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ORIGINAL ARTICLE

Application of DNA markers against illegal logging as a new tool for the Forest Guard Service

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Abstract

DNA markers are currently the most precise tool for forest tree species identification and can be used for comparative analyses of plant material. Molecular diagnosis of evidence and reference material is based on comparing the structure of DNA markers duplicated in the PCR reaction and estimation of the DNA profiles obtained in studied wood samples. For this purpose, the microsatellite DNA markers are the most suitable tool because of their high polymorphism and accurate detection of structural changes in the genome. The analysis of tree stump DNA profiles let avoid timely collection of data such as tree age, diameter, height and thickness, although such a piece of information may advantageous in wood identification process. For each examined tree species, i.e. *Pinus sylvestris* L., *Picea abies* (L.) Karst., *Quercus robur* L. and *Q. petraea* (Matt.) Liebl., *Fagus sylvatica* L., *Betula pendula* L., and *Alnus glutinosa* L., wood identification was possible *via* the DNA profiles established on a basis of minimum 4 microsatellite nuclear DNA loci, and at least one cytoplasmatic (mitochondrial or chloroplast) DNA marker. Determination of the DNA profiles provided fast and reliable comparison of genetic similarity between material of evidence (wood, needles, leaves, seeds) and material of reference (tree stumps) in the forest. This was done with high probability (approximately 98–99%).

Key words

stolen wood, wood molecular identification, PCR-RFLP markers, SSR, STS

Introduction

An analysis of the DNA structure in organic material is a method widely used in criminology and forensic medicine, where it provides conclusive evidence of doubt in the identification and origin of a sample, often collected in the volume trace (Bal 2006). So far, the identification of the illegal timber trade in Poland's State Forests has been primarily based on mechanoscopy reflecting the measurement of annual ring pattern of the wood tissue, similar to the methods used in dendrochronology. The other way of timber *versus* stump identification relies on an estimation of wood losses resulting from theft *via* determination of tree DBH (diameter at breast height), e.g. Scots pine (*Pinus sylvestris* L.), Norway spruce [*Picea abies* (L.) H. Karst.], white oak (*Quercus robur* L.), European beech (*Fagus sylvatica* L.), and black alder (*Alnus glutinosa* L.) (Bruchwald et al. 2002).

Previously applied methodology can be nowadays successfully completed by the identification of DNA

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polymorphism analysis, including nuclear and cytoplasmatic DNA structural variability. The advantage of DNA analysis derives from Mendelian inheritance of molecular markers and the fact that structural variation in the DNA sequence does not depend on age or morphological characteristics of trees (Eriksson and Ekberg 2001). Such DNA characteristics of genotype, defined on a basis of selected sequences of the genome, create a standard tool suitable for identification methods of wood coming from tree branches, stems or leaves, and helps to determine individual genotype with the accuracy of a "fingerprint". The forensic analysis basically relies on comparison between the DNA profiles of stolen wood and the material of origin (generally represented by a stump in the forest or forest tree plantation). This comparison does not require the collection of data such as tree age, diameter, height and thickness, although information obtained may be an advantage in wood identification. With DNA data, the only criterion of identification is based on the genetic profiles used for comparative analysis between suspected wood material (pieces of wood seized on-site) with the material of evidence (e.g. stump in the forest).

Among many DNA markers, the microsatellite sequences are currently classified as one of the most accurate markers for DNA polymorphism identification of any living organism. The microsatellite sequences, called also short sequence repeats (SSR or STR), are composed of long tandem repeats from 1 to 6 nucleotides, located mainly in noncoding regions of the genome (Li et al. 2002). The source of microsatellite DNA polymorphism derives from differences in the length of recurring motifs of nucleic bases, e.g. (ATC), (AT), (AC) and (GT). In comparison with other forest tree species, conifers include in their genome as much as 75% repetitive microsatellite sequences (Eriksson and Ekberg 2001). The huge advantage of SSR markers is their high degree of discrimination (even at a level of single nucleotide) between alleles and codominant inheritance, i.e. detection of both forms of alleles in heterozygous trees. Another advantage of these markers is fast implementation of DNA testing, which consists of three basic steps: DNA isolation, duplication of DNA fragments by PCR and the comparative analysis of marker profiles obtained.

