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A REVISED ITS NUCLEOTIDE SEQUENCE GIVES A SPECIFITY FOR *Smallanthus sonchifolius* (POEPP. AND ENDL.) AND ITS PRODUCTS IDENTIFICATION

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Yacon (*Smallanthus sonchifolius)* is an Andean crop which is very regarded for its benefits for people suffering from diabetes or various digestive or renal disorders. Because no specific *Smallanthus sonchifolius* identification DNA markers are still known the paper demonstrates ITS regions to be able to detect and differentiate among yacon species and the potential for specific food authentification purposes is reported, too. The newly sequenced ITS of yacon accessions originated in Peru, Ecuador and Bolivia analyse provide the unique sequence site that differs from all of the other yacon species and is recognized by DraIII restriction endonuclease. Restriction cleavadge of the PCR amplified ITSs of the twenty-eight yacon accessions was performed and in all cases the recognition site was confirmed as a typical for *Smallanthus sonchifolius* . Based on the nucleotide specifity of *Smallanthus sonchifolius*, ITS sequence the PCR method combined with the restriction clevadge protocol was developed for yacon identification.

Key words: Smallantus sonchifolius (Poepp. and Endl.) H. Robinson, yacon, ITS, Dra III digestion, PCR

INTRODUCTION

Yacon - *Smallantus sonchifolius* (Poepp. and Endl.) H. Robinson; (*Asteraceae*), originally cultivated in South America was considered by the early Andean inhabitants as a fruit and it has a relatively low energy value despite its juiciness and sweet taste. In South America, Bolivia, Brazil and Argentina, yacon roots and leaves are commonly consumed by people suffering from diabetes or various digestive or renal disorders and this ethnobotanical use was

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confirmed by recent scientific research (AYBAR *et al.* 2001; SIMONOVSKA *et al.* 2003). Recently, the interest in this crop has increased due to its good post-harvest life if managed properly (OHYAMA *et al.* 1990), exceptional qualities for low-calorie diets thanks to its abundant content of fructooligosaccharides that humans cannot digest in the colon, the absence of starch and medicinal properties (INOUE *et al.* 1995; AYBAR *et al.* 2001). GRAU and REA (1997) have summarized nowadays use of yacon as follows - in Bolivia, yacon is commonly consumed by diabetics and persons suffering from digestive problems. Properties to treat kidney problems and skin-rejuvenating activity also have been mentioned. Antidiabetic properties have been attributed to yacon leaves (KAKIHARA *et al.* 1996) in Brazil, where the dried leaves are used to prepare a medicinal tea. Dried yacon leaves are used in Japan, mixed with common tea leaves. Hypoglycemic activity has been demonstrated in the water extract of dried yacon leaves, feeding rats with induced diabetes (VOLPATO *et al.* 1997).

Yacon can be processed in different ways. The juice obtained from pressing the tuberous roots can be boiled and concentrated to produce solid dark-brown blocks called chancaca (NATIONAL RESEARCH COUNCIL 1989), similar to the product obtained from concentrating sugarcane juice. The juice also can be concentrated at low pressure, with the addition of sodium bisulphate to inhibit enzymatic darkening. The final product is a dense syrup similar to sugarcane syrup but with significantly lower energy value for humans (CHAQUILLA 1997). Another promising processing technique is the production of dry chips. In this case yacon tuberous roots are peeled and cut in thin slices. The slices are first dried in a plastic tunnel, then oven-dried at 60°C (KAKIHARA *et al.* 1996).

As in other very specific plants used for food or foodstuff production, for yacon, too, is very important to establish that the declared yacon compositions are not substitute, partial or entirely different species. MAFRA *et al*. (2007) rewiewed the analytical methods used for species identification and authenticity of foods as relying mainly on protein and DNA analysis. The protein-based methods include immunological assays (ZELEŇÁKOVÁ *et al*., 2010, 2011) and electrophoretical and chromatographic techniques (SOCACCIU *et al.,* 2009). More recently, DNA molecules have been the target compounds for species identification due to the high stability compared with the proteins, and also to their presence in most biological tissues, making them the molecules of choice for differentiation and identification of components in foods, and a good alternative to protein analysis (LOCKEY *et al*., 2000; VRÁBLIK *et al.,* 2012). Most DNA-based methods for species identification in foods consist on the highly specific amplification of one or more DNA fragments by means of polymerase chain reaction (PCR). This technique presents a high potential due to its fastness, simplicity, sensibility and specificity (WISEMAN, 2002; BAJZÍK *et al.,* 2010, HANÁK *et al*., 2012).

