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Short Communication

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Difference on ITS regions among Yacon genotypes and *Smallanthus* spp.

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Abstract

Yacon (*Smallanthus sonchifolius*) is an Andean crop which is very regarded for its medicinal properties. Yacon genetic profiles have been slightly studied using molecular markers. The aim of this study was to prepare the protocol for direct sequencing of the ITS (Internal Transcribed Spacers) regions of yacon DNA genome. The identified ITS regions in three Bolivian yacon's landraces (BOL 20, BOL 22 and BOL 24) were sequenced and then compared with ITS regions found in NCBI database, previously isolated in yacon and in other plants belonging to the genus *Smallanthus*. The analysed ITS regions of studied Bolivian landraces showed differences in the nucleotides 163-164 and 235-236 with the sequence previously isolated in yacon. A dendrogram was constructed by comparing the sequence presented in the manuscript and those in the NCBI database for *Smallanthus*, spp. In the dendrogram, the previously reported ITS sequence of *Smallanthus sonchifolius* was also used. Specific sites of *Smallanthus sonchifolius* ITS region gives a promising base for molecular approach identification of this underutilized crop.

Key words: ITS region, Landraces, Molecular markers, DNA sequence, Yacon

Introduction

Yacon [*Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson - Asteraceae] is a perennial herb, originating in the Andean region. Recently, greater attention has been focused on this plant due to its agronomical, nutritional and pharmacological characteristics (Valentová et al., 2001; Milella et al., 2011). This plant produces tuberous roots rich in inulin. Tubers are consumed as fresh fruit and have a pleasant sweet taste (Manrique et al., 2004) and they are also an excellent natural source of fructo-oligosaccharides (FOS), which can replace sucrose as sweetener for their low caloric value. In different yacon genotypes FOS content can sensibly vary, which offers the possibility of selection and their use for breeding purposes to obtain genotypes with high FOS content (Ishiki et al., 2000). Yacon roots accumulate about 10% of inulin-type FOS, which is characterized as a dietary supplement with positive health effects (Narai-Kanayama et al., 2007). For the assessment of genetic diversity molecular markers have been generally superior to morphological traits, and biochemical markers (Melchinger et al., 1991). Yacon leaf showed also several interesting pharmacological activities. In yacon leaves several compounds as sonchifolin, polymatin A and B, uvedalin, polyphenols and enhydrin were already isolated (Goto et al., 1995; De Pedro et al., 2003). These compounds possess antifungal and anti-inflammatory activities (Valentová et al., 2001; Hong et al., 2008). Yacon tubers also may have potential as a diet food. The human body has no enzyme to hydrolyze inulin, so it passes through the digestive tract unmetabolized, which means that yacon provides few calories. This could be an attractive marketing feature to dieters and diabetics (National Research Council 1989). In fact 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 ethno-botanical use was confirmed by recent scientific research (Valentova et al., 2006).

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Yacon was originally cultivated in South America and now has been introduced in New Zealand, Japan, Italy, Germany and the Czech Republic. The productivity and other valuable agronomic traits of yacon strongly suggest that it is a species with a great potential, moreover the cultivation of this plant needs almost no pesticides (Lin et al., 2003).

During yacon evolution, continued vegetative propagation and selection for root yield may have impaired flowering and fruit set. Although yacon is a clonal crop, it shows some morphological and physiological variations. However, this variation may reflect the phenotypic differences expressed due to its growing conditions, rather than genetic variation. On the other hand it is very difficult to differentiate some yacon landraces from a wide geographical range, from Ecuador to Argentina, when they were grown in the same environment (Fernandez et al., 2006; Lebeda et al., 2012).