The aim of the present study included:

- developing the most efficient method of obtaining DNA from tissues of wood species like *P. sylvestris*, *P. abies*, *Q. robur* and *Quercus petraea* (Matt.) Liebl., *F. sylvatica*, *Betula pendula* (L.), and *A. glutinosa*,
- determination of a set of DNA markers, both nuclear (SSR) and cytoplasmic (STS and PCR-RFLP), which were characterized by the greatest strength of discrimination (distinction) useful for comparison of genetic profiles of wood from the evidence and reference material,
- elaboration of detailed instructions for seizing of wood samples for forensic purposes.

Materials and methods

Wood material collection

A small amount of material collected in the field can be used for biochemical analyses. In general, an analysis of 100 mg pieces of wood, seeds, leaves or needles is required (Fig. 1, 2). Generally, unstable DNA molecules are very quickly degraded in desiccating or decaying tissues by proteolytic enzymes. Therefore, collected plant material (buds, leaves, needles, wood) should be stored in dry conditions and at temperatures below 0°C prior to further analysis.

Extraction of DNA from tissues

There are several techniques for DNA extraction, based on the lysis of cell walls to facilitate proper extraction of nucleic acids from plant, fungal and animal tissues (Sambrook and Russell 2001). Good performance of extraction is guaranteed by kits ready for DNA isolation, e.g. DNeasy Plant Mini Kit (QIAGEN®).

The first step in obtaining DNA molecules from wood tissues, is initial mechanical homogenization or grinding the material in liquid nitrogen. Liquid nitrogen damages mechanically cell walls, which allows easier access to DNA molecules, at the same time as their stability at low temperature (–176°C) is maintained. Efficiency of DNA extraction is then analyzed by spectrophotometry or by electrophoresis in agarose gel, followed by staining with ethidium bromide (50 mg·ml^{-1}). At the end, obtained DNA molecules can be stored in stabilizing buffer (pH 7.0) for a long term (even years) at –75°C.

