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IDENTIFICATION OF SWEET CHESNUT POLLEN IN BEE POLLEN PELLET USING MOLECULAR ANALYSIS

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ABSTRACT

Castanea sativa posses many characteristics that are used by human for different purposes, not only as a part of the food. One of them is the utilization of the sweet chesnut pollen for its pharmacological benefits. Actually, no information about the DNA based identification of the sweet chesnut exist. Here, an identification of *Castanea sativa* based on the specific DNA fragment amplification is described for the first time. Sweet chesnut identification was performed in the very complex sample of bee pollen pellets that were identified as to contain sweet chesnut pollen grains by morphological analysis. First, bioinformatic analysis was performed to find a *Castanea sativa* conservative part of galactol synthase gene. BLAST alignment of the CDS of GolS1 gene was performed by BLASTtn against plants nucleotide sequences in the NCBI database to ensure for the specifity or existing nucleotide differences. Then, specific primers were subsequently designed and PCR amplification was performed. All the PCRs have run in duplicates for pollen pellet sample and two independent samples of *Castanea sativa* pure pollen. Restriction cleavage of the PCR amplified fragment was performed to confirm the specifity of the obtained PCR product with the positive confirmation as the predicted three restriction fragments were obtained that fully correspond by the length to those from virtual clevage. Restriction endonuclease Hpy166II was used in restriction cleavage analysis. *Castanea sativa* pollen grains were confirmed reliable in multifloral pollen pellet by PCR and this approach has the potential to be used effectively for the authentication purposes of sweet chesnut.

Keywords: *Castanea sativa*; pollen; pollen pellet; identification; PCR

INTRODUCTION

Castanea sativa is reported as to have ecological and economical value **(Beyhan and Serdar, 2008; Lusini et al., 2014)**. It is cultivated throughout the whole Europe and historically, it is used for different purposes – it produces large amount of nectar, its fruits are edible and its pollen is valuable for the medicinal properties **(Hrga et al., 2010)**. *Castanea* pollen was described by **Peeters and Zoller (1988)** as small (18,3 x 12 μm), prolate and tricolporate with a smooth exine. **Maurizio and Grafl (1969)** reported the liberation time of *Castanea* pollen to be situated between 7-19 h, but to have two maxima at 10 h and 16 h. The pollination is entomophilous and anemophilous **(Hrga et al., 2010)**.

In spite of good knowledge about the genetic diversity and population structure of sweet chesnuts that was analysed using different biochemical and molecular markers, no information about the effective identification of the specie withing a complex samples – like bee pollen pellets, can be found. Different DNA markers were utilized for describing the variability of *Castanea sativa* – cpDNA **(Fineschi et al., 2000)**, RAPD **(Galderisi et al., 1998)** or microsatellites **(Martín et al., 2012; Mattioni et al., 2013; Lusini et al., 2014)**. **Hasegawa et al., (2009)** analyzed pollen genetic structure using microsatellite genotyping of pollen grains, seeds, and potential paternal trees in the self-incompatible monoecious tree species *Castanea crenata*.

Bee pollen is used in medicine as a part of supplementary nutrition and in alternative diets, because of its reported nutritional properties and health benefits. The components of bee pollen are: carbohydrates, crude fibers, proteins, lipids, minerals, trace elements, vitamins, carotenoids, phenolic compounds, flavonoids, sterols and terpenes **(Bogdanov, 2014)**.

Bee pollen is described as the perfectly complete food, because it contains all essential amino acids that humans need for their nutrition. The biochemical composition of bee pollen is affected by many factors such as plant source, geographic origin, climatic conditions, soil type or beekeeper activities **(Feás et al., 2012)**.

Single pollen identification was reported for the first time by **Suyama et al., (1996)** for the pollen grain from Pleistocene peat. **Matsuki et al., (2007)** modified this method and have used it for the purpose of multilocus genotyping of a single pollen grain by PCR. Using the PCR for pollen identification is now utilized in parentage analysis, discrimination of self-pollen, or evaluation of the genetic diversity of pollen grains **(Isagi, 2011)**.

