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HRM analysis targeting ITS1 and matK loci as potential DNA mini-barcodes for the authentication of *Hypericum perforatum* and *Hypericum androsaemum* in herbal infusions

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ABSTRACT

Hypericum species are among the medicinal plants that are being increasingly used due to their reported health benefits. *Hypericum perforatum* (St. John's wort) is well known for its anti-inflammatory, anti-depressive and anti-viral properties, as well as a healing agent. In Portuguese folk medicine, *Hypericum androsaemum* (Hipericão do Gerês) is largely used for its hepatic protective and diuretic properties. This work aimed at searching the potential use of two DNA mini-barcode candidates (ITS1 and *mat*K) for the authentication of *H. perforatum* and *H. androsaemum* in herbal infusions. The sequencing results from ITS1 and *matK* regions were the basis for the development of species-specific PCR and real-time PCR assays coupled to High Resolution Melting (HRM) analysis, as simple approaches for the reliable discrimination of both species. The barcode regions were successful in the species-specific PCR identification of each target. ITS1 region revealed some intra-species variability from sequencing results, which compromises HRM analysis, while *mat*K showed to be an adequate mini-barcode for the differentiation of both species by real-time PCR coupled to HRM analysis. The assays were effectively applied to commercial herbal infusions, enabling the consistent identification of two labels with non-declared *Hypericum* species.

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1. Introduction

Hypericum L. (Hypericaceae) is a large genus including approximately 450 species, from which 27 have been described to possess medicinal properties (Penso, 1983). Among those, *Hypericum perforatum*, also known as St. John's wort, is considered to be the most known and economically important *Hypericum* species due to its widespread use as a medicinal plant for the treatment of anxiety, depression and topical wounds (see review Guedes, Franklin, and Fernandes-Ferreira (2012)). Over the past decades, the consumption of St. John's wort has gained increased popularity for the symptomatic treatment of minor depressive episodes (European Medicine Agency, 2009; Sarris, 2007), being its phytotherapeutic activity demonstrated in different clinical trials, where it was found to be superior than the placebo and as effective as the commonly prescribed anti-depressive drugs (Linde et al., 1996; Schrader, Meier, & Brattstrom, 1998; Woelk, 2000). Currently, it is considered as one of the most requested and top selling herbal preparations in developed countries, being frequently included in traditional herbal medicines and plant food supplements (Egan, Hodgkins, Shepherd, Timotijevic, & Raats, 2011; Ernst & Izzo, 2003; Franz, Chizzola, Novak, & Sponza, 2011).

Other *Hypericum* species are also frequently used in folk medicine, namely *Hypericum androsaemum* (known in Portugal as Hipericão do Gerês), whose aerial parts are used for their diuretic and hepatoprotective effects (Almeida, Fernandes, Lima, Costa, & Bahia, 2009; Valentão et al., 2003, 2004). Since *H. androsaemum* is less abundant and simultaneously very popular in Portugal, its price is generally higher than the one of St. John's wort (Valentão et al., 2003). Due to the high demand of both species and considering that they are commonly sold after being dried and fragmented/powdered, these botanicals are particularly prone to

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be adulterated by the intentional substitution with other cheaper plants. Moreover, owing to the similarity of *Hypericum* species, unintentional swap of plants caused by the misidentification of wild collected specimens can also occur. For these reasons, there is an increasing need for reliable methods to authenticate both, raw botanical materials and final commercialised herbal products.

