jones et al., 1997) or even in different thermocyclers compared to more specific markers (*Table*).

*Table*

**Features of the molecular markers used in population genetic studies**

| Criterion | Molecular markers | | | | |
|---|---|---|---|---|---|
| | Allozymes | RFLP | RAPD | AFLP | SSR (microsatellites) |
| Mode of inheritance | cd | d | d | d | cd |
| Amount of generated data | + | + | +++ | +++ | +++ |
| Reproducibility | +++ | +++ | ++ | +++ | +++ |
| Resolution | ++ | +++ | ++ | +++ | +++ |
| Ease to use | +++ | + | +++ | ++ | + |
| Time needed for analysis | +++ | + | +++ | +++ | + |

Legend. cd – codominant, d – dominant, + – lowest value, +++ – highest value.

RAPD markers have similar to allozymes parameters of population genetic variability, especially when haploid tissue (endosperm) is used for the analysis (Szmidt et al., 1996; Aagaard et al., 1998). They have been successfully used to study closely related, cross-hybridizing, and low polymorphic species of conifers (Mosseler et al., 1992; Nkongolo, 1999), and to estimate the influence of forest restoration on genetic diversity of natural populations (Thomas et al., 1999).

Using fluorescently labeled primers and automated sequencers makes it possible to greatly increase resolution and throughput of this method.

AFLP (Amplified Fragment Length Polymorphism)

This method was designed in 1995 (Vos et al., 1995). Genomic DNA is digested with two restriction enzymes. The DNA fragments are ligated with adapters, which are short oligonucleotides that prevent repair of the restriction site. The adapters are the sites to which PCR primers then anneal.

Depending on the genome size, the processes of restriction-ligation generate thousands of fragments. In order to reduce their number and to increase reproducibility, selective primers are used. These are primers of the same sequence as the adapters plus 1–3 arbitrary nucleotides. These nucleotides reduce the number of the amplified fragments 4- , 16- , and 64-fold, respectively. Using different enzymes and primers makes it possible to amplify different sets of AFLPs and adjust the resolution of the method.

The amplified fragments are separated on a gel and compared. Until recently, the fragments were visualized using radioactively labeled primers, counted and compared manually. In such case, the resolution is sufficient for distinguishing between fairly distinct samples or for mapping relatively small portions of a genome. However, if the samples are genetically very similar or a large portion of a genome needs mapping, hundreds and even thousands of AFLP loci should be scored and statistically processed. In this case, fluorescently labeled primers and automated sequencer are used to increase throughput.

AFLP has an evident advantage over RFLP, because it does not require a large amount of DNA. It is also more technological. On the other hand, designing PCR primers is sometimes challenging. AFLP markers are random and dominant and thus similar to RAPD markers. However, AFLPs have a higher specificity and reproducibility (Jones et al., 1997).

Along with RFLPs and RAPDs, AFLPs are widely used in breeding for QTL mapping (Arcade et al., 2000). Recent data showed that AFLP markers are very useful for studying genetic variability of conifers (Harry et al., 1998; Jermstad et al., 1998; Lerceteau, Szmidt, 1999; Remington et al., 1999).

### Microsatellites

Microsatellites are short (2–6 nucleotides) non-coding DNA sequences, which may be repetitive (another name: SSR – simple sequence repeats). They are common in animal genomes, but are more rare in plant genomes (Lagercrantz, Ryman, 1990; Morgante, Olivieri, 1993). For example, $(CA)_n$ and $(GA)_n$ occur in plants with an average frequency one per 500–700 kb, which is about ten times less than in a human genome (Smith, Devey, 1994; Echt, May-Marquardt, 1997).

Microsatellites from one species may be used for studying related species, because their flanking sequences are often conserved. For example, microsatellites of chloroplast DNA may be amplified in several conifer species with the same set of primers (Powell et al., 1995). Microsatellites developed for radial pine (Smith, Devey, 1994) and eastern white pine (Echt et al., 1996), have been successfully applied to Scots pine (Karhu et al., 1996).

Like for RAPD, the main advantage of microsatellites over the other markers is their very high level of polymorphism. Since microsatellites occur mainly in non-coding regions, they are neutral markers. It is assumed they may be informative for monitoring of neutral mutations caused by environmental changes.

## CONCLUSIONS

As is seen from the above discussion, a perfect method for estimation of genetic variability and mutation rate should:

1. Be inexpensive and not laborious.
2. Generate multiple independent markers.
3. Give sufficient resolution.
4. Provide sufficient reproducibility.
5. Require small amounts of tissues or DNA for analysis.
6. Demand minimum experience in molecular genetics.
7. Need no preliminary information about a genome.

None of the above methods meet all these criteria. Therefore, choosing one or another should be based on specific research problems and resources of the laboratory. Currently there is a tendency to use primarily two groups of markers: AFLPs and microsatellites, due to their high reproducibility and resolution. Also, the last advances in technology make it possible to use automated sequencers to read and process AFLP and microsatellite data. It significantly increases throughput and accuracy of the methods. However, when estimating mutation rate, it is necessary to take into account that microsatellites represent mostly non-coding DNA. From this point of view, AFLPs are more preferred, because they allow screening of the whole genome, including its functional (coding) regions.

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*Надійшла до редколегії 05.11.03*