Wood Sci Technol (2017) 51:1377–1387
DOI 10.1007/s00226-017-0942-5

ORIGINAL

Analysis of DNA profiles of ash (*Fraxinus excelsior* L.) to provide evidence of illegal logging

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Received: 26 September 2016 / Published online: 15 July 2017

Abstract Illegal logging is a major problem in many European countries. Recent progress in molecular biology, however, has significantly improved the ability to accurately identify wood material. In this paper, the first application of microsatellite DNA markers is described in a case of illegal logging of European ash wood in Polish State Forests. The genetic fingerprints of seized ash wood samples were determined using six nuclear and four chloroplast microsatellite loci, characterized by sufficient stringency in forensic analyses. By comparing the DNA profiles obtained, the origin of one sample of ash wood used as evidence material was confirmed, from among three samples serving as reference material with 99.99999% of probability. This work demonstrates how DNA authenticity testing can serve as an important technical tool in monitoring the legality of the suspected ash timber and confirms the utility of these techniques in detecting illegally logged timber in general.

Introduction

European ash is an important species in biodiversity conservation of forest ecosystems and in economic terms, providing not only habitat for many fauna and flora, but also timber for the building and furniture industries. Among several negative factors impacting on forest ecosystems, human activities including poaching, vandalism or illegal logging all contribute to degradation. In addition,

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illegal logging is a major problem for many timber-producing countries and promotes both pest and pathogen spread and corruption, particularly in South-east-Asian, African and South American countries.

Trade in timber generated 37.8 billion euros in the European Union (European Union Trade Regulation 2013) in 2011, representing approximately 35% of global trade in this primary product. Although it is difficult to estimate the extent of illegal logging contributing to this trade, the European Commission is conscious of potential illegal sources of wood products arriving into EU markets. EU Regulation No 995/2010 of the European Parliament and of the Council, also known as the (illegal) Timber Regulation (EUTR), entered into force on 3 March 2013, defining obligations for operators who place timber and timber products on the market to possess legal certification of timber or wood provenance (European Union Trade Regulation 2013).

Poland is among the leading wood producers in the EU, with ca. 35 million m^3 of timber logged each year by the State Forests company (Gajdemski et al. 2016; Forestry statistics in detail 2015); illegal logging, however, is estimated to represent ca. 20,000 m^3 of annual loss in the State timber market. It is estimated that the 6500 cases of timber stolen from the State Forests reported in Poland between 2005 and 2011 led to losses of 1 million euros to the Polish economy (Nowakowska and Pasternak 2014).

To prevent illegally harvested timber and products derived from such timber appearing on the market, genetic tools based on DNA markers have been developed (Asif and Cannon 2005; Deguilloux et al. 2002, 2003; Tnah et al. 2012). These DNA markers are additional tools to be used alongside classical methods such as mechanoscopy and dendrochronology, which are often applied to investigate different forms of crime in forests (Yaman and Akkemik 2009); especially, when mechanoscopy or dendrochronology fails to provide sufficient evidence in investigations, i.e. in case of mechanical damages of trunks (by chipping or burning).

DNA profiling is commonly used in forensic science and forensic botany and provides conclusive evidence of the origin of a sample (Craft et al. 2007; Goodwin et al. 2011; Ng et al. 2016). In general, all comparative studies of material evidence (single tree, stump or piece of wood) based on DNA analyses rely on applying an optimized set of specific genetic markers providing the maximum discrimination possible (close to 100% accuracy). These methods can be used in molecular diagnostics at the level of the individual, the basis being the detailed DNA patterns, taking into account the random probability that an identical individual occurs in a forest stand.

Here, the results of the first application of microsatellite DNA markers are reported in a case concerning European ash timber stolen from the State Forests, the Forest District of Śnieżka in South-western Poland (*case number NS.2504.2.2015*). Total volume of stolen trees (*F. excelsior* L., *Quercus petraea* L., *Prunus avium* L.) was 5628 m^3. Material for laboratory analysis was obtained from three trunks of ash left in the stands, and the results were compared with the genetic profile of a single sample of ash found on the suspect. Those samples were preselected based on mechanoscopy hint (very likely match). The molecular

marker set applied to this case study has previously been proven to be highly polymorphic in population-level study of *F. excelsior* in Europe (Sutherland et al. 2010; Tollefsrud et al. 2016).