In spite of advancements in yacon morphological characterizations, the genetic diversity of the crop in molecular terms is still unknown (MANSILLA *et al*., 2006). Yacon belongs to the organisms where only a very limited information about the genome sequences are known (MILLELA *et al.,* 2005, MILLELA *et al*., 2011, ŽIAROVSKÁ *et al*., 2011). Because of this limited knowledge about the concrete DNA sequences, ITS (internal transcribed spacer) was chosen for PCR based *Smallanthus sonchifolius* effective identification. Nuclear rDNA are grouped into arrays consisting of hundreds to thousands of tandem repeats. This region includes two spacers – ITS1 and ITS2, that separate the SSU (small subunit, 18S), 5,8S and LSU (large subunit, 26S) of nuclear ribosomes genes (figure 1).

The aim of the study was to developed an ITS based method for *Smallanthus sonchifolius*, (Poepp. and Endl.) H. Robinson as a marker for PCR and restriction digestion protocol of yacon idenetification in a manner suitable for routine identification and authentification analyses.

MATERIALS AND METHODS

Plant Material

Six genotypes of *Smallanthus sonchifolius*, (Poepp. et Endl.) were chosen from the Institut of Tropics and Subtropics of the Czech university of Life Sciences for ITS sequencing and a collection of twenty-eight yacon landraces taken from the same institute were prooved by restriction digestion for the presence of the yacon ITS specific restriction site. Three Peruvian landraces (PER09, PER09 and PER11) and three Bolivian landraces (BOL20, BOL22, BOL24) were used in ITS sequence analysis. Plants were grown in field conditions on the experimental base of the Department of the Genetics and Plant Breeding, Slovak University of Agriculture. For the purpose of molecular analyses the parts of the leaves without insect or another damages were chosen.

DNA extraction and quantification

Young yacon leaves were homogenized in liquid nitrogen in a mortal and total genomic DNA was extracted by GeneJET™ Plant Genomic DNA Purification Mini Kit (ThermoScientific). DNA purification from lignified and polyphenol-rich plant tissues was used according the instructions of manufacturer. For quantity setting of the extracted DNA was used Nanodrop Nanophotometer™. Determination of DNA quality was done by agarose gel electrophoresis on 1,5 % agarose gel in 1xTBE buffer coloured by GelRed™.

PCR identification of ITS

Primers used for amplification of the region between large and small subunits of RNA (Figure 1) were those used firstly by WHITE et al. (1990). The sequence of forward primer is 5´ tccgtaggtgaacctgcgg 3´ and the sequence for revers primer is 5´ tcctccgcttattgatatgc 3´. PCRs were performed in 1X buffer containing 20 mM $MgCl_2$ and 0,2 mM dNTPs with 200nM forward and revers primer and 1U of Dream Taq Green DNA polymerase (Thermo Scientific) in a volume of 15 µl. The cycling profile was as follows - 95 °C; 3 min [95 °C; 40 sec - 55 °C; 40 sec, - 72 °C; 40 sec] 33x, 72 °C; 7 min. Primers for PCR identification of yacon ITS were synthetized by K-Trade.

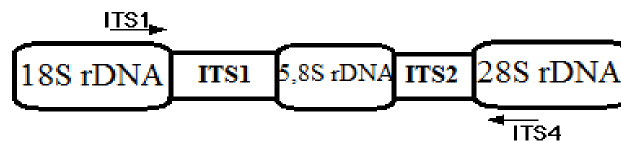

Figure 1. Localization of primers for PCR identification of ITS region. ITS1 – the forward primer used in this study; ITS4 – the reverse primer used in this study.

Sequencing of ITS

After the visual control of the PCR specifity in the 1,5 % agarose gel, the PCR product cleaning was performed to remove proteins, primers, primer-dimers and dNTPs. The procedure was performed by Agencourt AMPure XP solid-phase paramagnetic bead purification system (Beckman Coulter) following the manufacturer´s instructions.

Sequencing PCR reactions were designed for the molar ratio of primer to template of ~ 40:1 using the Dye Terminator Cycle Sequencing Genome Lab™ Kit (Beckman Coulter). The thernal cycling programm was as follows: 96 °C 20 sec; 50 °C - 20 sec and final extension 60 °C - 4 minutes. Purification of sequencing PCR was performed by Agencourt® CleanSEQ® solid phase reversible immobilization system (Beckman Coulter) following the manufacturer´s instructions. Sequencing of analysed landraces ITS was performed in CEQ ™ - Genetic Analyses System using the LFR-1 protocol. All the direct sequencing reactions were repeated twice.