The aim of the study was the identification of sweet chesnut pollen as to be a part of bee pollen pellet using molecular approach.

MATERIAL AND METHODOLOGY

Biological material

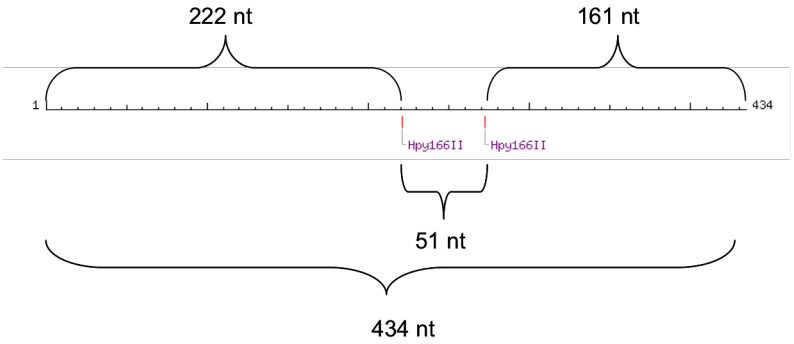

Figure 1 Restriction scheme for the obtained PCR fragment of the Go1 S1 gene of the *Castanea sativa*, L.

A pure pollen of *Castanea sativa*, Mill was collected through the chesnut flowering season during 2013 and 2014 in the form of biological duplicate. Samples were desicated and freezed in plastic aseptic container until further analysis. Pollen was obtained in the Radošina (Slovak republic), where a group of old trees of sweet chesnut can be found. Pollen pellets were collected by bee keepers in the same locality.

DNA extraction

Total genomic DNA from pollen and bee pollen pellet was extracted using the GeneJET™ Plant Genomic DNA Purification Kit (ThermoScientific) according to the manufacturer's recommendations. DNA quality and the concentration were quantified by spectrophotometer.

Bioinformatic alighment of Castanea sativa galactinol synthase (GolS1) gene and primer design

BLAST **(Zhang et al., 2000)** alignment of the CDS of GolS1 gene, (NCBI accession code JX512438) was done by BLASTtn against plants (taxid:3193) nucleotide sequences in the NCBI database to ensure for the specifity or existing nucleotide differences. Primer desing was performed in Primer-BLAST **(Ye et al., 2012)** in a manner to get *Castanea sativa* galactinol synthase specific amplification. Following primers were returned and used in the study: forward 5´ AAGCCCCTCCCTCTGGTATT 3´ (matching the nucleotides 4693 - 4712 of JX512438) and reverse 5´ GCTCAGGCATGGAAGCCATA 3´ (matching the nucleotides 5107 - 5126 of JX512438) with the amplification product length of 434 bp.

PCR conditions

PCR runs were performed in duplicates, each containing KAPA2G™ Robust HotStart Ready Mix (2x) (Kapa Biosystems); 300 nmol of forward and reverse primer 50 ng of template DNA, for 40 cycles using a BioRad C1000. Cycle conditions were as follows; 3 min. at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 15 s at 72 °C. The resulting reaction was run on a 2% agarose gel to confirm the product was unique and of the correct size.

Product specifity verification

Nucleotide sequence that was obtained by bioinformatic analysis was uploaded into the NEBcutter v 2.0 **(Vincze et al., 2003)** and the appropriate restriction enzyme was selected for the verification of the results of PCR. The amplified PCR product was inspected for the specifity using the Hpy166II restriction endonuclease that possess in total two restriction sites (figure 1) withing the amplified fragment of the Go1S1 gene of the *Castanea sativa*, L.

