Direct PCR as the platform of *Hedera helix*, L. genotypying without the extraction of DNA

Priama PCR bez použitia izolácie DNA ako platforma genotypovania *Hedera helix*, L.

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Abstract

Hedera helix, L. is a specie that is used for its ornamental and medicinal properties widely. In spite of its very good biochemical characterization, the knowledge about the DNA variability is very limited and no DNA markers were used to analyse the genomic variability of the ivy populations, up to date. Here, a direct PCR approach was used to evaluate a cytochrome P450 sequences based polymorphism by PBA technique. A set of 4 samples from Slovakia, 2 samples from Czech Republic, 2 samples originated in Poland, 2 samples from Croatia, 2 samples from Scotland and samples from Germany, Austria and Spain were used in analysis. Using a set of three PBA primers and their combinations, the number of amplified fragment levels obtained by individual primer combinations was for both of them 11 and the obtained polymorphism was 91% or 100% respectivelly. The PCoA analysis was performed and the genotypes belonging to a different geographic localities were grouped together.

Keywords: direct polymerase chain reaction, genetic diversity, *Hedera helix*, L.

Abstrakt

Brečtan popínavý je druh, ktorý je široko využívaný ako na okrasné účely, tak aj kôli svojím farmaceuticky zaujímavým vlastnostiam. Napriek svojej veľmi dobrej biochemickej charakterizácii sú však vedomosti o variabilite DNA zatiaľ limitované a doteraz neboli publikované práce zamerané na analýzu variability genómu brečtana DNA markérmi. V tejto práci je popísané využitie priamej PCR v analýzach vyhodnotenia dĺžkového polymorfizmu založeného na markéroch pre cytochróm P450 sekvencie pomocou PBA techniky. V analýzach boli použité genotypy z rôznych častí Európy, 4 vzorky zo Slovenska, 2 vzorky z Českej republiky, 2 vzorky boli pôvodom z Poľska, 2 vzorky z Chorvátska, 2 vzorky zo Škótska a vzorky z Nemecka , Rakúska a Španielska. Aplikáciou PBA primerov a ich kombinácií, bol počet amplifikovaných fragmentov získaných jednotlivými kombináciami primerov 11 a získaný polymorfizmus bol na úrovni 91% alebo 100% v závislosti od primerového

páru. Získané výsledky boli spracované PCoA analýzou, pričom hodnotené genotypy, ktoré patria do rôznych geografických lokalít boli zoskupené spolu.

Kľúčové slová: genetická diverzita, *Hedera helix*, L., priama polymerázová reťazová reakcia

Introduction

Hedera helix, L. belongs to the genus Hedera of the Araliaceae that comprises from thirteen species. It is a pharmaceutically interesting woody plant with evergreen leaves where a different morphological characteristics exist for youth and adults developmental stages (Green et al., 2011; Metcalfe, 2005). Ivy is reported to have a low tolerance to cold in winter what limit its distribution boundaries in Northeen and Eastern Europe (Metcalfe, 2005). *Hedera helix*, L. is a very popular decorative plant and has many cultivars that are ideal for winter gardens and gardens design. Beside its ornamental utilization, ivy is very important for its medicinal properties. *Hedera helix*, L. is known to contain the saponins - secondary metabolites with a number of pharmacological effects. It reduces cholesterol, has antibacterial, antifungal and antiviral influence (Bedir et al., 2000; Medeiros et al., 2002). Ivy is widely used in pharmacy for its expectorant and antitussive effect.