So far, different analytical methodologies, mainly based on chromatographic techniques, have been proposed for quality control purposes and authentication of *H. perforatum*. The antidepressive effect was firstly attributed to its composition in naphthodianthrones, namely hypericins and its derivatives (Butterweck, Petereit, Winterhoff, & Nahrstedt, 1998), though more recently, other major secondary metabolites, such as flavonoids and phloroglucinols, have also been suggested to contribute to its bioactivity (Butterweck & Schmidt, 2007; Russo et al., 2014). The use of these bioactive substances as marker compounds for *H. perforatum* (Conforti et al., 2005; Hosni, Msaâda, Taârit, Hammami, & Marzouk, 2010), in particular the hypericins, as it is only found in *Hypericum* species (Kitanov, 2001), has been suggested by different research studies, being also included in the European Pharmacopeia monographs for *H. perforatum* drug and extract. However, hypericins, phloroglucinols and flavonoids have been described in other *Hypericum* species besides St. John's wort (Linde et al., 1996; Valentão et al., 2003), making its use as marker compounds not adequate. Moreover, the amount of plant phytochemicals might vary depending on the edaphoclimatic conditions, part of the plant and harvest time, among other factors (Büter, Orlacchio, Soldati, & Berger, 1998; Nahrstedt & Butterweck, 1997; Poutaraud & Girardin, 2004).

In opposition to chemical profiling, the use of molecular biology techniques has proved to be well suited for unequivocal plant species identification since DNA molecules are found in all tissues and are not affected by external or physiological plant conditions (Sucher & Carles, 2008). Among different DNA-based techniques, the use of barcodes has been recently proposed as a powerful pharmacognostic tool for the identification of medicinal plants and their adulterants at species level (Chen et al., 2010; Newmaster, Grguric, Shanmughanandhan, Ramalingam, & Ragupathy, 2013; Techen, Parveen, Pan, & Khan, 2014; Zhang, Yang, Jiang, Lizhen, & Zhou, 2014). DNA barcoding relies on sequence variation within a short and standardised region of the genome, designated as a “barcode”, to provide accurate species identification (Hebert, Cywinska, Ball, & deWaard, 2003). However, DNA barcoding covers the amplification and sequencing of large DNA fragments (400–800 bp), which represents a major drawback for the analysis of highly processed samples. When analysing samples with degraded DNA, such as processed medicinal plants, poorly preserved plants and/or stored for a long period of time, DNA barcodes are probably not the best target sequences. In such cases, the use of species-specific primers based on barcodes, but targeting small size amplicons (e.g. DNA mini-barcodes) has been successfully applied for species identification (Fields, Abercrombie, Eng, Feldheim, & Chapman, 2015; Hajibabaei & McKenna, 2012; Little, 2014). The recent approach of high resolution melting (HRM) analysis coupled to specific barcodes represents a potential cost-effective tool to detect small nucleotide differences without requiring further sequencing (Xanthopoulou et al., 2016).

This work aimed at proposing a practical, specific and high-throughput tool to identify and differentiate two *Hypericum* species, namely *H. perforatum* and *H. androsaemum*. For that purpose, two candidate DNA barcode loci (ITS1 and *mat*K) were targeted by real-time PCR coupled to HRM analysis. The proposed methodology was further applied to authenticate different commercial herbal medicines labelled as containing *Hypericum* species.

2. Material and methods

2.1. Sample preparation

Voucher seeds from *H. perforatum* ($n = 3$) and *H. androsaemum* ($n = 2$) were gently provided by USDA-ARS Germplasm Resources Information Network (Beltsville, MD, USA) and by the Royal Botanic Gardens (RBG, Kew, Ardingly, UK). For cross-reactivity assays, 37 medicinal plant species were provided by National and International Germplasm Banks or obtained at herbal stores (*Cymbopogon citratus* (DC.) Stapf, *Foeniculum vulgare* Mill, *Taraxacum officinales* F.H. Wigg., *Equisetum arvense* L., *Melissa officinalis* L., *Matricaria chamomilla* L., *Camellia sinensis* (L.) Kuntze, *Salvia officinalis* L., *Mentha piperita*, *Cochlospermum angolense* Welw. ex Oliv., *Pterospartum tridentatum* L., *Malva sylvestris* L., *Senna alexandrina* Mill., *Tilia* spp., *Arctostaphylos uva-ursi* (L.) Spreng., *Geranium robertianum* L., *Cynara scolymus* L., *Passiflora incarnata* L., *Peumus boldus* Molina, *Verbena officinalis* L., *Crataegus monogyna* Jacq, *Valeriana officinalis* L., *Silybum marianum* (L.) Gaertn., *Origanum vulgare* L., *Ocimum basilicum* L., *Coriandrum sativum* L., *Curcuma longa* L., *Prunus avium* L., *Aloysia citrodora* Paláu, *Eucalyptus obliqua* L'Hér., *Ginkgo biloba* L., *Aesculus hippocastanum* L., *Vitis vinífera* L., *Rosmarinus officinalis* L., *Olea europaea* L., *Ficus carica* L., *Juglans regia* L.). Herbal infusion samples ($n = 13$) were acquired at the retail market or at specialised herbal stores. Seeds were grounded with mortar and pestle, while herbal infusions and medicinal plants, mostly consisting of leaf material, were grounded and homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). All containers and material used during this procedure were previously treated with DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany).