RESULTS AND DISCUSSION

Molecular markers are reported by **Allendorf et al. (2010)** as an efficient tool for characterization of long-life species, mainly for its facilitation of conservation decisions. DNA based markers that were applied in sweet chesnut analysis have aimed in past mainly on mapping studies for disease resistance genes or population studies. Different DNA markers were utilized for this purpose, such as RAPDs, RFLPs or ISSRs **(Fineschi et al., 2000; Casasoli et al., 2001; Goulão et al., 2001)**. In nucleotide databases, a total of 55 differrent microsatellite loci are stored for *Castanea sativa*, Mill. **Martín et al. (2012)** used 7 microsatellite loci for genetic structure analysis of sweet chesnut in Spain and **Lusini et al., (2014)** used 6 of the same plus 1 different locus for genetic structure analysis of sweet chesnut in Bulgaria. Both of the analysis showed a high level of genetic diversity. That is why finding of reliable conservative sequences is important for identification studies of *Castanea sativa*, Mill.

First, bioinformatics alighment was performed for GolS1 gene of *Castanea sativa*, Mill.. Any of returned alightments don´t posses the relevant similarity with the Go1S1 gene (table 1).

Based on the obtained data, *Castanea sativa* galactinol synthase specific amplification primers were designed and used in subsequent PCR analysis. PCR amplification was performed in duplicates for pollen pellet sample and two independent samples of *Castanea sativa* pure pollen. Optimization of annealing temperature and primer concentration was performed, were 150, 300 and 450 nmol primers were tested. A combination of 300 nmol primers and 60 °C annealing temperatures resulted in fully reproducible PCR product for both of pure pollen samples. Optimized PCR conditions were used for the analysis of pure *Castanea sativa* pollen, pure *Betula verucosa* pollen and tested pollen pellet sample.

A single 434 bp long PCR product was obtained (figure 2) for all the samples except the pure pollen sample of *Betula verrucosa* that was used as negative control in the study. The analysis was repeated one more time with the same results.

The analytical methods used for species identification and authenticity rely mainly on protein and DNA analysis.

Table 1 Alightment characteristics for similar sequences for Gol1S gene.