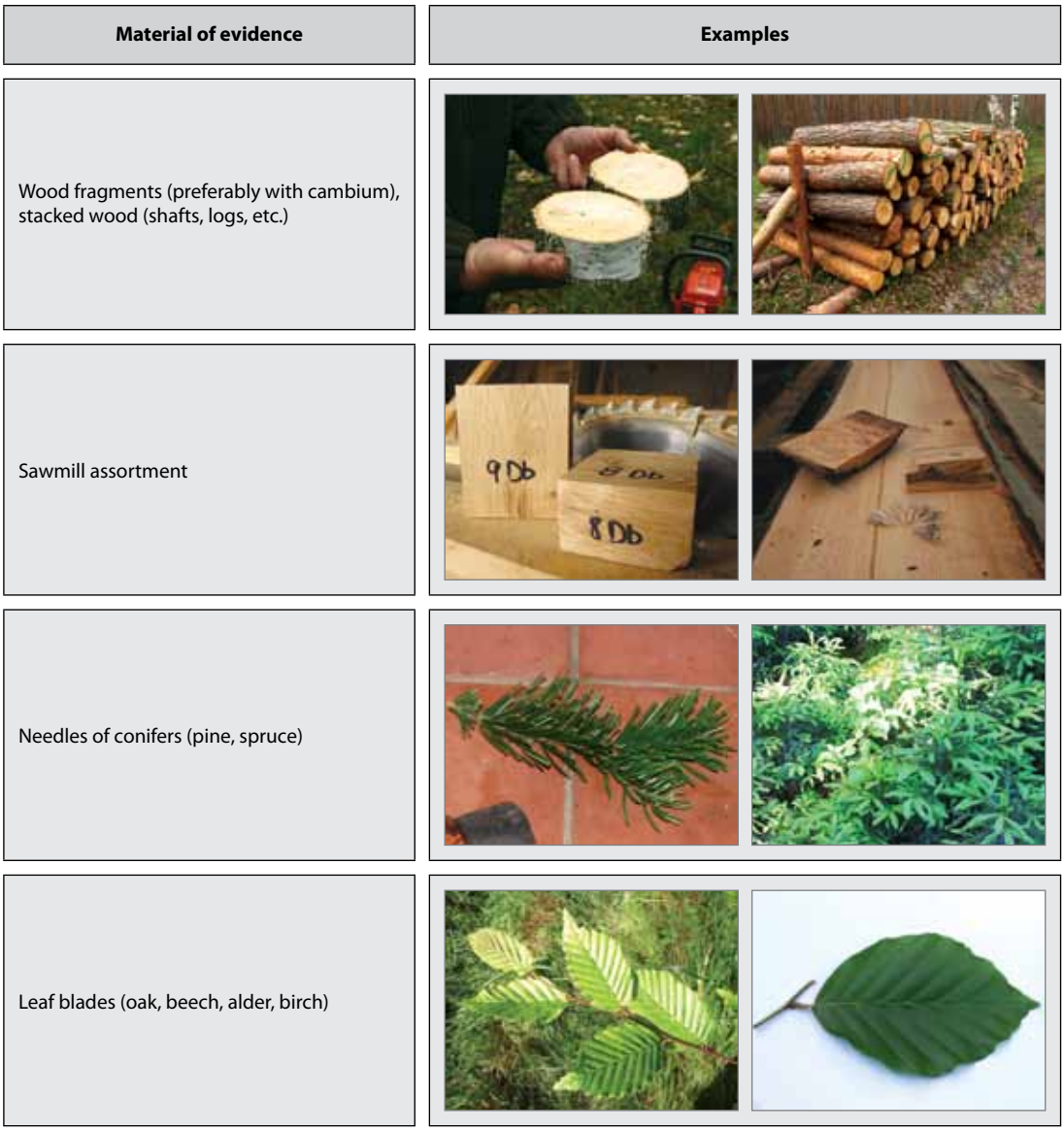

Fig. 1. Examples of evidence material taken for DNA analysis

Genotyping

Among billions of DNA sequences located in each forest tree species genome, only some of them can differentiate species at a single tree level. Such sequences are called DNA markers, and constitute a great tool for any identification purposes in forensic science.

Most of DNA techniques are based on amplification of the genomic DNA fragments by polymerase chain reaction (PCR) thanks to a thermostable enzyme *Taq* polymerase deriving from thermophilic bacteria *Thermus aquaticus*. The PCR reaction involves the DNA-matrix in the following reaction mixture: oligonucleotide primers (from 10 to 24 base pairs length), four types of free nucleotides (dATP, dGTP, dCTP and dTTP), magnesium ions (Mg^{2+}), reaction buffer and *Taq* polymerase. The reaction proceeds in DNA multiplication in a thermocycler programmed for multiple cycles (average of 30 to 40). The first stage of amplification takes about a few minutes and leads to double-stranded DNA template denaturation at 94°C. Then the stage of

