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Genomics for authentication of plant food supplements: the case of artichoke (*Cynara scolymus*)

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ABSTRACT

Artichoke (*Cynara scolymus* L.) is a medicinal plant frequently included in herbal infusions and plant food supplements (PFS) marketed for weight-loss. In both types of products, *C. scolymus* can be substituted by other plants, either due to deliberate adulteration or to the accidental swap of plants owing to misidentification. In this work, a molecular approach based on real-time PCR coupled to high resolution melting (HRM) analysis to discriminate *C. scolymus* from other *Cynara* species was developed and applied to herbal infusions and PFS labelled as containing artichoke. All herbal infusions and three PFS were positive for the presence of *Cynara* spp. based on the qualitative PCR assay. The application of HRM analysis confirmed the unequivocal presence of *C. scolymus* with high level of confidence (>98.8%) in the tested samples. To our knowledge, this is the first successful attempt for the rapid discrimination of *C. scolymus* in PFS.

1. INTRODUCTION

The consumption of plant food supplements (PFS), especially those marketed for weight-loss, has been growing since they are generally considered as being "natural" and safe. PFS are legally considered as foods under EU Directive 2002/46/EC [1], thus they are not submitted to any safety assessment prior to commercialisation, being more susceptible to adulteration issues. Possible botanical adulterations include the accidental swap of plants or deliberate substitution of high value plant material by other species of lower cost. Therefore, to ensure consumer's safety, the development of analytical methods for plant species identification in complex matrices has become crucial. For its high specificity, DNA-based methods have been reported as the most adequate tools for plant authentication [2]. Moreover, DNA molecules are very stable, not affected by the plant's age or environmental factors, in opposition to chemical markers.

Considering that artichoke is frequently used in PFS for weight-loss, the main goal of the present study was to develop a new DNA approach based on high resolution melting (HRM)

analysis to discriminate *C. scolymus* from other *Cynara* spp. in PFS. In view of previous studies reporting that excipients used in PFS can hamper DNA extraction [3], this work also intended to propose a new strategy to release DNA from PFS.

2. MATERIAL AND METHODS

2.1 Sampling and DNA extraction

Voucher specimens of *C. scolymus*, *C. cardunculus*, *C. humilis* and *C. syriaca* were obtained from germplasm banks and botanic gardens, while samples of herbal infusions (n=6) and PFS (n=9) were acquired in specialised stores. PFS included 8 samples labelled as containing artichoke and 1 declaring milk thistle (*Silybum marianum*), a plant species morphologically identical to artichoke.

DNA from plant material and PFS was extracted using the NucleoSpin Plant II kit. For PFS, a pre-treatment with phosphate buffer (pH 8; 15% de ethanol) was performed prior to extraction.

2.2 Qualitative PCR and real-time PCR

Qualitative PCR was carried out in 25 µL of total reaction volume, containing 2 µL of DNA extract, 67 mM of Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Germany), 3.0 mM and 1.5 mM of $MgCl_2$ (for CyC-F1/CyC-R1 and 18SRG-F/18SRG-R, respectively) and 280 nM of each primer CyC-F1/CyC-R1 and 240 nM of each primer 18SRG-F/18SRG-R (Table 1). The reactions were performed in a thermal cycler MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, USA) with a set of temperature specific for each primer.

The amplifications by real-time PCR targeting *Cynara* spp. sequences were carried out in 20 µL of total reaction volume containing 2 µL of DNA extract, 1x of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, USA) and 400 nM of each primer (Table 1). The reactions were performed on a thermal cycler CFX96 Real-Time System (Bio-Rad Laboratories, USA) using the following conditions: 95ºC for 5 min; 45 cycles at 95ºC for 10 s followed by 57ºC for 20 s, with collection of fluorescence signal at the end of each cycle. For HRM analysis, temperature was increased at 0.2ºC each 10 s until 95ºC. Data were collected and processed using the softwares Bio-Rad CFX Manager 3.1 and Precision Melt Analysis 1.2 (Bio-Rad Laboratories, USA).

Table 1. Oligonucleotide primers used in this study.

Species	Primer	Target gene	Sequence 5'- 3'	Amplicon (bp)	GenBank accession no.
Cynara spp.	CyC–F1 CyC–R1	CELMS-57 microsatellite	TCGATGGTAGGATAGTGGCCTACT TGCTGCCTTCCTTGGATGTGGTA	130	EU744973.1
Eukaryotes	EG-F EG-R	18S rRNA	TCGATGGTAGGATAGTGGCCTACT TTGGATGTGGTAGCCGTTTCTCA	109	AF412275

3. RESULTS AND DISCUSSION

After DNA extraction from voucher plants, infusions and PFS, the amplificability of all extracts was tested targeting a universal eukaryotic sequence (18S rRNA gene) (Table1). The extracts from plant material and infusions were all amplified. Regarding PFS, previous studies have shown that some pharmaceutical excipients used in PFS formulations can hamper DNA extraction due to adsorption phenomena [3]. Therefore, a pre-treatment with phosphate buffer (pH=6, 15% ethanol) to compete for DNA adsorption sites was tested. Compared to the non-modified extraction protocol, better results were obtained with the proposed pre-treatment since only 1 from 9 PFS extracts did not amplify with universal EG-F/EG-R primers (Figure 1).

