FPS COST Action FP1202

Strengthening conservation:
a key issue for adaptation of marginal/peripheral populations of forest trees to climate change in Europe (MaP-FGR)

SHORT TERM SCIENTIFIC MISSION REPORT

Natural genetic variability of a marginal population of Populus x canescens

Ing. Eva Žižková, Ph.D.

Dr. Eva Žižková
Young researcher
Forestry and Game Management Research Institute
Prague, Czech Republic

Dr. Berthold Heinze
Head of the DNA laboratory at the Department of the Genetics
Federal Research and Training Centre for Forests, Natural Hazards and Landscape
Vienna, Austria

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Abstract

Populus x canescens (grey poplar) is a spontaneous hybrid of *P. alba* and *P. tremula* with a fast growing ability and early reproductive maturity. Marginal population of grey poplar (*Populus x canescens*) is uniquely located in floodplain forest at Dyjákovice village (South Moravia, Czech Republic) which belongs among the most threatened ecosystem in Europe. To protect and extend this grey poplar population, characterization of genetic variation among the individuals and gene introgression of parentals was followed.

Data from nuclear microsatellites analysis revealed clonal identity in all individuals of grey poplar with the heterozygous genotypes from Dyjákovice village. The polymorphic character of analysed loci have been confirmed base on comparison with another population of grey poplar.

The investigation of chloroplast microsatellite markers suggested in accordance to nuclear microsatellites analysis genetic divergence of parental species with the more conserved DNA polymorphic loci detected in *Populus tremula*.

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1. INTRODUCTION

Domestic species of poplars represent unrecoverable components in forest ecosystem although they are not commonly in the main interest of forest management due to their limited utilization. Grey poplar (*Populus x canescens*) occur generally in the tree layer of floodplain forests which belong among the most threatened ecosystem in Europe (Čížková *et al*., 2007). In the Czech Republic, populations of grey poplars occur rather marginally. Therefore to sustain function of floodplain ecosystems it is important to adapt the local forest management and introduce the suitable woody species like poplars.

In the Czech Republic, there is the only evidence of ecotypes and ecodems for *Populus tremula* and *Populus alba* species (Macků *et al*., 1995). In this study, we focused more on natural marginal population of *Populus x canescens* uniquely located in floodplain forest in South Moravia region (Czech Republic). The population of grey poplar is characterized by older individuals with a relatively lower recovery. Unfortunately, up to date it is not clear whether vegetative or generative reproduction prevails in the population. Moreover, newly established artificial underbrushes of alder (*Alnus* sp.) and ash (*Fraxinus* sp.) are affected by fungi pathogens like *Phytophthora sp*. and *Chalara sp*. both limiting the optimal forest environment and development. To protect and extend marginal population of grey poplar in floodplain forest, there is strong necessity to distinquish poplar individuals based on genetic studies.

Tandem repeat regions or simple sequence repeats (SSRs) often show higher levels of allelic variation in nuclear, chloroplast or mitochondrial genome (Wheeler *et al*., 2014). Previously it has been reported, that microsatellite analysis is suitable method for detection of DNA polymorphism patterns for poplars (Fossati *et al*., 2005; Santos-del-Blanco *et al*., 2013 and Yin *et al*., 2009). In this study, we used several nuclear and chloroplast microsatellite markers as powerfull tool for discrimination of genetic variation among the grey poplar individuals and their parentals, white (*Populus alba*) and aspen (*Populus tremula*) poplars.

2. OBJECTIVE OF THE STSM

The main objectives of STSM were:

- determination of genetic variability inside the population of *Populus x canescens*
- assesment of clonality among the grey poplar individuals from Dyjákovice village
- determination of *Populus alba* and *Populus tremula* introgression
- data comparison with population of grey poplar from Mladá Boleslav and confrontation with the literature

3. LINKS WITH COST ACTION FP1202 MaP FGR

Marginal and peripheral forest populations (MaP) became under environmental conditions and management strategies fastly changing. To study adaptive processes in MaP populations it is important to determine characteristics of population like genetic variability and introgression of parentals. As COST Action FP1202 highly encouraged collaboration with institution or laboratory in another Participating COST Country, determination of genetic diversity in marginal population of grey poplar uniquely located in floodplain forest was assessed in Federal Research and Training Centre for Forest, Natural Hazards and Landscape, Department of Forest genetics, group of Dr. Berthold Heinze, in Vienna (Austria).