The main objective of the work reported here was to determine the genetic fingerprints of European ash wood samples in a specific forensic botany case using microsatellite loci developed for European ash foliage with proven accuracy of discrimination based on high level of variability and power of discrimination of the markers used.

Materials and methods

Following a request from the Śnieżka Forest District in Poland, the Laboratory for Molecular Biology of the Forest Research Institute in Sękocin performed genetic fingerprint analyses of 4 wood samples originating from 60- to 80-year-old-ash trees (*Fraxinus excelsior* L.). Three samples (3a, 4a and 5a) represented reference material and were collected from trunks remaining in the forest in Śnieżka Forest District (50°48′N, 15°49′E). One piece of evidence (number 1) was collected from the person suspected of stealing the timber. Further analysis of the DNA profiles was designed to preclude or confirm the resemblance between the evidence and the reference material.

Small wood shavings were cut with a razor blade from the timber samples, with two independent replicates. DNA was extracted using NucleoSpin® Plant II (Macherey–Nagel, Düren, Germany) kits, following the manufacturer's instructions, with minor modifications. The changes included adding 600 µl PL2 buffer and 150 µl PL3 at the lysis stage to remove contaminants such as polysaccharides, pigments and other polymerase chain reaction (PCR) inhibitors; 900 µl PC buffer was also added at the DNA binding. The final elution was also performed with 50 µl of buffer. The quantity and quality of DNA obtained was checked using a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA).

PCR was performed using six nuclear microsatellite (nSSR) and four chloroplast (cpSSR) loci, split over two multiplex reactions, using the Multiplex PCR Kit (Qiagen®, Hilden, Germany). In the first "A" multiplex, the five loci amplified were Femsatl-4, Femsatl-8, Femsatl-19, ccmp3, and ccmp6, whereas in the second "B" multiplex the loci were Femsatl-11, Femsatl-16, M2-30, ccmp7 and ccmp10 (developed by Sutherland et al. 2010, for leaf tissue). PCRs were optimized and carried out in 10 µl reaction volumes, with 1 µl of ash DNA template, 10 pmol of each primer and 5 µl of Multiplex PCR Master Mix. Each forward primer was labelled with fluorescent WellRED dyes (Sigma-Aldrich, St Louis, USA). PCR programming in Verity 96 Thermal Cycler apparatus (Life Technologies™, USA) thermocycler was based on that of Sutherland et al. (2010) for both multiplex reactions ("A" and "B"). Each PCR sample was analysed twice in a CEQ™8000 sequencer (Beckman Coulter®, Fullerton, USA), and precise allele lengths determined using the CEQ™8000 Series Genetic Analysis System 9.0 (Beckman Coulter®, Fullerton, USA).

The set of markers "A" and "B" was preliminary checked according to their polymorphism in a large population scale. For this, fresh leaf tissues were collected

from 278 single ash trees across Poland, i.e. 127 trees from Białowieża Primeval Forest (52°42′N, 23°52′E), 96 trees from Kozienice Forest District (51°34′N, 21°32′E) and 55 trees from Chojnów Forest District (51°17′N, 15°56′E). Sampled trees were between 60 and 90 years old, randomly located in each stand to avoid close kinship. Plant material was processed as described above, applying the same set of nuclear and chloroplast DNA markers. General genetic variation among the sampled trees was computed as average over the loci with GeneAlEx v. 6.5 (Peakall and Smouse 2012): allelic richness (*Ar*), an observed and expected number of alleles per locus (n_a and n_e, respectively), the observed and expected heterozygosity (H_O, H_E); gene diversity (H) for haploid loci, inbreeding coefficient (F_{IS}) and coefficient of genetic differentiation among populations (F_{ST}, Nei 1987).

To evaluate the discriminatory power of the 10 markers used, the power of discrimination was calculated according to formulae by Hedrick (2011).

Results and discussion

Genetic fingerprints of European ash wood in the forensic case from Śnieżka FD

It was demonstrated that genetic variation at applied microsatellite loci was sufficient enough to characterize European ash trees at the population level (Tables 1 and 2). *F. excelsior* is diploid, with 2C = 46 chromosomes (Sollars et al. 2017), and in Poland the probability of hybridization with other ash species in forest stands is null.