Sequence	Max score	Total score	Query cover	E value	Ident	NCBI Accession
Theobroma cacao Galactinol synthase 4	231	231	5%	9,00E-57	81%	XM_007044611.1
Ricinus communis conserved hypothetical protein	228	336	7%	1,00E-55	82%	XM_002525318.1
Predicted: *Nelumbo nucifera* galactinol synthase 1-like	220	428	9%	2,00E-53	83%	XM_010252922.1
Manihot esculenta galactinol synthase (GolS3)	211	211	4%	1,00E-50	84%	KJ722606.1
Predicted: *Sesamum indicum* galactinol synthase 1-like	209	209	4%	4,00E-50	82%	XM_011072789.1
Predicted: *Nelumbo nucifera* galactinol synthase 1-like	200	371	9%	2,00E-47	80%	XM_010244766.1
Predicted: *Nelumbo nucifera* galactinol synthase 1-like	200	371	9%	2,00E-47	80%	XM_010244765.1
Camellia sinensis galactinol synthase	196	196	5%	3,00E-46	80%	JX624168.1
Camellia sinensis cultivar Longjin43 galactinol synthase 1	195	195	4%	1,00E-45	81%	KF472133.1
Pop. alba x *Pou. grandidentata* galactinol synthase I	171	171	3%	2,00E-38	81%	JF499886.1
Amborella trichopoda hypothetical protein	169	169	4%	7,00E-38	78%	XM_006840664.1
Solanum tuberosum cultivar Umatilla putative galactinol synthase (GolS)	150	150	4%	3,00E-32	77%	GU596453.1
Solanum commersonii cultivar PI243503 putative galactinol synthase (GolS)	148	148	4%	9,00E-32	78%	GU596447.1
Solanum commersonii cultivar PI243503 putative galactinol synthase (GolS) gene	148	148	4%	9,00E-32	78%	GU596446.1
Predicted: *Solanum tuberosum* galactinol synthase 2-like	145	145	4%	1,00E-30	77%	XM_006346903.1
Arabidopsis thaliana chromosome 1 sequence	141	141	3%	2,00E-29	80%	CP002684.1
Arabidopsis thaliana chromosome I BAC F25P12 genomic sequence	141	141	3%	2,00E-29	80%	AC009323.4
Arabidopsis thaliana glycosyltransferase (AT1G56600)	135	135	3%	7,00E-28	79%	KJ138960.1
Arabidopsis thaliana galactinol synthase 2	135	135	3%	7,00E-28	79%	NM_104537.2
Arabidopsis thaliana At1g56600/F25P12_16 mRNA,	135	135	3%	7,00E-28	79%	AY058238.1
Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTSIL64ZG05	135	135	3%	7,00E-28	79%	BX818170.1
Arabidopsis thaliana At1g56600/F25P12_16 mRNA, complete cds	135	135	3%	7,00E-28	79%	AY050410.1
Arabidopsis thaliana AtGolS2 mRNA for galactinol synthase	135	135	3%	7,00E-28	79%	AB062849.1
Populus trichocarpa galactinol synthase family protein	128	128	4%	1,00E-25	77%	XM_002319437.2
Populus trichocarpa galactinol synthase 2-2	128	128	4%	1,00E-25	77%	HQ727694.1
PREDICTED: *Populus euphratica* galactinol synthase 2-like	122	122	4%	5,00E-24	76%	XM_011025526.1
Populus trichocarpa putative galactinol synthase family protein (POPTR_0002s19230g)	121	121	4%	2,00E-23	75%	XM_002301495.2
Populus trichocarpa galactinol synthase 8 (GolS8)	121	121	4%	2,00E-23	75%	KF496091.1
Populus trichocarpa x *Populus deltoides* galactinol synthase 4 (GOLS4)	121	121	4%	2,00E-23	75%	EU305724.1
Arabidopsis thaliana At1g56600/F25P12_16 mRNA	115	115	3%	9,00E-22	79%	AF412094.1
Solanum lycopersicum chromosome ch02, complete genome	111	111	4%	1,00E-20	75%	HG975514.1
Populus alba x *Populus grandidentata* galactinol synthase II (GolSII)	111	111	4%	1,00E-20	75%	JF499887.1
Populus trichocarpa galactinol synthase 2-1 mRNA	111	111	4%	1,00E-20	75%	HQ727693.1
Predicted: *Solanum lycopersicum* galactinol synthase 2-like (LOC101244339)	106	106	4%	5,00E-19	74%	XM_004233483.2
Populus trichocarpa galactinol synthase family protein (POPTR_0013s00720g)	106	106	4%	5,00E-19	75%	XM_002319436.2
Ajuga reptans mRNA for galactinol synthase, isoform GolS-2	73.1	73.1	1%	6,00E-09	79%	AJ237694.1
Predicted: *Camelina sativa* galactinol synthase 5-like	62.1	62.1	0%	1,00E-05	88%	XM_010437916.1