Hedera helix, L. was designed as an example of a genomic plasticity which occurs during typical developmental changes from juvenile to adult phase (Obermayer, 2000) and thirteen different haplotypes were detected by Grivet and Petit (2002) in Europe. Up to date, a very limited information exist about the ivy genome variability in natural populations based on the DNA markers. The first analysis of genetic variability of *Hedera* was performed by Vargas et al. (1999) based on the ITS sequences variability and chromosome counts. Valcárcel et al. (2003) have defined four chlorotypes of ivy that differ by *trnT–L* nucleotide substitutions and two insertions of 50-bp and 30-bp. This results have provided the evidence for evolutionary biogeography of ivy. Chloroplast polymorphism together with the polymorphism of ITS regions was used by Grivet and Petit (2002) to describe the phylogeography of *Hedera helix*, L. in populations from Great Britain, Sweden, Germany, France, Spain, Italy, Croatia, Slovakia, Greece and Morocco. Two ITS types and nine haplotypes of ivy were defined throughout the Europe. Up to date, only RAPD markers were used to analyse the genome variability of *Hedera helix* (Clarke et al., 2006) and a selection of different DNA markers for the analysis of *Hedera helix*, L. was reported by Žiarovská et al. (2016).

Here, a direct PCR approach was applied to analyse a cytochrome P450 marker variability in the Europe populations of *Hedera helix*, L. Amplification of the target sites of plant genome by the direct PCR is a method where no DNA extraction is needed prior the PCR. It is possible to use both, crude plant extract as well as the small pieces of plant tissues without steps of isolation and purification of total genomic DNA (Chum et al., 2012). Omission of the step of DNA isolation process brings benefits mainly in terms of utilization of samples without loss, saving time and reducing the cost of analysis. Direct hits PCR has the potential to facilitate routine genotyping widely, even its application is not widespread in plant analysis yet as in animal tissues. This is caused mainly by a different composition of plant cells where

much more PCR contaminats exist. The approach of direct PCR was used firstly for the amplification of *Nit1* gene of *Arabidopsis thaliana*, L. (Young et al., 2007). The combination of commercially available extraction buffer and in laboratory prepared specific PCR buffer was used for this purpose with the result of effective amplification of the target even in the presence of both, endogenous and exogenous contaminats of the *Arabidopsis* leaves.

The aim of the study was to analyse the variability of PBA markers in a set of 15 populations of *Hedera helix*, L. from different localities of Europe and constructing a scattergram to visualized the relationships among them.

Material and methods

Juvenile healthy leaves of *Hedera helix*, L. were collected *in situ* in different localities of Slovakia (4 different localities in total), Czech Republic (2 different localities), Poland (2 different localities), Croatia (2 different localities), Scotland (2 different localities), Germany, Austria and Spain. A total of ten leaves were collected randomly in the shrub of *Hedera helix*, L. in the concrere locality. All the samples were treated immediatelly with etanol for the purpose of the surface desinfection. When transporting them to the laboratory, they were stored under the -20°C for the further processing.

Direct PCR was performed by commertial kit Phire Plant Direct PCR Kit (Thermo Scientific) followed the manufacturer´s instructions with minor modification in the thermal profile of the PCR. To obtain an appropriate piece of tissue, a 0,35 mm cutter was used. Fistly, annealing temperature for the used primer pairs was tested in gradient PCR and subsequently the following thermal and time profile was used: 95°C - 3 min; 35 cycles of : 95°C 1 minute; 58°C 2 minutes; 72°C 3 minutes with final 72°C 10 minutes.

Primers used for the PBA marker´s amplification was used as reported by Yamanaka et al. (2003). Amplified products were analysed in 2% agarose gels and scored for the binary matrices - presence of band was denoted as 1 and its absence as 0. Principal component analysis was performed in SYNTAX software using a Nei and Li coefficient of similarity (Nei and Li,1979) to obtain a scattergram of different PBA profiles of analysed samples of *Hedera helix*, L.