Table 1 Genetic parameters of nuclear microsatellite markers used for European ash wood identification calculated based on 278 diploid individuals

Nuclear SSR loci	Sequence motif	Allele size range	n_a	n_e	Ar	H_O	H_E	F_{IS}	F_{ST}
Femsatl-4	$(CA)_{17}(AG)_{24}$	158–242	37	9.056	36.023	0.739	0.890	0.180*	0.026
Femsatl-8	$(GT)_{23}$	131–197	33	14.189	32.608	0.641	0.930	0.272*	0.017
Femsatl-11	$(GA)_{20}(TA)_4$	178–230	25	9.687	23.908	0.788	0.897	0.098*	0.025
Femsatl-16	$(CA)_3(CG)(CA)_{10}(TA)_2(CA)_3$	176–242	13	2.305	13.000	0.252	0.566	0.553*	0.017
Femsatl-19	$(CA)_6CGGC(CA)_{13}$	142–218	30	10.297	29.024	0.752	0.903	0.131*	0.042
M2-30	–	146–282	41	24.477	39.378	0.887	0.959	0.047*	0.027
Mean		–	30	11.669	28.990	0.676	0.857	0.214*	0.026

n_a observed allele number per loci, n_e expected allele number per loci, *Ar* allelic richness, H_O H_E observed and expected heterozygosity, F_{IS} inbreeding coefficient (the coefficients denoted with asterisk (*) are significantly greater than zero, $p < 0.05$), F_{ST} coefficient of genetic differentiation among populations (Nei 1987). Allele sizes for each locus are given in base-pairs

Table 2 Characteristics of the chloroplast microsatellite markers detected in 278 European ashes

Chloroplast SSR loci	Allele size range	n_a	n_e	H
ccmp3	94–102	8	2.674	0.626
ccmp6	93–101	9	4.355	0.770
ccmp7	116–123	6	1.874	0.466
ccmp10	92–105	10	3.438	0.709
Mean	–	8	3.085	0.643

n_a and n_e observed and expected allele number per locus, H gene diversity (Nei 1987)

The mean heterozygosity level obtained for 287 Polish ash trees for the nuclear markers ($H_E = 0.857$) was similar to 0.871 reported by Tollefsrud et al. (2016) for the same set of markers used to assess 1099 ashes sampled across Europe. Further, Ruņģis et al. (2016) reported similar genetic variation for Lithuanian ash trees examined with five Femsatl4, Femsatl10, Femsatl16, Femsatl19 and M2-30 loci. The inbreeding coefficient ($F_{IS} = 0.214$) was significantly different from zero. Low F_{ST} values obtained strongly suggest that sample sites in these areas essentially form a single uniform population, with little indication of localized population differentiation (Table 1). The same level of genetic similarly was found in a Scottish population of *F. excelsior* L. analysed with the same set of markers (Sutherland et al. 2010).

A high level of expected heterozygosity ($H_E = 0.857$) was found in all nSSR loci compared to cpSSR loci ($H = 0.643$), probably because of the higher complexity of the nuclear genome compared to chloroplast DNA (Arumuganathan and Earle 1991; Finkeldey et al. 2010). The chloroplast markers used were less polymorphic (Table 2), with on average 8 haplotype variants in each locus. The Polish ash stands studied with cpDNA markers revealed some moderate differentiation due to the presence of only two haplotypes of ccmp genes (Heuertz et al. 2006).

The above nuclear and chloroplast microsatellite markers enabled to establish a genetic profile of the evidence samples with high certainty. Sample 1, the evidence (a fragment of an ash wood shaft) had a genetic profile matching reference 4a (a sample of comparative material derived from trunk number 4) (Table 3), but was distinct from reference sample 3a at the 5 nuclear DNA loci. In comparison with reference sample 5a, only the locus Femsatl4 matched the profile sample 1. The 4 chloroplast SSR markers were identical in all samples of wood examined; their sizes ranged from 95 to 117 base-pairs (Table 3). The mean frequencies of the nuclear SSR markers obtained for wood samples ranged from 0.2% in locus Femsatl11 to 60.9% in locus Femsatl16 (Table 4). In chloroplast SSR loci, the frequency shifted between 12.3% in ccmp6 locus to 18.5% in ccmp3 locus (Table 5). Few rare alleles were denoted for nSSR loci, i.e. in Femsatl-4 and M2-30 loci (data not shown).