2.2. DNA extraction

In this work, two different extraction procedures were used, namely the CTAB-PVP method and Nucleospin Plant kit (Macherey–Nagel, Düren, Germany), respectively for the DNA extraction from leaves and seeds. The CTAB-PVP method was performed as described by Costa, Melo, Santos, Oliveira, and Mafra (2015) using an initial amount of 100 mg of ground leaf material.

DNA from 100 mg of ground seeds was extracted with the commercial DNA extraction kit Nucleospin Plant II (Macherey–Nagel, Düren, Germany), according to manufacturer's instructions with minor alterations. Briefly, DNA extraction followed the protocol using buffer PL2 (pre-heated at 65 °C) and an incubation of 1 h at 65 °C with continuous stirring (1000 rpm). After incubation, the protocol was performed according to manufacturer's instructions. DNA extracts were immediately kept at −20 °C until further analysis.

2.3. Quality of DNA

Yield and purity of extracts were assessed by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

2.4. Target gene selection and oligonucleotide primers

For each locus, ITS1 and *matK*, sequences from *H. perforatum* and *H. androsaemum* were retrieved from the NCBI database, with the accession numbers presented in Table 1. In each locus, one set of

Table 1
Oligonucleotide primers used for the amplification of *Hypericum* spp.

Target	Primers	Sequence (5′→3′)	Amplicons (bp)	Reference or GenBank accession no.
ITS1 sequencing				
H. androsaemum/	ITS1-F	TGAACCTGCGGAAGGATCATT	297/298	AY573012.1/
H. perforatum	ITS1-R	AGATATCCGTTGCCGAGAGT		EU796888.1
ITS1 species-specific				
H. androsaemum	HA-ITS1-F2	TTTATCCCCGTAACCCCCGTG	116	This work
	HA-ITS1-R2	AGGTTCCTTGGCGCGTGCC		
H. perforatum	FO2	CATAAGAAGTGTAAGGCTCCCGG	85	Crockett et al. (2004)
	HRI-S	AGAGTCGTTATTGTTATGAACAGAAGGAG		
matK sequencing				
H. androsaemum/	matK-F	ATCCAACCATTTTCGGGTTC	358	HM850934/
H. perforatum	matK-R	TCGTATCGCTGAAGGAGTGA		HQ331618
matK species-specific				
H. androsaemum	HA-matK-F1	TACTATAGAGTTGTTCAAGGACCC	92	This work
	HA-matK-R1	TCATTAAAAGTGGCGTACTCCTG		
H. perforatum	HP-matK-F1	TTAATATACAGTTGTTCAAGGACC	92	This work
	HP-matK-R1	CATTATAAGTGGCGTACTCCTT		

primers was firstly designed to produce amplicons with adequate size to allow the sequencing of *Hypericum* genus (*H. perforatum* and *H. androsaemum*). After sequencing, new primers were specifically designed for each species in order to enable their unequivocal identification. All primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.5. Qualitative PCR