4. MATERIALS AND METHODS

During spring/ summer 2015, apical buds of grey poplar (n=90) were collected in five small regions from floodplain forest in the north at Dyjákovice village while young leaves were obtained from *Populus alba* (n=31), *Populus tremula* (n=31) and population of grey poplar (n=32) from Bezděčín located close to Mladá Boleslav. Total genomic DNA was isolated and quantified at home institution (Forest and Game Management Research Institute, Prague, Czech Republic). Set up of polymerase chain reactions (PCR) was given according to the Type-it Microsatellite PCR Kit (Qiagen) and annealing temperatures (T_m) of primers combined into the multiplex. Nuclear variation was analysed by twelve microsatellite loci whereas we focused on nine of them in more details (primers WPMS14, WPMS16, WPMS17, WPMS20, PTR8, Yin2, ORPM344, ORPM86 and GCPM1894). The fragment size was determined using capillary gel electrophoresis a CEQ8000 sequencer (Beckman-Coulter, USA). Data was gathered and summarized into the table based on allele sizes. Repetition of analyses have been done for several samples while any adequate data were included into the evaluation dataset.

Chloroplast microsatellite markers have been designed especially for *Populus alba* and *Populus tremula* and gently provided by Dr. Berthold Heinze. The parameters of markers are summarized in Table 1. The PCR conditions were set up as follow: 25µL reactions contained 1 µL of DNA (10 ng μL^{-1}), 5 x Phire reaction buffer and Thermo Scientific Phire Hot Start II DNA Polymerase enzyme (Thermo Scientific), 50 mM $MgCl_2$, 10 mM dNTPs and 4 µM of each primer. Cycling conditions were set at 94°C – 3 min, 9 cycles of 94°C – 5 min and 70°C – 1 min, 34 cycles of 94°C – 30 s, 55°C (depending on the Tm, Table 1) – 50 s and 70°C – 2 min, then 4°C until recovery. Subsequently, polymorphic DNA loci were tested according to Joseph and Lexer (2008) by three candidative genes: transcription factor from AP- 2 family (AP2), phenylalanine ammonia lyase (PPAL-P1) and cellulose synthase gene (Cse-A2) in both *Populus alba* and *Populus tremula* species using the same PCR conditions as previously described. The number of alleles, their frequencies, observed and expected heterozygosity and Shannon´s informative index have been evaluated using software GenAlEx version 6.4 (Peakall and Smouse, 2006).

shortcut	name of gene	Forward primer "F"	name of gene	Reverse primer
A	trnS(GCU)-Ham	GCCGCTTTAGTCCACTCAGC	trnG2-r	TTACCACTAAACTATACCCGC
B	ccSSR-03-F	CCAAAAGCTGACATAGATGTTA	trnR-Doyle	GTCCTATCCATTAGACAATGG
C	ccmp5-F	TGTTCCAATATCTTCTTGTCATTT	rpoC2-R1	CATTTATAGGATTTAACGATTCCT
D	ORF62-P	CTTGCTTTCCAATTGGCTGT	trnG-M	AACCCGCATCTTCTCCTTGG
E	psbE-f1	TATCGAATACTGGTAATAATATCAGC	petL-R1	CCAAAATAACTAGTTAGAGTGGG
F	rps18-f Grivet-mod	GCTCGTATTTTATCTTyrTTACCC	rpl20-R1	GGAATTGCCTTTATCTGATTTCC
G	petB-F1	CGTAAGCAAGGTATTTCGGG	petD-pop-R1	AATCCACACTTTTCTCTTAGG
H	ycf1-F1	GAACCAAAAATGGAGGATCG	ndhF-R1	GTAGGAGGTGGTCGCATCTC
CH	ndhF-F1	TAACATAGGGACTGGAAGTG	trnL(UAG)-r1	CTGCTTCCTAAGAGCAGCGT

Table 1: Chloroplast microsatellite markers for *Populus alba* and *Populus tremula*.