Although the nuclear microsatellite DNA markers are considered to be the most powerful tool in identification of biological material, chloroplast loci can also provide suitable barcode markers for timber (Deguilloux et al. 2002; Rachmayanti et al. 2006; Finkeldey et al. 2010; Ng et al. 2016). The chloroplast genome is present

Table 3 Comparison of genetic fingerprints based on molecular markers used for materiel of evidence and reference with original naming of samples after Forest Service Guard

No (after Forest Service Guard)	Samples, material type	Loci															
		Femsatl4 [D-4]		Femsatl8 [D-2]		Femsatl19 [D-3]		M2-30 [D-4]		Femsatl11 [D-2]		Femsatl16 [D-3]		ccmp3 [D-3]	ccmp6 [D-4]	ccmp7 [D-2]	ccmp10 [D-4]
1	**Evidence**[a]	**164**	**174**	**151**	**161**	**164**	**186**	**204**	**238**	**202**	**208**	**184**	**186**	**99**	**99**	**117**	**95**
3a	Reference	166	174	163	163	166	192	226	248	188	204	184	184	99	99	117	95
4a	**Reference**	**164**	**174**	**151**	**161**	**164**	**186**	**204**	**238**	**202**	**208**	**184**	**186**	**99**	**99**	**117**	**95**
5a	Reference	164	174	153	153	176	186	220	226	192	228	184	184	99	99	117	95

The numbers correspond to the DNA fragment length (in base-pairs). For each marker the fluorochrome symbol was given according to WellRed (Sigma, Germany)

[a] Wood materials with identical DNA profiles are highlighted in bold

Table 4 Alleles frequency for each nuclear SSR locus investigated in the case of illegal logging of ash timber from Table 3

	Femsatl-4 (D4)	Femsatl-8 (D2)	Femsatl-11 (D2)	Femsatl-19 (D3)	Femsatl-16 (D3)	M2-30 (D4)
Allele size						
151		0.115				
153		0.015				
161		0.013				
163		0.126				
164	0.273			0.092		
166	0.102			0.032		
174	0.044					
176				0.031		
184					0.609	
186				0.155	0.238	
188			0.021			
192			0.195	0.137		
202			0.030			
204			0.055			0.017
208			0.015			
220						0.040
226						0.032
228			0.002			
238						0.057
248						0.043
Nb of samples*	249	234	264	262	206	265

* Number of ash trees which shared a given DNA marker

Table 5 Alleles frequency for each chloroplast SSR locus investigated in the case of illegal logging of ash timber from Table 3

Haplotype size	ccmp3 (D3)	ccmp6 (D4)	ccmp7 (D2)	ccmp10 (D4)
95				0.199
99	0.185	0.123		
117			0.168	
Nb of samples*	271	277	274	276

* Number of ash trees which shared a given DNA marker

with high-copy number in the amyloplasts found in wood, and it is highly conserved due to low heteroplasmy (i.e. intra-individual variation) and a lack of recombination (Saranpää 1988; Navascués and Emerson 2005). The cpSSR loci present some advantages over other loci, for example their low mutation rates and high species

specificities (Provan et al. 1999). The usefulness of cpDNA was reported in the identification of woods of China-fir (*Cunninghamia lanceolata*), Manchurian catalpa (*Catalpa bungei* C.A. Mey), white ash (*Fraxinus chinensis*) and the Diptero-carpaceae (Rachmayanti et al. 2009; Tang et al. 2011). Chloroplast DNA was also applied to trace the geographical origin of oak timber in France, and one tropical timber in Peninsular Malaysia (Deguilloux et al. 2003; Tnah et al. 2009).

Generally, the higher rate of evolution in nuclear microsatellites versus chloroplast microsatellites allows conclusions to be drawn on the genetic identity of plant material based solely on nuclear markers. Because of the lack of cpSSR variation found in the ash samples examined, the chloroplast markers were treated as auxiliary to the case study. At 6, the number of nuclear markers used in the present case was not large but sufficient to perform matching of the DNA profiles. Considering the total number of nuclear SSR markers used (Femsatl4, Femsatl8, Femsatl9, M2-130, Femsatl11 and Femsatl16), the profile of evidence sample 1 consistently matched the profile of reference sample 4a, but differed from reference samples 3a and 5a (samples of comparative material derived from trunk number 5).