For sequencing ITS1 and *matK* loci, PCR amplifications were carried out in 25 μL of total reaction volume, containing 2 μL of DNA extract (20 ng), 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, Ulm, Germany), 1.5 mM or 2.5 mM (only for HA-ITS1-F2/HA-ITS1-R2 primers) of $MgCl_2$ and 100 nM (ITS1-F/ITS1-R and matK-F/matK-R) or 200 nM (HA-matK-F1/HA-matK-R1, HP-matK-F1/HP-matK-R1, HA-ITS1-F2/HA-ITS1-R2 and FO2/HRI-S) of each primer (Table 1). The reactions were performed in a MJ Mini thermal cycler (BioRad, Hercules, CA, USA).

For sequencing primers, the following program was used: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 61 °C or 59 °C for 45 s and 72 °C for 1 min, respectively for primers ITS1-F/ITS1-R or matK-F/matK-R (Table 1); and a final extension at 72 °C for 5 min. The other PCR amplifications were performed using the following programs: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 65 °C, 64 °C, 62 °C or 59 °C for 30 s, respectively for primers FO2/HRI-S, HA-ITS1-F2/HA-ITS1-R2, HA-matK-F1/HA-matK-R1, or HP-matK-F1/HP-matK-R1 (Table 1), and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

The amplified fragments were analysed by electrophoresis in 1.5% agarose gel containing 1 × Gel Red (Biotium, CA, USA) for staining and carried out in 1 × SGTB buffer (GRISP, Porto, Portugal) for about 20–25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (BioRad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (BioRad Laboratories, Hercules, CA, USA).

2.6. Real-time PCR and HRM analysis

The real-time PCR assays were carried out in 20 μL of total reaction volume, containing 2 μL of DNA (20 ng), 1x of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 250 nM of each primer (Table 1). The reactions were performed in a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 95 °C for 5 min; 50 cycles at 95 °C for 10 s and 65 °C, 64 °C, 62 °C or 59 °C for 30 s, for primers FO2/HRI-S, HA-ITS1-F2/HA-ITS1-R2, HA-matK-F1/HA-matK-R1 or HP-matK-F1/HP-matK-R1, respectively (Table 1). The collection of fluorescence signal was performed at the end of each cycle and data were processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 65 °C, 64 °C, 62 °C or 59 °C for 3 min, for primers FO2/HRI-S, HA-ITS1-F2/HA-ITS1-R2, HA-matK-F1/HA-matK-R1 or HP-matK-F1/HP-matK-R1, respectively, in order to allow the correct annealing of the DNA duplexes. These two steps were followed by melting curve ranging up to 95 °C with temperature increments of 0.02 °C/s. The fluorescence data were acquired at the end of each melting temperature and further processed using the Precision Melt Analysis Software 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for easier visual identification of clusters. Melting curve shape sensitivity determines the stringency used to classify melting curves into different clusters, while temperature of melting (Tm) difference threshold is a parameter that determines the lowest amount of Tm difference between samples. In both cases, cluster detection parameters were set to high sensitivity and threshold yields aiming at providing more heterozygote clusters. Therefore, melting curve shape sensitivity was adjusted as a default value of 50% and Tm difference threshold was set as 0.20.

DNA extracts of voucher seeds and herbal infusions were analysed in replicates ($n = 3$) in two independent assays.

2.7. Sequencing of PCR products

PCR products of *H. androsaemum* and *H. perforatum* amplified with ITS1-F/ITS1-R and matK-F/matK-R were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components. The purified products were sent to a specialised research facility (STABVIDA, Lisbon, Portugal) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences of high quality.