5. RESULTS

Nuclear microsatellite analysis

Selected nuclear microsatellites used in this study were tested for *Populus x canescens*, *Populus alba* and *Populus tremula* individuals to determine genetic variability within and between poplar populations. Firstly, five subpopulations (TPE01 – TPE05) of grey poplar from South Moravia region were analysed and evaluated separately. Allele frequencies and allelic patterns across the subpopulations revealed clonal identity in all studied samples (Figure 1) whereas the variance in allele sizes is suppose to be caused by a shift during next analyses. Expected and observered heterozigosity (H_E = 0.518 and H_O = 1.0) excluded presence of homozygous genotypes in the population (data not shown). These data clearly indicates identical genotypes in grey poplar subpopulations from Dyjákovice village.

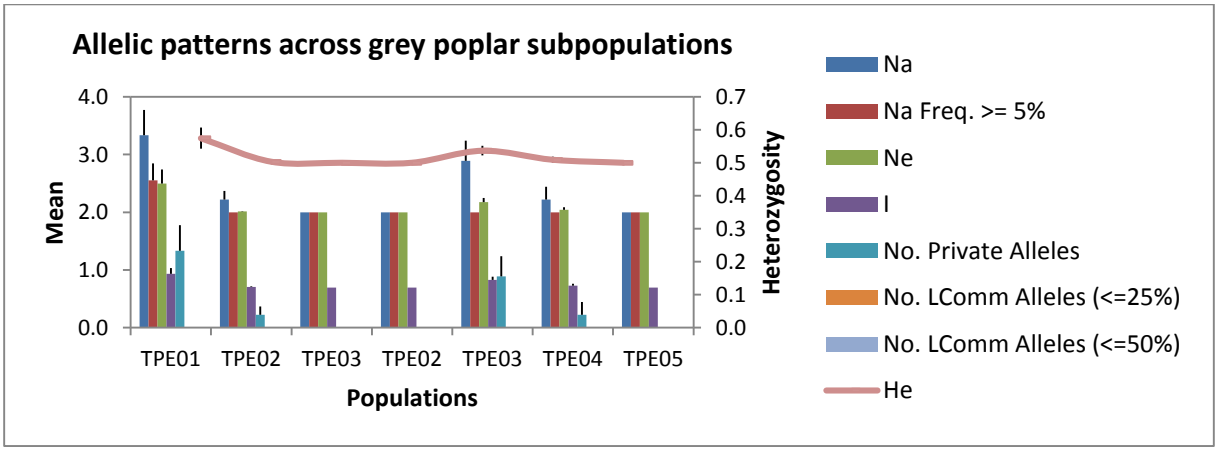


Figure 1: Distribution of alleles in the subpopulations of grey poplar. Number (No.) of different alleles (Na), No. of different alleles with a frequency ≥ 5% (Na Freq. ≥ 5%), No. of effective alleles (Ne), Shannon´s information index (I), No. of alleles unique to a single population (No. Private Alleles), No. of locally common alleles (Freq. ≥ 5%) found in 25% or fewer populations (No. LComm Alleles ≤ 25%),

No. of locally common alleles (Freq. ≥ 5%) found in 50% or fewer populations (No. LComm Alleles ≤ 50%), expected heterozigosity (H_E).

To confirm polymorphic patterns of selected nuclear microsatellite loci, we assessed genetic variability and estimated primary characteristics in two populations of grey poplar, population from Bezděčín village (*Populus x canescens* II) compared to population from Dyjákovice village (*Populus x canescens* I). Our results shown an average of 4.6 and 4.8 alleles per locus in a total number of 90 and 32 samples in *Populus x canescens* (I) and (II), respectively. The highest frequencies of alleles by population and locus were specified by WPMS17, GCPM1894 and ORPM86 nuclear markers (Figure 2). Interestigly, ORPM86, ORPM344 and PTR8 resulted in single allele detection in *Populus x canescens* (II) determining homozygotous genotypes in the population oppositely to the clearly observed heterozygosity through GCPM1894 nuclear microsatellite marker in both grey poplar populations (Table 1).