Results and discussion

In spite of an active pharmaceutical use of *Hedera helix*, L. its molecular diversity based on the DNA markers is practically unknown. The only information about the applications of polymorphic markers are reported for ITS, cpDNA and RAPD (Clarke et al., 2006; Green et al., 2011, 2013; Valcárcel et al., 2003). RAPD marker was not used directly to analyse the polymorphism of *Hedera helix*, L. but an invading populations of *Hedera hibernica* (Kirchner) Bean and *Hedera helix*, L. were identified based on the RAPD data (Clarke et al., 2006). The first screening for the microsatellite, retrotransposon and miRNA based markers for the *Hedera helix*, L. reported by Žiarovská et al. (2016). In this study, retrotransposon based types of markers – IRAP and iPBS were prooved to be an excellent for the ivy diversity analysis, microsatellite markers were prooved to be successfull based on the type of the repetition and miRNA as a novel types of DNA markers were prooved to be

a very promising for ivy diversity analysis (Žiarovská et al., 2016). Here, a cytochrome P450 sequences were used to analyse the diversity of European ivy populations by PBA technique. The technique was reported firstly by Yamanaka et al. (2003) as to be very effective in the functional genomic studies of plants. It can be used universaly, as the P450 gene-primer sets were designed to cover the analogue fragments of this gene. The polymorphism of P450 was analysed for *Hedera helix*, L.. using a set of three PBA primers and their combinations, The number of amplified fragment levels obtained by individual primer combinations was for both of them 11 and the obtained polymorphism was 91% or 100% respectivelly (table 1).

Table 1. Amplification characteristics of the primer sets used in the study
Tabuľka 1. Amplifikačné charateristiky párov prajmerov použitých v analýzach

Primer pair	Number of totally amplified loci levels	% of polymorphism
CYP1A1F-CYP2B6R	85	91
CYP2B6F-CYP2B6R	88	100

The number of amplified PBA loci has ranged from 3 up to the 8 for both of the used primer combinations (figure 2 and 3). For the group of Slovak samples were the average number of amplified loci 12 and the samples from Poland have 14 and 15 amplified loci (figure 3). For both of these groups it was the highest achieved number of amplified PBA loci.

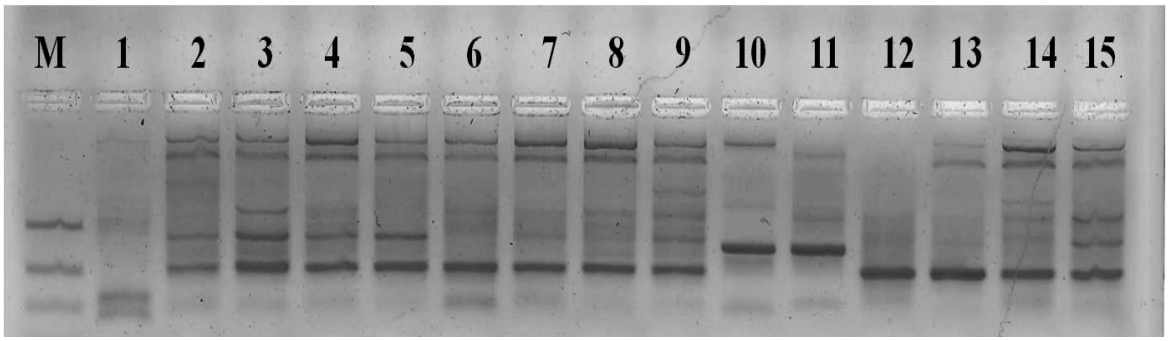


Figure 1. Amplified PBA loci for primer combination CYP1A1F and CYP2B6R.
Obrázok 1. Zmnožené PBA lokusy pre kombináciu CYP1A1F and CYP2B6R.

Cytochrome P450 mono-oxygenases were widely sequenced throughout all the kingdoms and in higher plants they are a part of oxidative detoxification and the secondary metabolites biosynthesis (Ohkawa et al., 1998). Using a functional genomic markers such as PBA for the analysis of genetic variation overcome the problems of the analysis of the genetically neutral regions such as RAPD or ISSR (Yamanaka et al., 2003), but the neutral markers are still an important part of the variability analysis of different plant genetic resources (Balážová et al., 2014; Milella et al., 2005; Musilová et al., 2013; Štefúnová et al., 2015; Vivodík et al., 2015).