3. Results and discussion

DNA barcoding has been recently advanced as a widely used and effective tool for the rapid and accurate identification/differentiation of species (Li et al., 2015). Although the cytochrome oxidase I

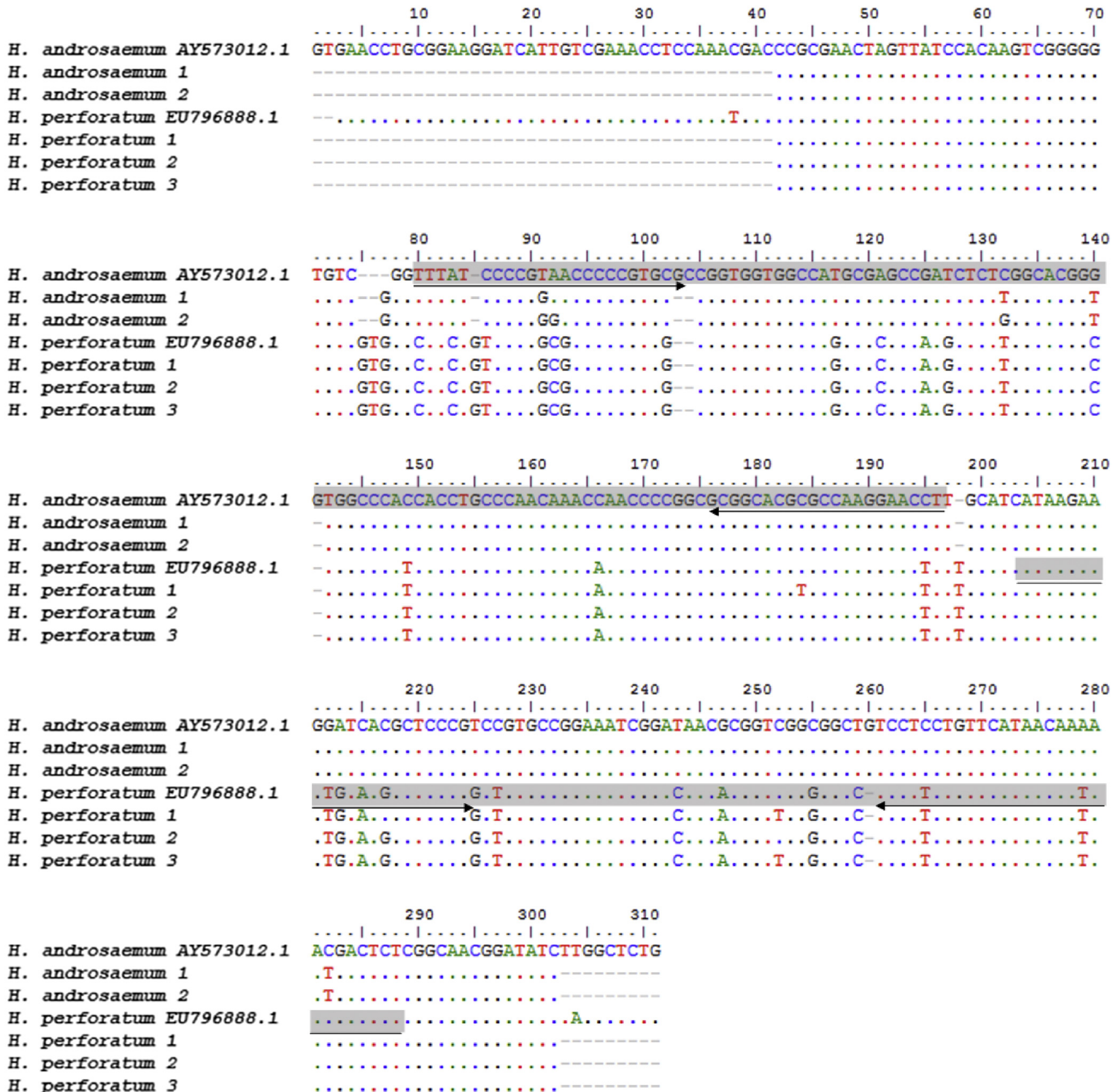

Fig. 1. Alignment of ITS1 locus of sequencing products of *Hypericum* spp with the consensus sequences from NCBI database. Legend: *H. androsaemum* 1–2 (accessions PI618712 and 0122906, provided by USDA-ARS Germplasm (Beltsville, MD, USA) and RBG (Kew, Ardingly, UK), respectively); *H. perforatum* 1–3 (accessions PI325351, Ames27495 and PI664858, respectively provided by USDA-ARS Germplasm (Beltsville, MD, USA)).