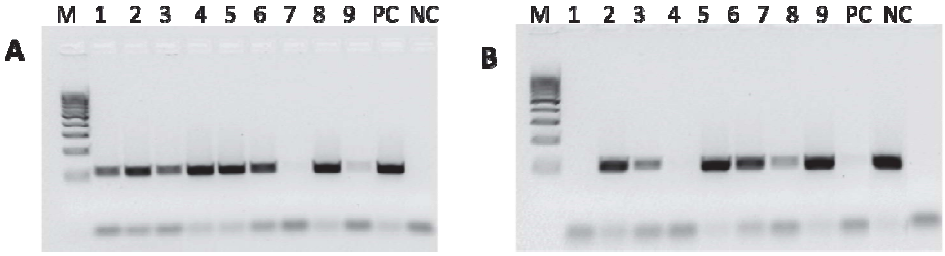

Figure 1. Agarose gel electrophoresis of PCR products from PFS extracts amplified using EG-F/EG-R primers, DNA extraction with (A) and without (B) phosphate buffer pretreatment; Legend 1 to 9 – PFS samples; M –100 bp DNA Ladder (Bioron, Germany); PC- positive control; NC –negative control.

For *Cynara* spp. differentiation, new primers were designed on a microsatellite region of *C. cardunculus* (Table 1). The qualitative PCR results showed positive amplification for all tested *Cynara* spp., thus evidencing that the new primers can be used for *Cynara* genus identification. Further development of real-time PCR coupled to HRM analysis showed that the tested *Cynara* spp. were grouped in three distinct clusters, with a level of confidence above 99.4%, enabling the discrimination of C. *scolymus* from the other tested species (Figure 2).

Also, the specificity of the developed PCR assay was tested against several different medicinal plants frequently used in herbal mixtures and PFS, including milk thistle, to evaluate any possible cross-reactivity. From the 39 plant species assayed, no cross-reactivity was observed, confirming the adequate specificity of the assay for *Cynara* spp. identification.

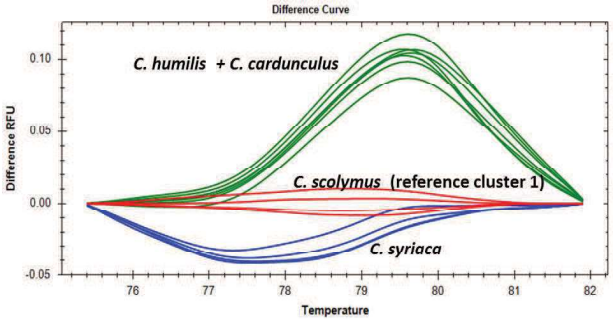

Figure 2. Difference curves obtained by HRM analysis after real-time PCR amplification with CyC-F1/CyC-R1 primers applied to *Cynara* spp. voucher specimens.

The developed methodology was applied to commercial samples, allowing detecting *Cynara* spp. in 3 out of 8 samples labelled as containing artichoke (Figure 3). Real-time PCR coupled to HRM analysis allowed to unequivocally confirm the presence of *C. scolymus* in the tested samples, with high level of confidence (>98.8%), thus confirming their labelling (Figure 4).

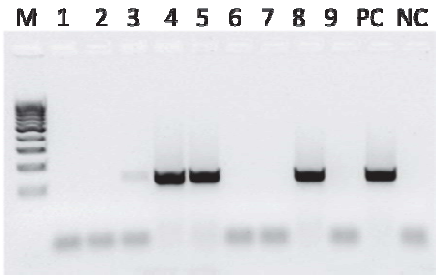

Figure 3. Agarose gel electrophoresis of PCR products from PFS extracts amplified using CyC-F1/CyC-R1 primers, Legend: 1 to 8, artichoke PFS samples; 9, milk thistle PFS; M,100 bp DNA Ladder (Bioron, Germany); PC, positive control; NC, negative control.

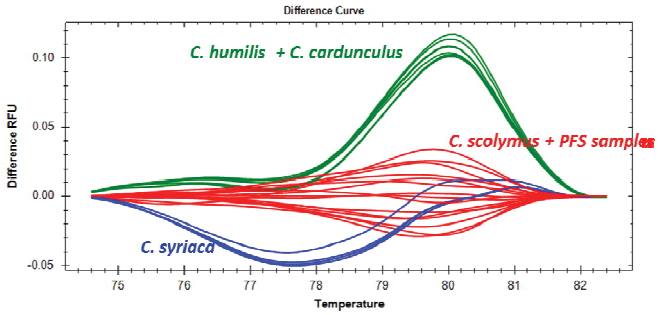

Figure 4. Difference curves obtained by HRM analysis after real-time PCR amplification with CyC-F1/CyC-R1 primers applied to *Cynara* spp. voucher specimens and PFS declaring artichoke.

4. CONCLUSION

In this work, a modified protocol for DNA extraction, more adequate for the complex matrix of PFS, was successfully proposed. A reliable and fast DNA-based methodology for the unequivocal identification of *C. scolymus* was also developed. To our knowledge, this is the first successful attempt for the rapid discrimination of *C. scolymus* in PFS.

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