At the same time molecular markers demonstrated to be useful, in several crops, in order to investigate their genome structure (Milella et al., 2006; Ovesna et al., 2013), germplasm characterization (Garcia-Mas et al., 2000; Žiarovská et al., 2012), or to define crossing combinations and cultivar identification (Karp et al., 1996). Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Karp et al., 1996). Molecular Marker based Genetic Diversity Analysis (MMGDA) also has potential for assessing changes in genetic diversity over time and space. Nowadays, molecular research activities connected to yacon germplasm are focused on the development of different mapping techniques for its genomic characterization. Yacon intraspecific variability was characterized using isozyme polymorphism method and also using some DNA markers. Unlike the morphological and biochemical analysis, molecular characterization of yacon showed to be more efficient (Milella et al., 2011). In fact molecular markers as random amplified polymorphic DNA (RAPD) and amplified fragment length (AFLP) were presented as suitable methods for the analysis of intraspecific genetic variability in *Smallanthus sonchifolius* (Mansilla et al., 2006; Milella et al., 2007). Both methods demonstrated their ability in differentiating among analyzed genotypes and their relationships with yacon phenolic content (Milella et al., 2011). It was demonstrated that phenolic content depends, in this case, not on environmental condition but rather on the genome of a particular landrace (Milella et al., 2011). Svobodová et al. (2011) characterized 14 cultivars native yacon from Peru, where certain polymorphic loci showed a close genetic distance between genotypes. These results demonstrated as molecular markers play an important role in breeding, cultivation and industrial use purposes.

No studies at the moment have investigated Internal Transcribed Spacers (ITS) sequence variation in yacon. The purpose of this study was to develop a protocol for the identification and direct sequencing of ITS regions in yacon genomic DNAs and to compare obtained sequences with those previously reported for *Smallanthus* spp.

Material and Methods

DNA extraction

Three different landraces of yacon [*Smallanthus sonchifolius,* (Poepp. et Endl.) H. Robinson; *Asteraceae*] named BOL 20, BOL 22 and BOL 24, were collected in Bolivia and selected for their different morphological traits. BOL 20 was collected in San Pedro area (2800 m. - Potosí-Bolivia), BOL 22 was collected in Tuquiza (2900 m - Potosí - Bolivia) and BOL 24 was collected in El Locotal (2 500 m- Cochabamba - Bolivia) (Fernández et al., 2005, 2006). The 3 genotypes were then grown under the same environmental conditions in the Czech Republic (Institute of Tropics and Subtropics of the Czech University of Life Sciences in Prague) after vegetative propagation, were their germplasm is deposited.

Genomic DNA was extracted from approximately 500 mg of young leaf tissue. Leaf tissue was ground to a fine powder in liquid nitrogen. Total genomic DNA was extracted using a protocol described by Milella et al. (2011). DNA concentrations were estimated by spectrophotometric assay and then diluted to a final concentration of 25 ng/ul.

PCR reaction and sequencing

Primers were selected for their putative amplification of the DNA sequences that codify for small (18S, **SSU**) and large subunit (28S, **LSU**) ribosomal RNA (rRNA). Primers were selected for their previous use, they are considered as standard primers for the amplification of these regions in various species of fungi and higher plants (White et al. 1990; Gardens and Burns, 1993; Smolik et al. 2011a). Several primer combinations have been selected but only one gave the expected results in the direct sequencing (FWD ITS1 nucleotide sequence 5 '- tccgtaggtgaacctgcgg - 3' REV ITS2 5 '- tcctccgcttattgatatgc - 3'). The polymerase chain reaction (PCR) amplifications of ITS regions were

carried out in an MJ Research PTC-200 thermal cycler under the following conditions: 1X buffer solution containing $MgCl_2$ (2 mM), dNTP (0.2 mM), sense and antisense primer (200 nM) and 1U Taq Green Dream DNA polymerase (Thermo Scientific) in a volume of 15 µl. Following cycling conditions were used: 95°C, 3 min; [95°C, 40 sec; 55°C, 40 sec; 72°C, 40 sec] 33x; 72°C, 7 min. PCR products were run on agarose gel (1.5%), PCR products were purified with Agencourt ® kit Ampure ® XP (Beckman Coulter) according to the producer's instructions. The sequencing reaction took place under the following conditions: 96°C - 20 s, 50°C - 20 s and 60°C 4 min. Free nucleotides purification was performed with Agencourt ® CleanSEQ ® kit (Beckman Coulter) according to the manufacturer instructions. The ITS region sequencing was carried out on the CEQ TM Genetic Analyses System (Beckman Coulter, Inc. Fullerton, California) under standard conditions.