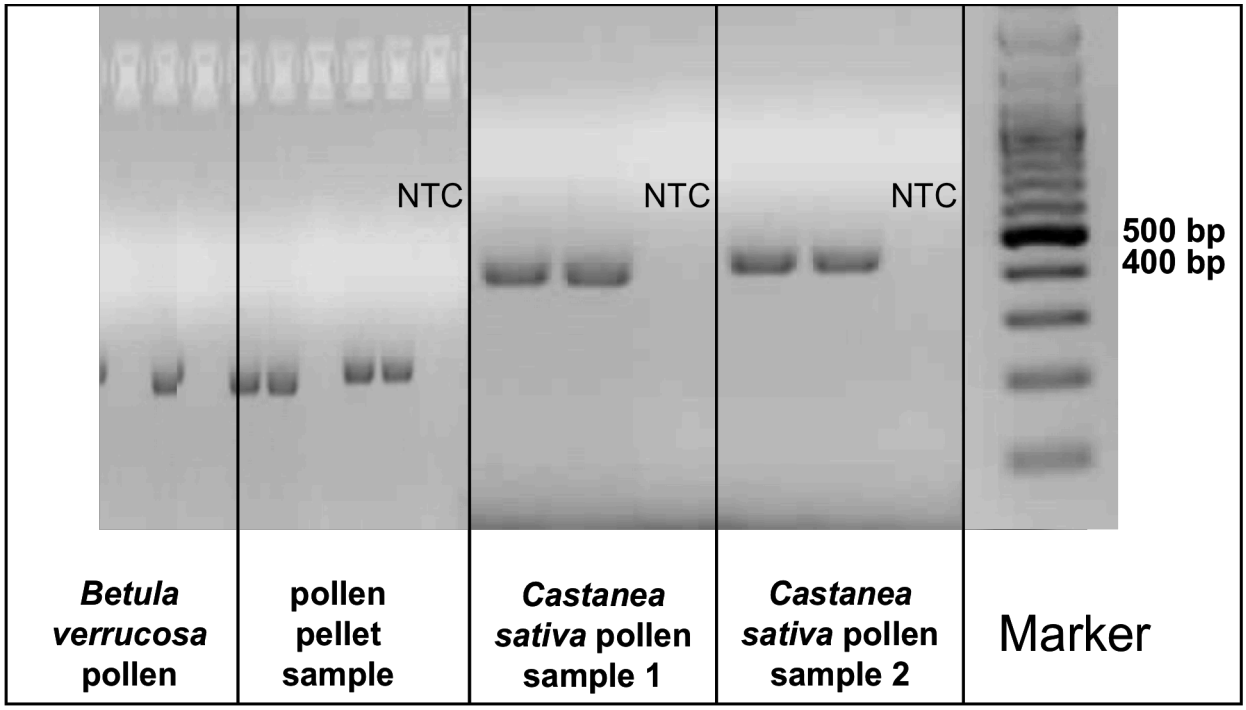

Figure 2 PCR amplification of conservative part of the Go1S1 gene.