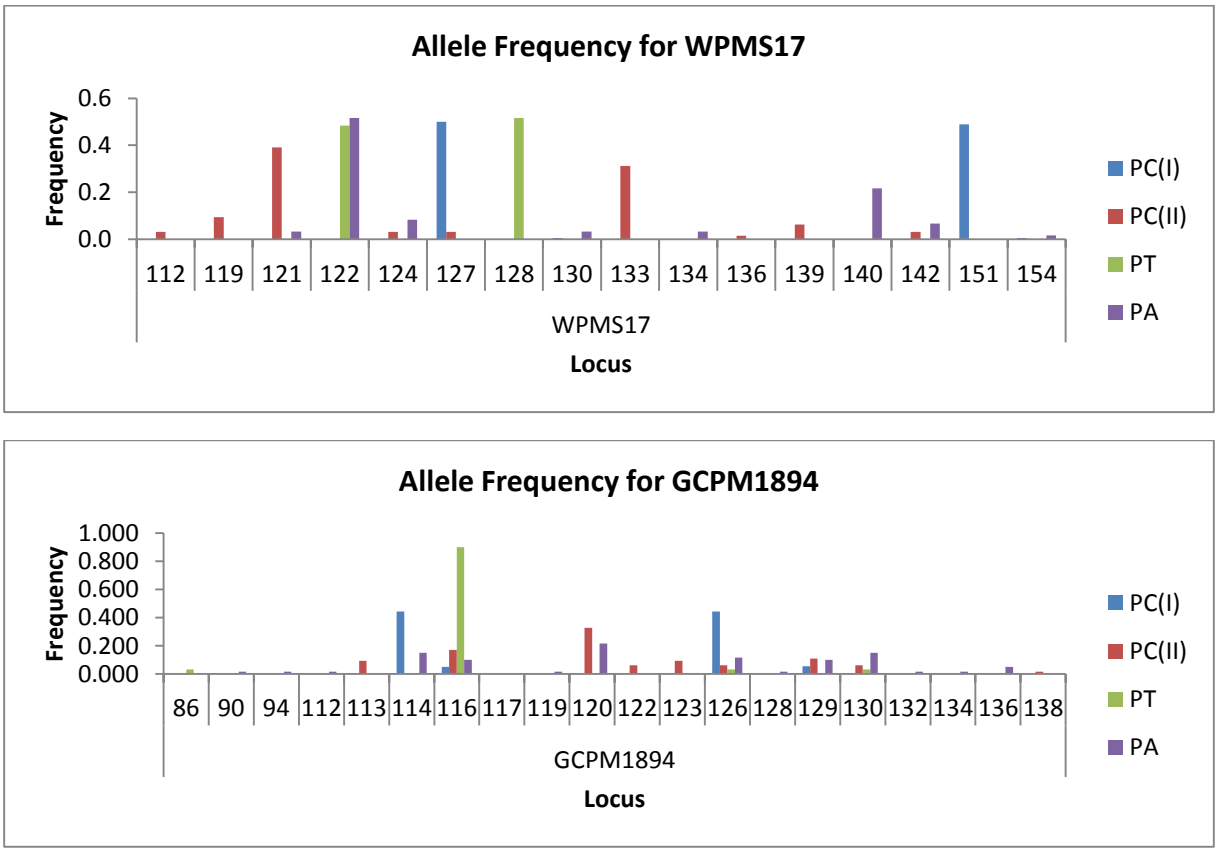


Figure 2: Allele frequencies for nuclear microsatellite markers WPMS17 and GCPM1894 in poplar populations. PC(I) - *Populus x canescens* from Dyjákovice village, PC(II) - *Populus x canescens* from Bezděčín village, PT – *Populus tremula*, PA – *Populus alba*.