Dendrogram construction

Dendrogram was constructed by hierarchical clustering of selected ITS sequences of *Smallanthus*, spp. and newly sequences one. For the dendrogram construction, Neighbor Joining method was used and the construction was performed fully withing a NCBI software.

Results and Discussion

Currently ITS regions and their sequences have broad application in molecular systematic (molecular phylogenetics) of fungi, to determine relationships among species or within a species, as well as in plants. For ITS region amplification of our DNAs were chosen primer pairs considered as reference and very effective for molecular analysis in previous studies (Alvarez and Wendel, 2003; Feng et al., 2007; Smolik et al., 2011b). 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 1) then eluted and sequenced as described above.

In total, 3 sequences for each of the accession were obtained in 3 repeated reactions. Any of the sequences didn't show nucleotide difference among them. On the other hand their comparison with *Smallanthus sonchifolius* ITS region previously deposited in the NCBI database (AF465902) showed differences in two nucleotides. More in detail a specific variability in nucleotide 163-164 and 235-236 (Figure 2) was observed. In the data source AF465902 sequence, it is not mentioned the country of origin of sequenced yacon variety but it showed to be different from our germplasm.

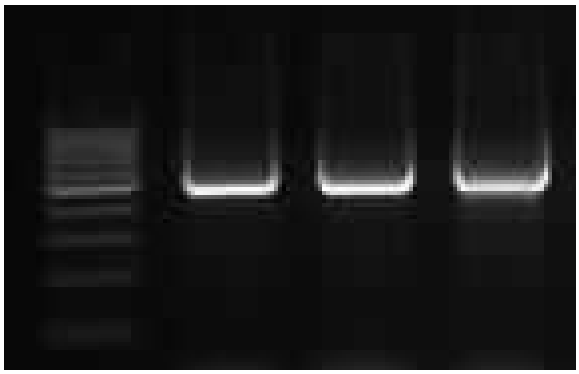

Figure 1. PCR amplification products of ITS regions.

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 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). In our case the ITS products were not sufficient for differentiation among analyzed genotypes but they resulted as a useful tool for differentiation between bolivian yacon genotypes and the one present in NCBI database. Sequences obtained in our study were also compared with other sequences deposited in NCBI database and obtained from other plant species belonging to the genus *Smallanthus* (Figure 3). For comparison purposes registered NCBI database sequences were used and their sequences were compared with the BLAST algorithm. The dendrogram obtained from BLAST analysis depicting ITS region sequence comparison within the same genus is shown in Figure 3.

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Query  1    TCGAATCCTGCACAGCAGAGCCACTCGTGAACTAGTACTACAAACAGGGCTTAGCGGGGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654  TCGAATCCTGCACAGCAGAGCCACTCGTGAACTAGTACTACAAACAGGGCTTAGCGGGGA  595

Query  61   TCAAGGCTTCTGTTTTGATCCTTGTGAAGCCTCGCTGGCATGCGTTCATGGGCCTCTTTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  TCAAGGCTTCTGTTTTGATCCTTGTGAAGCCTCGCTGGCATGCGTTCATGGGCCTCTTTG  535

Query  121  GGGCGTCATGGACGTAAGTTAGCACAACAACAACCCCCGGCAC-GCATGTGCCAAGGAAA  179
            |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||
Sbjct  534  GGGCGTCATGGACGTAAGTTAGCACAACAACAACCCCCGGCACGGCATGTGCCAAGGAAA  475

Query  180  GCTAAACTTCAAGACCGCCCGTGCCATGTTGCCCCGTTTTTGGTGTGCACATTGTCGGTG  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  474  GCTAAACTTCAAGACCGCCCGTGCCATGTTGCCCCGTTTTTGGTGTGCACATTGTGCGTG  415

Query  240  GCTTCTTTGTAATCTAAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  GCTTCTTTGTAATCTAAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAA  355

Query  300  GAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAATCATCGAGTTTT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAATCATCGAGTTTT  295

Query  360  TGAACGCAAGTTGCGCCCGAAGCCATCCGGTTAAGGGCACGTCTGCCTGGGCGTCACGCA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  TGAACGCAAGTTGCGCCCGAAGCCATCCGGTTAAGGGCACGTCTGCCTGGGCGTCACGCA  235