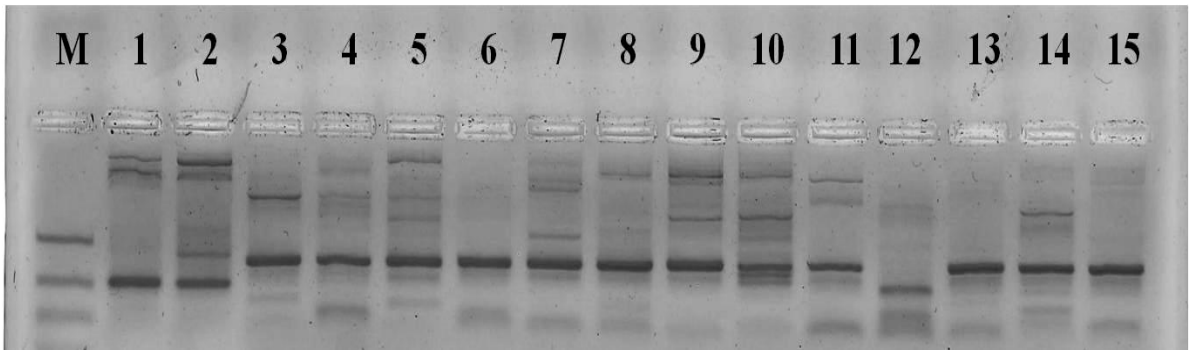
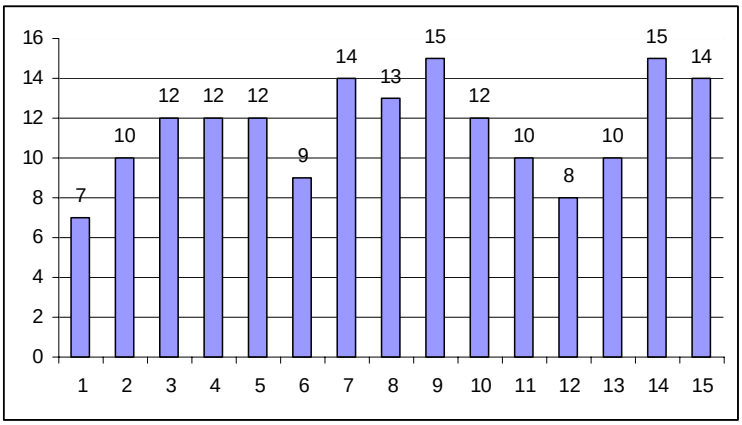


Figure 2. Amplified PBA loci for primer combination CYP2B6F and CYP2B6R.
Obrázok 2. Zmnožené PBA lokusy pre primerovú kombináciu CYP2B6F a CYP2B6R.

PBA markers were sucessfully used for 42 different plant species by Yamanaka et al. (2003) when testing the newly developed method for *Oryza sativa* (Yamanaka et al., 2011), for *Eleusine coracana*,L. (Panwar et al., 2010) and *Sechium edule* (Jacq.) Sw. (Machida-Hirano et al., 2015). For Mexican chayote from 9 primer combinations used in the PBA analysis, 8 were polymorphic and the reported total number of polymorphic bands was 88 (Machida-Hirano et al. 2015). Panwar et al. (2010) reported 55% polymorphism for *Eleusine coracana*,L and ten primer pair combinations used.



Origin of the samples: 1 – Spain; 6,7,9,13 – Slovakia; 2 – Austria; 3,4 – Croatia; 5,8 – Czech Republic; 10,11 – Scotand; 12 – Germany; 14,15 – Poland
Pôvod jednotlivých genotypov: 1 – Španielsko; 6,7,9,13 – Slovensko; 2 – Rakúsko; 3,4 – Chorvátsko; 5,8 – Česká Republika; 10,11 – Škótsko; 12 – Nemecko; 14,15 - Poľsko

Figure 3. Number of total amplified PBA loci for individual analysed genotypes.
Obrázok 3. Počty zmnožených PBA lokusov pre jednotlivé analyzované genotypy.