Observed heterozygosity in parentals from Dyjákovice village ranged from 0.067 to 1.0 in *Populus alba* (PA) and 0.1 to 1.0 in *Populus tremula* (PT) suggesting occurence of homozygous as well as heterozygous genotypes within the populations (Table 2). The most effective alleles

(N_e = 7.692 and N_e = 2.839) were exposed by GCPM1894 and WPMS16 in PA and PT, respectively. In summary, our data suggested polymorphic characters of tested markers in population of *Populus x canescens* (II) and thus confirmed monoclonal stands of *Populus x canescens* (I). Moreover, nuclear microsatellites revealed divergent pattern of genotypes across the parentals of *Populus x canescens* (I) with the prospective contribution of gene admixture.

	Populus x canescens (I)				***Populus x canescens (II)***			
locus	*A*	H_o	H_E	*I*	*A*	H_o	H_E	*I*
WPMS14	3	1.000	0.505	0.724	5	0.500	0.579	1.085
WPMS17	4	0.989	0.511	0.754	9	0.688	0.733	1.624
WPMS20	2	1.000	0.500	0.693	3	0.063	0.119	0.277
GCPM1894	5	1.000	0.599	1.060	9	0.938	0.821	1.939
ORPM344	4	0.967	0.562	0.937	2	0.031	0.031	0.080
WPMS16	6	1.000	0.522	0.815	5	0.125	0.328	0.716
PTR8	6	1.000	0.522	0.815	4	0.031	0.251	0.525
Yin2	5	1.000	0.516	0.785	4	0.094	0.203	0.463
ORPM86	7	1.000	0.566	0.976	2	0.000	0.061	0.139

	Populus alba				***Populus tremula***			
locus	*A*	H_o	H_E	*I*	*A*	H_o	H_E	*I*
WPMS14	4	0.400	0.528	0.991	4	0.800	0.585	1.106
WPMS17	8	0.467	0.671	1.469	2	0.567	0.499	0.693
WPMS20	4	0.067	0.157	0.375	4	0.367	0.317	0.634
GCPM1894	14	1.000	0.870	2.239	4	0.133	0.187	0.435
ORPM344	4	0.100	0.157	0.375	6	0.800	0.638	1.184
WPMS16	5	0.067	0.214	0.495	5	1.000	0.648	1.172
PTR8	3	0.067	0.238	0.468	6	0.333	0.638	1.235
Yin2	3	0.100	0.096	0.230	6	0.600	0.508	1.004
ORPM86	3	0.500	0.493	0.742	2	0.100	0.095	0.199

Table 2: Genetic variability for 9 nuclear microsatellite loci for poplar populations. *Populus x canescens* sampled at Dyjákovice (I) and Bezděčín (II) village. Parentals of *Populus x canescens* (I), *Populus alba* and *Populus tremula*.

Chloroplast DNA microsatellites

The set of chloroplast primers designed by Dr. Heinze given in Table 1 were used for detection of chloroplast DNA polymorphism within the fragments of *Populus alba* and *Populus tremula*. The primer combination listed in Table 1 derived amplification products in a robust manner. Variability in fragment sizes was easily visible for primers A, B, C, E and F (Figure 3). Other primers (D, G, H and CH) yielded less polymorphisms in chloroplast DNA regions (Fig. 3).

Interestingly, more conserved fragments in length were apparent in *Populus tremula* samples (Fig. 3, data not shown). Altogether, these results show differences in fragment lenghts of *Populus alba* and *Populus tremula* individuals, indicating potential of gene flows among the *Populus x canescens* population.