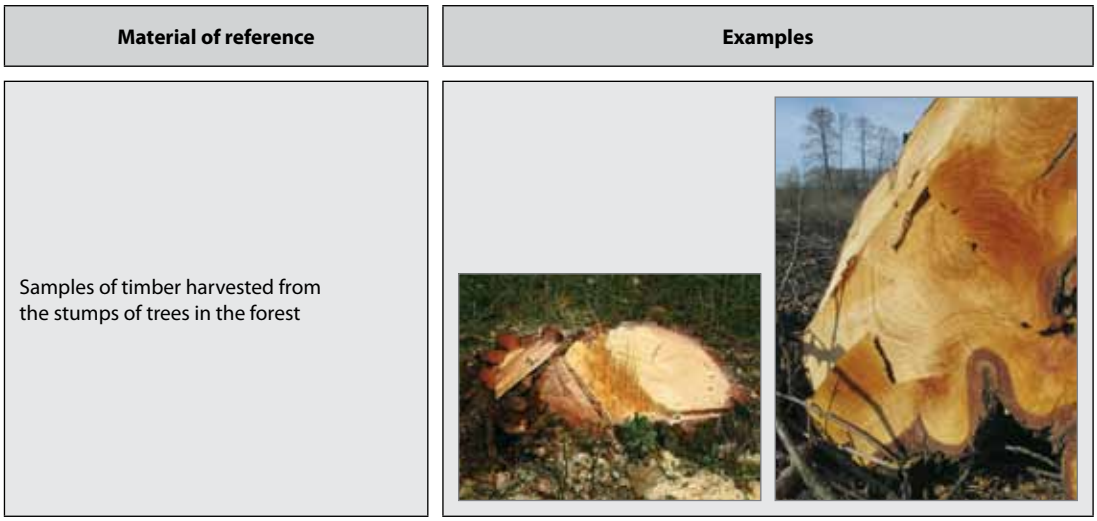

Fig. 2. Comparative material – tree stumps in the wood

annealing at 32–42°C comes, lasting from 30 sec. to a few minutes, when the formation of complementary DNA strand to the matrix (at 72°C) occurs. The temperature and duration of each stage depends on many factors, mainly on the G/C and A/T content in primers and the size of duplicated DNA fragments. Efficiency and precision of PCR is very high, and theoretically it allows reproducing the output-template DNA molecules present in the extract up to 10^9 copies (Sambrook and Russell 2001).

After the PCR, amplified DNA fragments are separated using electrophoresis on agarose gel or polyacrylamide gel performed in automated sequencer (Beckman Coulter®) of a DNA-chip in Bioanalyser (Agilent®). During electrophoresis, negatively charged DNA molecules migrate in the direction to a voltage positive electrode of the gel at a speed proportional to their size and molecular weight. The separated DNA fragments are then analyzed using computer software.

Computer analysis

Overall, genetic diversity is defined as the probability of occurrence of the identical genotype among randomly chosen trees in a forest stand. The picture of electrophoretic separation of DNA fragments is converted to numerical data, using software such as GelDoc™ 2000 (BioRad), Bio-Profil Bio-Gene v.99.05 Windows Application (Vilber Lourmat) and GelPro-Analyzer™ (Agilent®).

Results and Discussion

The findings provided the latest and most modern tools in addition to mechanoscopy and dendrochronology, to fight with illegal logging of wood. The results were directly linked to development of DNA identification methods for prevention of the illegal timber trade in the Forest State Holding in Poland. Methods involving isolation, DNA amplification and identification allowed to develop a new tool based on the nuclear and cytoplasmatic DNA markers to determine the origin of stolen assortment in the forest.

On the basis of developed methodology (Tab. 1), it was found that cambium and adjacent sapwood tissues were the most suitable for DNA isolation, because of the best preservation of DNA molecules in these tissues.

For each examined tree species, i.e. *P. sylvestris*, *P. abies*, *Q. robur* and *Q. petraea*, *F. sylvatica*, *B. pendula*, and *A. glutinosa*, wood identification was possible *via* the DNA profiles established on a base of minimum 4 microsatellite nuclear DNA loci, and at least one cytoplasmatic (mitochondrial or chloroplast) DNA marker. The variability of these sequences was very high (from 78% to 98%) among individuals of one species, which allowed to distinguish almost any single tree (Nowakowska 2010a).