BLAST alignment of sequenced products and restriction digest specifity designing

ITS sequences of analysed landraces obtained by direct seguencing were compared using the BLAST search to the ITS sequence of yacon existing in the NCBI (National Center for Biotechnology Information, Bethesda, Maryland, USA) under the accession number AF465902. The obtained ITS sequences of six analysed landraces were used as query and in all of them the difference within the nucleotides 234-235 was found.

For restriction cleavage of the ITS site that showed the difference the sequence was screened for restriction digestion in NEBcutter V2.0 software (Figure 2) for finding a restriction endonuclease suitable for fast and effective identification of *Smallanthus sonchifolius* , (Poepp. et Endl.). The Dra III restriction endonuclease was found as suitable for a revised yacon ITS sequence confirming and identification, too (Figure 2).

Figure 2. The cleavage site for Dra III endonuclease that correspond to the 234-235-th nucleotide of the yacon ITS presented in this study showed on the strength of the nucleotides ranged from 180 up to the 239 of the ITS.

Subsequently the alignment of the revised ITS sequence was done by BLATtn against all green plants (taxid:33090) nucleotide sequences in the NCBI database to compare nucleotide difference that was found. A total of 100 hits were found with the maximum identity ranged from 84 up to the 99%. For all of the sequences, the unigue of the Dra III recognized site was confirmed for revised ITS of *Smallanthus sonchifolius*, (Poepp. et Endl.). A selection of the results for others of the *Smallanthus* species is summarized in the Table 1.

Table 1. Comparison of nucleotides 226 – 237 among Smallanthus species and revised ITS of Smallanthus sonchifolius, (Poepp. et Endl.).

Species	Accesion number	Nucleotides of revised *Smallanthus sonchifolius* ITS	Nucleotides of corresponding position
Smallanthus sonchifolius	AF465902		AC**CG**ACAATGTG
Smallanthus microcephalus	AF465896		AC**A**CACAATGTG
Smallanthus jelskii	AF 465893		AC**A**CACAATGTG
Smallanthus pyramidalis	AF 465898		AC**A**CACAATGTG
Smallanthus oaxacanus	AF 465897		AC**A**CACAATGT**T**
Smallanthus siegesbeckius	AF 465901	AC**GC**ACAA**TGTG**	ACGCACAATG**C**G
Smallanthus connatus	AF 465891		ACGCACAATG**C**G
Smallanthus fruticosus	AF 465892		ACGCACAATG**C**G
Smallanthus maculatus	AF 465894		AC**A**CACAATG**C**G
Smallanthus uvedalius	AF 465903		ACGC**R**CAATG**C**G
Smallanthus riparius	AF 465900		AC**A**CACAATG**C**G
Smallanthus meridensis	AF 465895		AC**A**CACAATG**C**G
Smallanthus quichensis	AF 465899		AC**A**CACAATG**C**G

R=A or G

Confirmation of the reported difference of the revised yacon ITS to those in the NCBI database was done by Dra III restriction endonuclease (New England Biolabs® Inc.) for the germplasm of twenty-eight yacon landraces using the following restriction protocol - 10 µl of PCR product, 3 µl of 10NEB4 buffer, 0,5 µl of DRA III enzyme and 16,5 µl water. Restriction digestion was performed in a total volume of 20 µl.

RESULTS AND DISCUSSION

The internal transcribed spacer (ITS) is intercalated in the 16S-5.8S-26S region separating the elements of the rDNA locus (Fig. 1). The ITS region consists of three parts: the ITS1 and ITS2 and the highly conserved 5.8S rDNA exon located in between. The total length of this region varies between 500 and 750 bp in angiosperms while in other seed plants it can be much longer, up to 1,500–3,500 bp (POCZAI and HYVŐNEN, 2010).

In this study reported nucleotide specifity of *Smallanthus sonchifolius*, (Poepp. et Endl.) provides a base for simple and effective PCR combined with restriction digestion identitification of *Smallanthus sonchifolius,* (Poepp. et Endl.) in the cases where the authentification of the product is needed.