Power of discrimination of the markers

Based on the Mendelian inheritance of markers in forest tree species, there remains a possibility that the same DNA profile could occur in two non-parentally linked trees. This inconvenience can be resolved by calculating the probability of accidental identity (P_{ID}) (Hedrick 2011), which illustrates the probability that two individuals drawn at random from a population will have the same genotype at multiple loci and is generally used to assess the confidence of the marker system for individual identification. The value of P_{ID} for the trees examined in this work allowed determination of the discriminatory power in each DNA marker for distinguishing individual specimens. The probability of the DNA profile of the 278 European ash samples from three different forest stands in Poland showing a random match with the samples taken from the evidence samples was very low, equivalent to 1 in a ten million for the 6 nuclear SSR loci ($P_{ID} = 0.0000001$). These results clearly demonstrate the high power of a single sample discrimination equal to 0.9999999 (99.99999%) that can be attributed to the 6 nuclear markers used in this work on European ash wood.

Work on other forest tree species showed that P_{ID} values ranged from 0.0001 for wood of *Pinus sylvestris* (L.) assessed with 10 nSSR markers to 0.0102 for *Larix decidua* (L.) examined with 4 nSSR loci. These data provided evidence supporting two forensic cases based on genetic profiles of pine and larch (wood from branches or logs) and reference material (a stump in the forest) which were estimated to be 99.98 − 98.99% identical (Nowakowska et al. 2015).

In a similar approach, four nuclear microsatellite markers were used in DNA profiling of sand live oak (*Quercus geminata*) foliage and produced physical evidence linking a suspect to a crime scene (Craft et al. 2007): the average probability of identity of live oak leaves was 2.06×10^{-6}.

Expert opinion based on genetic fingerprinting methods now suggests that these techniques provide a level of basic proof acceptable in criminal cases in line with

the Penal Code; in forestry, this evidence enables those people who take felled trees away from a forest for the purpose of appropriation to be subject to the same liability as if they had engaged in stealing. Courts now accept that genetic profiles based on nuclear or plastid markers provide rapid and robust tools for comparison between evidence (foliage, wood) and material of reference (tree stumps) in the forest (Tnah et al. 2012; Nowakowska and Pasternak 2014). It should be emphasized, however, that a minimum of 4–6 highly polymorphic nuclear markers, that possess between five and 15 alleles each, are required to provide suitable discriminatory power in forensics (Butler 2005; Goodwin et al. 2011). Age of the wood material and stage of the decomposition process of biological material are crucial for optimizing genetic fingerprinting of wood samples (Fengel 1970; Dumolin-Lapègue et al. 1999; Deguilloux et al. 2002; Rachmayanti et al. 2009; Tnah et al. 2012).

Based on the results presented here and previously published data on illegal logging investigations, the low probability of finding an apparently identical genotype (P_{ID}) among randomly chosen trees in a forest stand supports the high relevance of utilizing several nuclear and organelle DNA markers in evidence for wood; the technique has now been proven with *Pinus sylvestris* L., *Picea abies* L. Karst., *Abies alba* L., *Larix decidua* L. and *L. kaempferi* Lamb. Carr., *Fagus sylvatica* L., *Quercus robur* L., *Q. petraea* Matt. Liebl., *Q. geminata* Mill., *Alnus glutinosa* Gaertn. and *Betula pendula* Roth. (Deguilloux et al. 2002; Craft et al. 2007; Finkeldey et al. 2010; Nowakowska 2011; Nowakowska et al. 2015).

Conclusion

It was demonstrated that 6 microsatellite markers previously developed for European ash: (1) can be applied successfully to forensic botanical investigations for wood tissues, and (2) these markers have a very high power of discrimination ($P_{ID} = 0.0000001$). In a particular court case of illegal logging of European ash from Śnieżka FD, it was proven that evidence sample 1 (seized in a private estate) was genetically identical to reference sample 4a (from a stump in the forest) on the basis of 6 polymorphic nuclear markers: Femsatl4, Femsatl8, Femsatl9, M2-130, Femsatl11 and Femsatl16. The average probability of randomly finding a genetically identical tree in the forest when using these 6 loci was one in 10,000,000. These results demonstrate that forensic genetic profiling can provide robust evidence in tracing illegal logging activities, particularly when based on at least 6 nuclear SSR markers.

Acknowledgements The present work formed part of a research project supported by the General Directorate of State Forests (Grants BLP-333 and BLP-384). We gratefully acknowledge the Forest Guard staff from Śnieżka Forest District for their efficient cooperation. We also thank Małgorzata Gorzkowska from the Laboratory of Molecular Biology FRI Poland, who assisted with processing of plant material in the laboratory.

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