In the case of Scots pine wood, the following sequences gave the best DNA discrimination between samples: four nuclear DNA markers (SPAG 7.14,

Table 1. Modification of DNA extraction protocol from the woody tissues

Step No.	Description
1.	Remove the outer layer of the wood surface with sterile scalpel
2.	Drill approximately 100 mg of tissue drill bar cooled in ice and rinse in ethanol 70%
3.	Grind the sample collected in liquid nitrogen or in a homogenizer (cooling parts in liquid nitrogen) and triturate for 5 min at 75th scale. Then cool again the tubes in liquid nitrogen, and repeat the homogenization for 5 min. Store samples at -20ºC before further analysis.
4.	To each tube, add 500–800 ml AP1 buffer (DNeasy Plant Mini Kit), followed by 1 mg of PVP and 6 ml Rnase
5.	Vortex and incubate at 65°C overnight (about 12h, preferably in an automatic mixer)
6.	After incubation, add 163–260 ml of buffer AP2 and mix thoroughly (Vortex)
7.	Incubate for 15 min at –20ºC
8.	Centrifuge for 5 min at 20000 g
9.	Transfer the supernatant to the columns of QIAshredder Mini Spin 2 ml tube (QIAGEN®)
10.	Centrifuge 2 min at 20000 g
11.	Proceed according to protocol DNeasy Plant Mini Kit 250 (QIAGEN®)

SPAC 12.5, PtTX3025 and SsrPt-ctg4363; Chagné et al. 2004; Elsik et al. 2000; Nowakowska 2010b), and eight chloroplast markers (PCP45071, PCP4113, PCP30277, PCP71987, PCP26106, Pt30204, PCP87314 and Pt71936; Provan et al. 1998; Vendramin et al. 1996).

For Norway spruce wood – four nuclear markers: SPAG-C1, SpAC1-H8, SpA-G2, SpAG-D1 (Pfeiffer et al. 1997; Yazdani et al. 2003), and one mitochondrial second intron of the *nad*1 gene (Sperisen et al. 2001) generated the best DNA profiles identifying a single tree. Moreover, the mitochondrial marker was helpful to determine the region of origin of illegal timber (northern or southern Poland).

DNA identification in white oak wood fragments was performed using five nuclear SSR markers: MSQ4, MSQ13, ssrQrZAG7, ssrQrZAG9, 102 (Muir and Schlötterer 2005; Steinkellner et al. 1997), and two chloroplast markers: "DT" and "CD" (Demesure et al. 1995), with the emphasis on nuclear SSR markers accuracy.

The most suitable DNA markers for genetic profiles of European beech wood samples were four nuclear SSR markers: FS1-03, FS1-25, FCM5 and mcf11 (Pastorelli et al. 2003; Vornam et al. 2004), and three chloroplast SSR markers: ccmp4, ccmp7 and ccmp10 (Grivet et al. 2001).

A wood comparison study performed for both common birch and black alder revealed five nuclear SSR markers: L2.3, L2.7, L5.5, L7.8 and L022 (Kulju et al. 2004), and three chloroplast DNA markers: trn1, psb1 and matK (Zhuk et al. 2008) which were most informative.

The DNA profiles created by all these markers for each tree species, *i.e.* pine, spruce, oak, beech, alder and birch, were composed of different alleles (DNA fragments) occurring in different loci of nuclear and cytoplasmatic DNA. The comparative analysis of DNA profiles was performed on a basis of minimum two samples – evidence (Fig. 1) and reference (Fig. 2) materials, simillarly to the methods of DNA-based determining paternity.

Because of Mendelian inheritance of DNA molecules (markers) in forest tree species, there is still the probability of finding the same genotype (DNA profile) between two no-parentally linked trees. This inconvenience may be resolved by calculation of the probability of accidentual identity (P_{ID}) between two individuals (Hedrick 2000).