Primers used for amplification of the region between large and small subunits of RNA (Figure 1) are routinelly used in analysis of phylogenetic history and molecular systematics at the species level, and even within species (WHITE *et al,* 1990, SMOLIK *et al,* 2011a). Research on determination of phylogenetic relationships between taxons is focused mainly on amplification of the ITS region and sequencing of the obtained product. The bioinformatic analysis of the

obtained sequences enables to draw conclusions about phylogenetic relationships between subjects (SMOLIK *et al,* 2011 b). But not only as a phylogenetic marker can ITS be used. PAVÓN *et al.* (2011) has reported study, where ITS was used as a marker of fungal contaminats what confirm the effectivess of ITS for the food oriented research.

Here, we report the great potential of bioinformatic tool for easy and effective searching for differences that can be directly used for specific foodstuff plant compounds identification or confirmation. For the purpose of the ITS region amplification the previous reported pair of primers (WHITE *et al.*, 1990) was used. After optimization of PCR reaction conditions it was obtained specific monomorphic fragment with an approximate size of 650 bp. The size of the amplified product corresponds to the size of the ITS yacon region previously registered in the NCBI database (AF465902). Amplicons were run on 1.5% agarose gel (Figure 3) then eluted and sequenced as described above.

Broadening the effectiveness of ITS regions by PCR is based on multicopy structure. Of equal importance, the ITS regions (ITS1 +5.8 S + ITS2) is the product of an approximate length of 700 bp, which is optimal for the specific determination of the chemicals and thermal parameters for PCR reaction. According to many authors ITS regions, thanks to their variability, gave sufficient molecular information, which can be directly used as identifiers or molecular markers in phylogenetic analyses (ALVAREZ and WENDEL, 2003, NALINI *et al.*, 2007, SMOLIK, 2011a).

Figure 3. PCR amplification products of BOL20, BOL22 and BOL24 ITS regions.

All the obtained sequences were BLASTed against the ITS sequence of *Smallanthus sonchifolius* region previously registered in the NCBI database (AF465902). In all sequences was confirmed differrencies in nucleotides 163-164 and 235-236 where instead of A163-C164-GC.. reported in previously registered region was found ..ACGGC.. and instead of ..GT235-C236-GGT was found a sequence of .GTGCGT... (Figure 4). When screened for restriction sites of the newly sequenced ITS of yacon, the ..GTGCGT.. site was found as an end of the recognition site for DraIII restriction endonuclease (Figure 2). The presence of the GC bases in the 235-236 nt instead of CG bases provides a specific site for restriction cleavadge by DraIII and can be used when *Smallanthus sonchifolius*, (Poepp. et Endl.) confirmation is needed, because none of the other ITSs of Smallanthus species has the DraIII recognition site here.

Restriction cleavadge of the PCR amplified ITSs of the twenty-eight yacon accessions from the Institut of Tropics and Subtropics of the Czech university of Life Sciences was performed subsequently and in all cases the recognition site was confirmed by visualisation of

two DNA fragments in agarose electrophoresis. The first fragment was of the lenght of approximately 237 bp and the second of about 400 bp confirm the presence of the CACNNNGTG cleavage site of the ITS, that is specific for *Smallanthus sonchifolius*, (Poepp. et Endl.).

The main advantage of species identification by genetic markers is the relative stability of the genotype rather than the phenotype. Nucleic acids and proteins act as a fingerprint of a species, allowing a more accurate identification. DNA has all the characteristics of an ideal molecular marker - is a relatively stable molecule; is present in all living organisms (except some viruses); contains genetic information; possess a semi-conservative structure of a double helix with complementary pairing of nucleotides and can be easily manipulated.