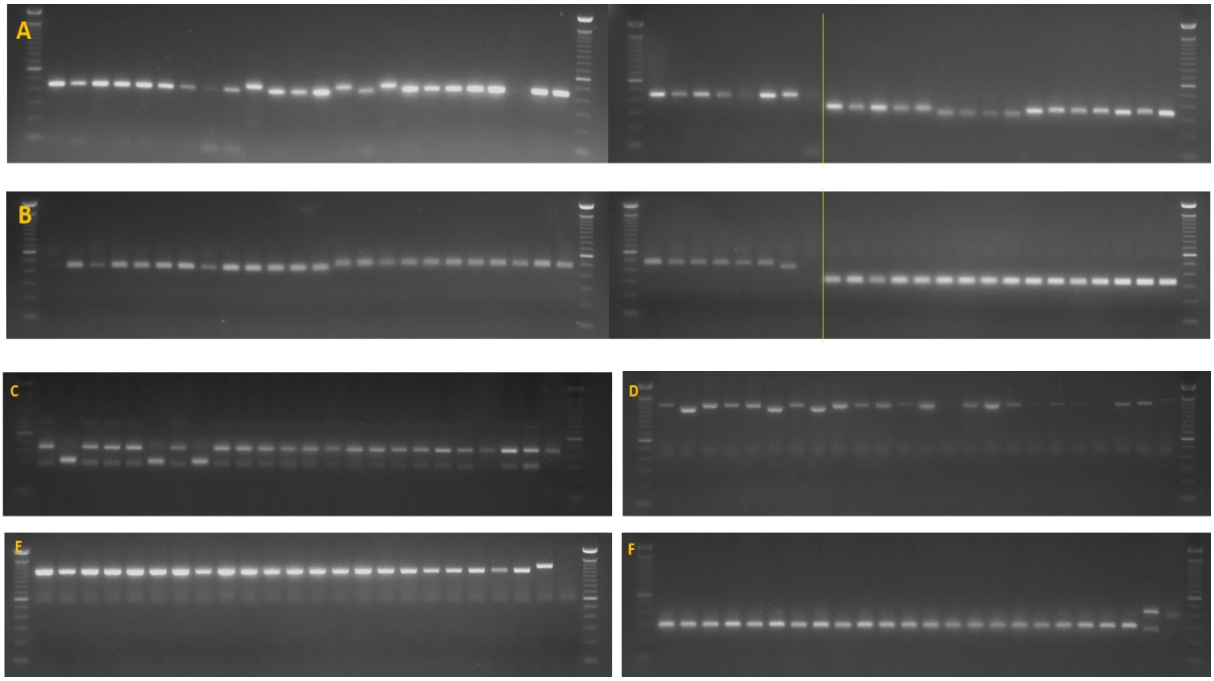
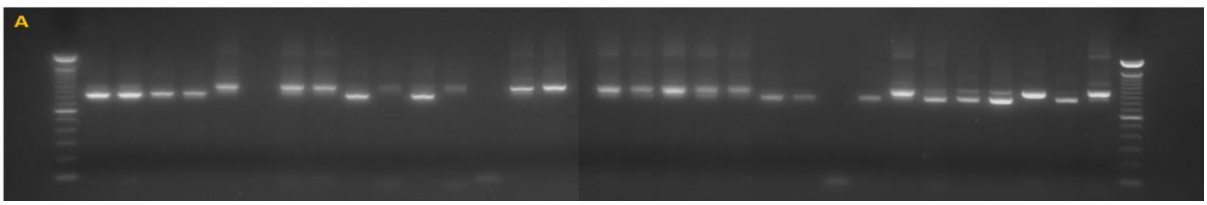


Figure 3: Two chloroplast DNA regions in *Populus alba* (A) and *Populus tremula* (B). DNA fragments amplified with chloroplast primers "B" (from the left) and "C" (after yellow line). Polymorphic patterns in *Populus alba* (C and D) and more conserved patterns in *Populus tremula* (E and F) detected by chloroplast markers "E" and "F", respectively.

DNA polymorphisms within cadidative genes

Three markers of candidative genes including transcription factor from AP-2 family (AP2), phenylalanine ammonia lyase (PPAL-P1) and collulose synthase (CseA2) involved in different physiological processes in plants have been tested for DNA polymorphisms in *Populus alba* and *Populus tremula*. Optimalization of PCR reactions was done only for CseA2. In both species, amplificated fragments were observed whereas unspecified products were formed in several samples. The highest DNA polymorphisms were identified for PPAL-P1 (Figure 4) whereas the other tested markers exhibited lower levels of genetic variability (data not shown). Interestingly, *Populus tremula* exhibited more conserved DNA locus for all tested markers in comparison to *Populus alba* as indicated Figure 3 and 4.