The binary matrix was prepared and used to calculate a similarity matrix based on Nei and Li coefficient of genetic similarity (Nei and Li , 1979) that was calculated in SYNTAX as a dissimilarity form. The coefficient of dissimilarity ranged from 0,083 (Croatian samples) up to the 0,778 (Scotish sample with the Germany one). When evaluating the samples from the same region, slovak samples has the coefficient of dissimilarity of 0,28; scotish samples 0,273, poland samples 0,103, Czech samples

0,200 and croatian samples 0,083. All these values show the high similarity of analysed populations. The PCoA analysis of cyt P450 gene based PBA polymorphism was performing (figure 4) where the genotypes belonging to a different geographic localities were grouped together. The pattern of grouping of the analysed samples according a specific region was reported by Panwar et al. (2010) for finger millet.
Grivet and Petit (2002) have reported that european ivies belong to eight different chloroplast haplotypes with the following characteristics: scotish ivies are of haplotype C1; slovak ivies are of A2, G, D and C1; croatian are of A1, A2 and H; german ivies are of haplotype A1, C1 and A2 and spanish are of E and G. This distribution is visible in the results of PBA based polymorphism, as the Spanish sample is the most distinct to the others and the common haplotype A2 is visible in the grouping of Slovak and Croatian samples and the haplotype C1 differ the samples from Scotland and Germany.

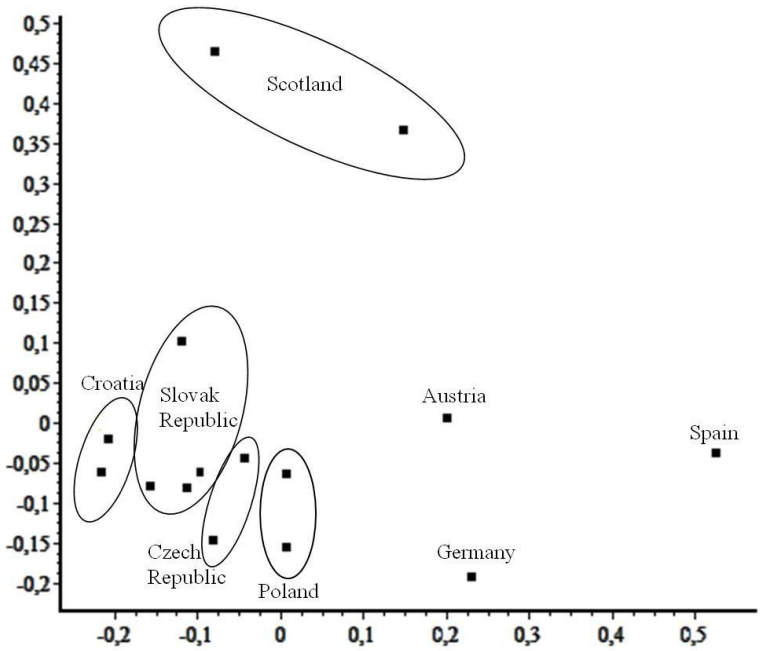


Figure 4. Distribution of PBA polymorphism in the analyzed populations of ivy
Obrázok 4. Distribúcia PBA polymorfizmu v analyzovaných populáciách brečtanu

Direct PCR was used to evaluate PBA polymorphism. This method was reported to be effective in plant genome analysis by Belstedt et al. (2010). The authors confirmed its success and effectivity for 32 different plant families. They have used the specific extraction and PCR buffer that were fully function in the case of species, where a wide range of PCR contaminants exist in their cells - *Vitis vinifera*, L; *Coffea arabica* L.; *Laurus nobilis* L. or *Thymus vulgaris* L.. Here, the application of direct PCR for the PBA analysis of *Hedera helix*, L. is reported for the first time and was prooved as an efficient method for the screening of the genetic variabilty.

Conclusions

Using of direct PCR approach in the marker based analysis of plants is quit a novel method as many of PCR contaminants are present in the plant cells. Here, the direct PCR protocol was prooved as to be applicable for the fast screening of PBA polymorphism of *Hedera helix*, L. PBA profiles were obtained as correlating with the genotypes provenience.

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