Query  420  TCACGTCGCCCCCATAAAGTATCTCTTCAAGGGACGCGTTGGGCGGGGCGGAGATTGGTC  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234  TCACGTCGCCCCCATAAAGTATCTCTTCAAGGGACGCGTTGGGCGGGGCGGAGATTGGTC  175

Query  480  TCCCATGCATGTTGCGTGGTTGGCCTAAATAGGAGTCTCCTCAAGAGGGACGTACGACTA  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  TCCCATGCATGTTGCGTGGTTGGCCTAAATAGGAGTCTCCTCAAGAGGGACGTACGACTA  115

Query  540  GTGGTGGTCGAAGTTACTGTCGTCTCGTGTCGTGCGTTTTGATCCTTGAGGAAGAAACTC  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114  GTGGTGGTCGAAGTTACTGTCGTCTCGTGTCGTGCGTTTTGATCCTTGAGGAAGAAACTC  55

Query  600  TTAAGATACCCTGTCGTGTCATCTTCTGATGATGCTTCGATCGC  643
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  54   TTAAGATACCCTGTCGTGTCATCTTCTGATGATGCTTCGATCGC  11
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Figure 2. ITS sequence variation between ITS sequences of genotypes analyses and NCBI sequence AF465902.

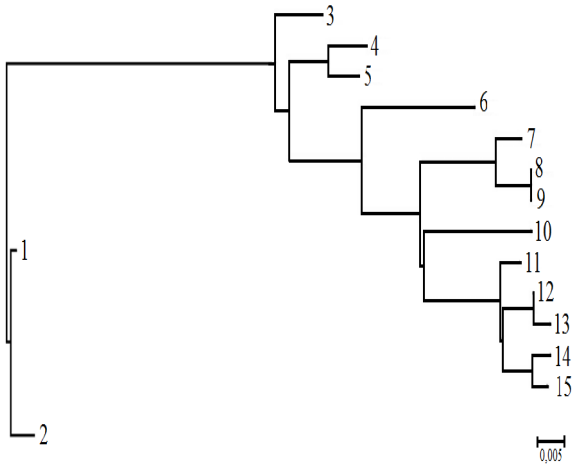

Figure 3. Dendrogram displaying the similarity of ITS sequences obtained from the native cultivars BOL 20 BOL BOL 22 and 24 with gender ITS sequences *Smallanthus*.
Coding ITS region of: 1 *S. sonchifolius* – (sequenced in this study), 2 - *S. sonchifolius* X; 3 - *S. microcephalus*; 4 - *S. jelskii*; 5 - *S. pyramidalis*; 6 - *S. oaxacanus*; 7 - *S. siegesbeckius*; 8 - *S. connatus*; 9 - *S. fruticosus*; 10 - *S. uvedalius*; 11 - *S. maculatus*; 12 - *S. riparius*, 13 - *S. meridensis*; 14 - *S. maculatus*; 15 - *S. quichensis*. Sequences from 2 till 15 were obtained from NCBI database.

The 100% similarity of our ITS sequences (yacon cultivars native from Bolivia) was compared with the existing ITS *Smallanthus sonchifolius*, the homology showed to be 99%. On the other hand the homology with other ITS sequences from 13 species of the genus *Smallanthus* showed to be lowerer, from 92 to 97%. Baldwin et al. (1995) indicate as base substitution ITS regions are a frequent source of polymorphism in a sequence. Results obtained in the yacon ITS region sequencing confirms data previously reported by Baldwin et al. (1995), but for our genotype. On the other hand it is possible to underline as knowledge of nucleotide differences in ITS regions could represent a wider possibility of differentiation and identification of species with a fast and easy PCR screening.

Conclusions

Direct sequencing of PCR products currently represents a useful tool in molecular genetics that present advance in the analysis of anonymous markers. It can result in a high and accurate determination of plant genome characteristics directly at nucleotide sequence level instead their whole genome. ITS regions demonstrated to be able to detect and differentiate among species and also between our germplasm and other germplasm

previously analyzed. The variability in yacon ITS sequences compared with other ITS sequence of plants belonging to the genus *Smallanthus* opens the possibility of a more focused and accurate molecular analysis directed to intraspecific and interspecific *Smallanthus* spp. characterization.

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