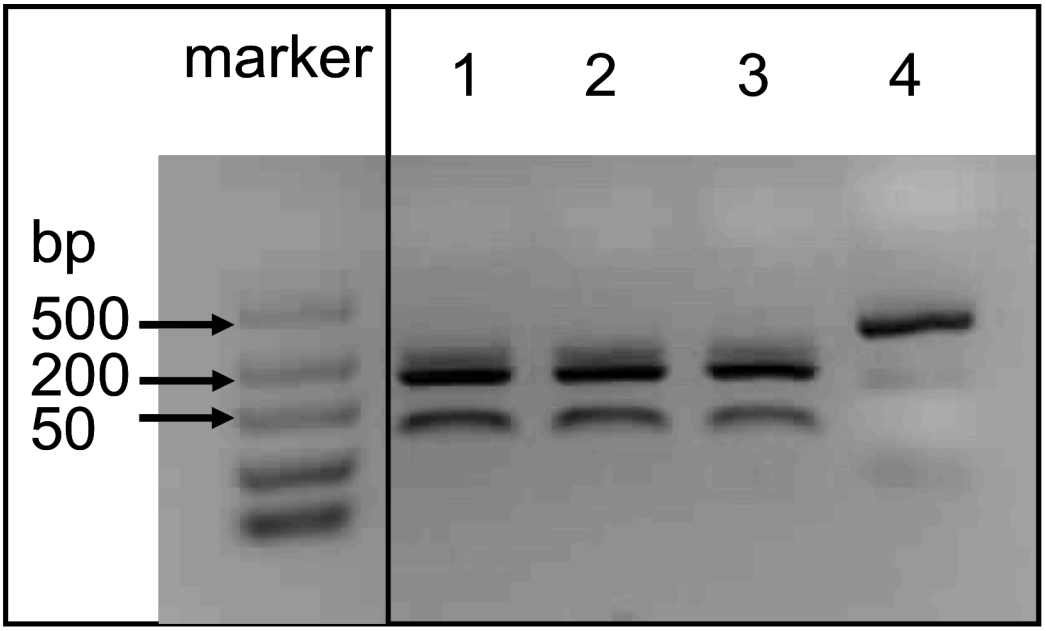

Figure 3 Restriction analysis of amplified fragment of Gos1S1 gene. 1 – pollen pellet sample; 2 – *Castanea sativa* pollen sample 1; 3 – *Castanea sativa* pollen sample 2; 4 – unrestricted fragment of pollen pellet sample.

The specific amplification of one fragment by PCR followed by gel electrophoresis for fragment size verification is the simplest strategy to evaluate the presence of a species **(Mafra et al., 2000)**. In the standart PCR a single internal primer mismatch does not affect the PCR results seriously when other PCR conditions are optimized **(Kwok et al., 1990; Christopherson et al., 1997)**. But chosen of the perfectly matched primers is one of the crutial step in pollen DNA analysis, as the amount of template DNA can be extremely low and even a small mismatch can cause failing of the PCR **(Ito et al., 2008)**. For *Chenopodiace* **Zhou et al., (2007)** used internal transcribed spacer sequence (ITS) for DNA based identification of the pollen in the soil samples on the level of species. Here, using a bioinformatic alignment, specific primers were possible to be designed for the purposes of *Castanea sativa* identification in the DNA targed sequences extracted from pollen samples.

The specifity of amplified fragments of the galactinol synthase (GolS1) gene of Castanea sativa was performed using the Hpy 166II restriction endonuclease. In total, three restriction fragments were obtained with the length

that corresponded to those of predicted by NEBcutter software (figure 3).

Nowadays, molecular and DNA based identification of different, not only plant species is a well-established method **(Kántor et al., 2014; Židek et al., 2012)**. Molecular analysis that utilized the knowledge based on molecular genetics became an inevitable part of the research in many areas. Plant genome analysis are performed in a wide range of different approaches ranged from DNA markers based analysis **(Petrovičová et al., 2015; Oslovičová et al., 2014; Trebichalský et al., 2013; Milella et al., 2011)** up to the specific analysis of plant allergens detection or their expression **(Revák et al., 2014; Ražná et al., 2014)**.

Pollen and pollen pellet samples are in the centre of scientific research in a wide range of different analysis – from antioxidant **(Fatrcová-Šramková et al., 2013)** through protein **(Longhi et al., 2009; Bryce et al., 2010)** and animal food supplements **(Haščík et al., 2014)** up to the allergenic ones **(D'Amato et al., 2007; Alche´, 2012; Ražná et al., 2014)**. Genotyping methods for pollen and single pollen are utilized in many areas including ecology, evolutionary and genomic research **(Isagi et al., 2011)**. In plant population analyses, this technique will allow the estimation of pollen flow by directly tracking the actual movement of individual pollen grains **(Paffetti et al., 2007)**. In genomic studies this technique will enable direct analysis of the haploid DNA sequence of single pollen grains **(Zhou et al., 2007)**.

Here, a PCR based identification of Castanea sativa, Mill. was prooved as effective when used in multifloral pollen pellets.

CONCLUSION

Bioinformatic approach can be used well in pollen DNA based species identification. It provides an efficient method for rapid species specific primers designing that work in PCR reliable. *Castanea sativa* pollen can be detected in complex pollen pellets samples by specific PCR amplification of galactinol synthase. Such as identification of *Castanea sativa* galactinol synthase is efficient in multifloral pollen pellet for *Castanea sativa* authentification purposes.

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