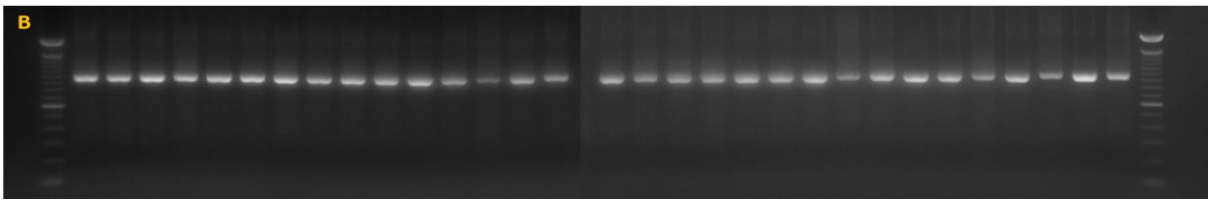


Figure 4: Polymorphic locus of phenylalanine ammonia lyase (PPAL-P1) gene in *Populus alba* (A) and *Populus tremula* (B).

6. DISCUSSION

Genetic diversity of poplar populations is commonly characterized by a range of molecular techniques. Among them, nuclear and chloroplast microsatellites substantially contribute to assessment of differences in the nucleotide sequences (Heinze, 1998 and Liesebach *et al*., 2010).

In this study, nine chloroplast (Table 1) and nine nuclear microsatellite loci (WPMS14, WPMS16, WPMS17, WPMS20, PTR8, ORPM344, ORPM 86, GCPM1894 and Yin2) were applied and tested on a collection of poplar populations. Natural marginal population of *Populus x canescens (I)* has been characterized by a clonal genetic structure with the heterozygous genotype (Figure 1, Table 2). Surprisingly, the individual subpopulations uniquely located in floodplain forest at Dyjákovice village were sampled separately with the highest distance approx. 1 km. It is therefore speculative which way of grey poplar propagation is more prevalent. Clonal growth of *Populus alba* in the Mediterranean islands was explained by human introduction rather than autovegetative propagation (Fussi *et al*., 2012). Clonality of *Populus x canescens* within the Danube Floodplain National Park in Austria was suggested to be established by vegetative reproduction as seedling mortality is often high while the roots are long enough to reach the water table (van Loo *et al*., 2008). Futher, we hypothesise that apomixis can be one of possible strategy in poplar species reproduction due to prevailance of female inflorescence at this locality.

Recently, new approach for characterization of clonal population was established. Plant biodiversity in clonal population of *Populus alba* was followed based on DNA epigenetic status. Decrease number of population clusters in relation to their geographic position suggesting that DNA methylations substantially contribute to increase of genetic diversity within the clone individuals (Guarino *et al*., 2015).

Divergent pattern of chloroplast and nuclear genome across the parentals of grey poplar subpopulations (*Populus x canescens I*) was studied. Generally, it has been shown that *Populus tremula* exhibited more conserved DNA loci for all tested markers in comparison to *Populus alba* (Table 3 and 4) wich is in accordance to Liesebach *et al*. (2010). Similarly with published data by Joseph and Lexer (2008), DNA polymorphisms within AP2, PPAL-P1 and CseA2 genes was observed for *Populus alba* and *Populus tremula* (Table 4). Unfortunately, to precisely identify the level of introgression in grey poplar species, additional analyses using chloroplast markers for grey poplar samples are essential in this case.

7. CONCLUSIONS

Based on the results of this study, we determined clonal genetic structure of grey poplar subpopulations located in South Moravia region. This relevation is very important for several following steps including especially the forest management strategy for this marginal grey poplar population. Moreover, relevation of divergent patterns of nuclear genetic diversity in parental species (*Populus alba* and *Populus tremula*) suggests the potential of gene flow into the young forest tree generation. It is therefore essential for the future step to estimate the level of introgression within the grey poplar population.

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