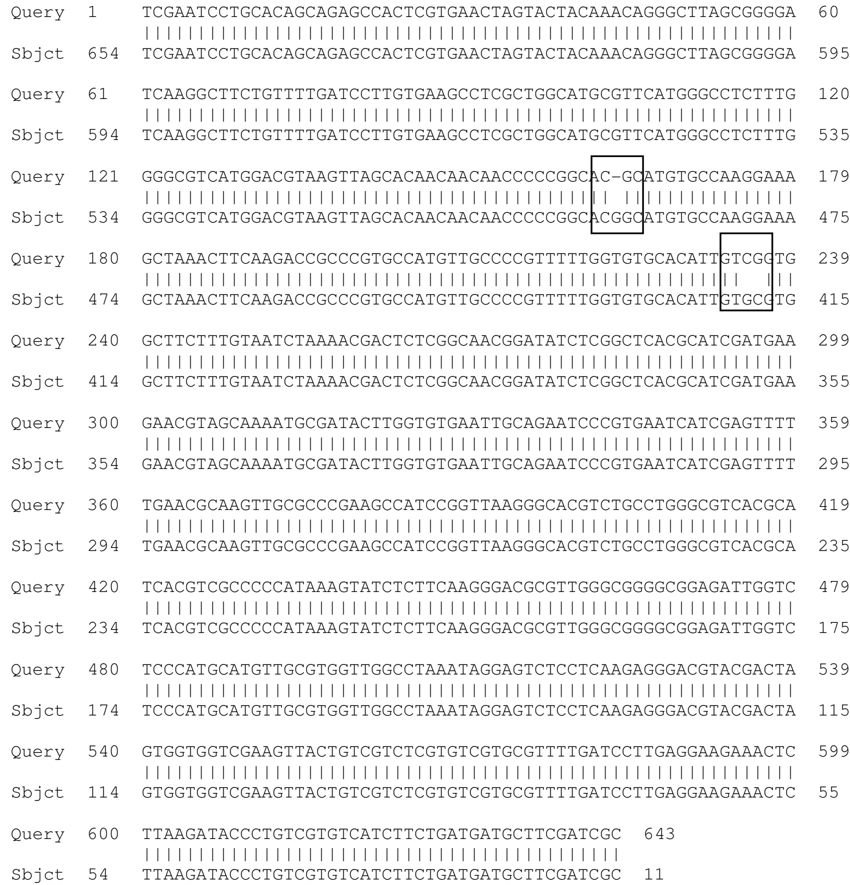

Figure 4. Differences of yacon ITS when comparing AF465902 sequence in NCBI database (query) and reverse primer sequenced ITS of PER11 landrace (sbjct).

Internal transcribed spacers was used as a marker of fungal contaminats by PAVÓN *et al.* (2011). As the *Alternaria* is considered one of the most important fungal contaminants of vegetables, fruits, and cereals, the effective identification is needed. Conventional methods for *Alternaria* identification and enumeration are laborious and time-consuming, and they might not detect toxigenic molds inactivated by food processing. PAVÓN *et al.* (2011) dveloped a PCR method for the rapid identification of *Alternaria* spp. DNA in foodstuffs, based on oligonucleotide primers targeting the internal transcribed spacer (ITS) 1 and ITS2 regions of the rRNA gene. The applicability of the method for detection of *Alternaria* spp. DNA in foodstuffs was assessed by testing of 100% spoiled tomato samples, 8% of tomato products, and 36.4% of cereal-based infant food samples analyzed.

CONCLUSION

Using of well defined DNA based markers in species identification is a very useful tool for PCR and restiction clevadge based methods for food and foodstufs analyses. Developing this type of methods can result in a high and accurate determination of tested or analysed genomes characteristics and their identification directly through the nucleotide sequence level instead the whole genome. ITS regions demonstrated to be able to detect and differentiate among species with the potential for specific food authentification purposes. Based on the reported *Smallanthus sonchifolius*, (Poepp. et Endl.) ITS sequence specifity the PCR method combined with the restriction clevadge was developed for yacon identification.

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IZMENJENA ITS SEKVENCA NUKLEOTIDA DAJE SPECIFIČNOST ZA *Smallanthus sonchifolius* (Poepp. and Endl.) I IDNETIFIKACIJU PRODUKTA

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Izvod

Smallanthus sonchifolius, biljka koja uspeva na Andima je vrlo značajna za ishranu ljudi koji boluju od šećerne bolesti i različitih poremećaja varenja i renalnih problema. Kako još uvek ne postoje DNK marker za identifikaciju genotipa u radu su prikazani ITS (Internal Transcribed Spacer) regioni koji mogu kako da detektuju i utvrde razlike između *Smallanthus sonchifolius* vrsta kao id a se koriste u autentifikaciji hrane za specifične namene. Analizom novih genotipova, identifikovani korišćenjem nove izmenjene sekvencionirane ITS sekvence, koji potiču iz Perua, Ekvadora i Bolivije dobijeno je unikatnao mesto sekvence koje se razlikuje od svih drugih *Smallanthus sonchifolius* vrsta a utvr]eno je korišćenjem Dra III restrikcione endonukleaze. Izvršena je digestija ITS fragmenta iz dvadeset osam vrsta i njihovo umnožavanje PCR metodom i u svim slučajevima je utvrđeno da je prepoznato mesto tipično za *Smallanthus sonchifolius.* Na osnovu dobijenih podataka o nukleotidnoj specifičnosti ITS sekvence, razvijen je protok za identifikaciju *Smallanthus sonchifolius* i proizvoda dobijenih od tog genotipa.

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