The average value of the probability of identity (P_{ID}) calculated for examined trees allowed determination of the strength of discrimination of any DNA marker, which revealed its power to distinguish the individuals. For all studied tree species P_{ID} ranged from 0.0011 (in birch wood samples) to 0.0255 (in white oak samples), which meant that the probability of identical genotypes among 100 randomly examined trees in the stand was very small (from 0.11% to 2.55%). Thus, genetic profiles read for the evidence (*e.g.* branches, logs) and ref-

erence material (stump in the forest) were identical in 97.45–99.89%. Comparatively to forensic science used data, the markers with 94.91% of probability were generally retained as suitable for identification study and paternity tests (Konarzewska et al. 2006).

The most important issue in forest practice against illegal logging concerns the proper manner of wood material collection. Well seized wood material on-site guarantees successful DNA isolation and adds to the quality of further analyses at a laboratory level. In order to carry out the identification and comparative analysis of illegal timber trade for the cause of action by the Forest Guard Service, a handy set of "DNA IBL-1" was developed (Fig. 3). It consists of basic tools for collecting wood material in the forest, such as saw, ax, paper bags, tape measure, electric torch, camera, magnifying glass, two pieces of waterproof markers to write, waterproof adhesive tape, rubber gloves, ruler, plastic packaging film, wrapping tape, knife, and a set of procedural forms (Fig. 3).

Fig. 3. Handle set "IBL DNA -1" for collection of wood material for DNA analysis

Detailed instructions for seizing wood samples for DNA analyses were presented in a video training film entitled "DNA analysis of wood in combating timber illegal trade" (www.ibles.pl). The film acquaints society with current identification methods of wood (mechanoscopy, dendrochronology), and the potential use of DNA markers for the purpose of genetic DNA identification of illegal timber trade. It illustrates how to sample wood parts on the ground, and shows the possibility of the use of DNA testing in practice related to lawsuits (Nowakowska and Górniak 2010).

Overall, the obtained results are important for practice against harmful activities related to forests. It is estimated that timber theft annually generates the greatest losses incurred by Poland's State Forests along with other forms of crimes, and thieves are unpunished for such losses because they are becoming more organized, equipped with professional devices. The way they protect traces of crime make it impossible to prove them guilty in court. Methods for identifying timber based on the developed set of DNA will then provide new evidence in litigation by the Forest Guard Service.

The research on DNA markers can be considered as an additional tool to the classical methods like mechanoscopy and dendrochronology, applied to investigate illegal logging in Polish forests. They may contribute to the international actions like FLEGT[1] focusing on wood trade from Asia, Africa and South America or other processes such as LULUCF[2] and REDD[3]. The main objective of these actions is monitoring illegal timber trade between overseas countries and Europe (FLEGT), as well as promoting wood from certified sustainable managed forests (MCPFE, REDD).

Conclusions

Presented investigation in wood DNA marker study for forensic purposes resulted in:

- successful methods of identifying the six major forest-forming species: Scots pine (*P. sylvestris*), Norway spruce (*P. abies*), oak (*Q. robur* and *Q. petraea*), beech (*F. sylvatica*), black alder (*A. glutinosa*) and birch (*B. pendula*). These methods can be used for practical molecular diagnostics of individuals based on detailed DNA patterns, taking into account the random probability of an identical individual existing in the woods,
- DNA markers, both nuclear (SSR) and cytoplasmatic (STS and PCR-RFLP), characterized by the greatest strength of discrimination (distinction between

[1] FLEGT – Forest Law Enforcement, Governance and Trade .
[2] LULUCF – Land Use and Land Use Change and Forestry.
[3] REDD – Reducing Emissions from Deforestation and Forest Degradation in Developing Countries.

trees reaching 99.89%) being useful for comparison of genetic profiles from the evidence and reference material,
– determination of a quick set "IBL DNA-1" comprising procedural forms and the most useful mechanical tools to collect wood material in field conditions,
– guidelines for practice described step-by-step procedures for collecting and preservation of wood samples from the evidence and comparison material for forensic purposes.

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