(COI) has been recommended as the universal DNA barcode for animal identification (http://www.barcodeoflife.org), the same region DNA region is not suitable for plants (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005). From numerous available loci, none works across all plant species, thus disabling their universal identification. So far, several plastidial and mitochondrial genes have been evaluated, but there is still no consensual opinion concerning the best candidate markers for plant DNA barcoding (Ferri et al., 2015). Presently, a multi-locus approach, combining the identification of noncoding intergenic spacers (e.g. ITS, trnH-psbA) and plastidial coding sequences (e.g. *mat*K, *rbc*L), is considered the best option for the correct differentiation of plant species (Selvaraj et al., 2013). Among them, two DNA regions, namely the *mat*K and the *rbc*L genes, have been approved as excellent plant DNA barcodes. In addition to plastid genome, the internal transcribed spacer, which is flanked by the coding sequence of 18S and 5.8S rRNA or 5.8S and 28S rRNA, respectively for ITS1 and ITS2, has also been classified as potential barcode regions (Howard et al., 2009).

In the present work, based on the available information regarding DNA barcoding of plants, aiming at identifying the botanical origin of different species of *Hypericum*, namely the *H. perforatum* and the *H. androsaemum*, two genomic regions were selected: the ITS1 that is considered a powerful phylogenetic marker for species with high levels of interspecific divergence; and

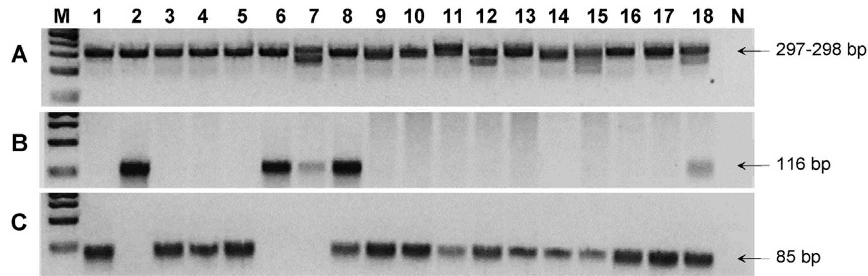

Fig. 2. Agarose gel electrophoresis of PCR products targeting ITS1 region for *Hypericum* spp. (A), *H. androsaemum* (B) and *H. perforatum* (C). Lane 1, 100% *H. perforatum* (commercial herbal infusion); Lane 2, 100% *H. androsaemum* (commercial herbal infusion); Lanes 3–5, *H perforatum* (accessions PI325351, Ames27495 and PI664858, respectively provided by USDA-ARS Germplasm (Beltsville, MD, USA)); Lanes 6,7, *H. androsaemum* (accessions PI618712 and 0122906 provided by USDA-ARS Germplasm (Beltsville, MD, USA) and by RBG (Kew, Ardingly, UK), respectively); Lane 8, 100% *H. androsaemum* (commercial herbal infusion); Lanes 9–18, commercial herbal infusions (plant mixtures labelled as containing *H. perforatum*).

the plastidial gene *mat*K, which has a high evolutionary rate, suitable length, interspecific divergence and a low transition/transversion rate (Li et al., 2015).

The quality assessment of DNA extracts from plant material (herbal infusions and other medicinal plants) showed adequate yields (89–355 ng/μL) and purities (1.8 ± 0.2). The extracts from seeds exhibited low DNA yield (<5 ng/μL), but with good purities (1.9 ± 0.2). These results could be related to the rigid nature of the material (seed casing) that was probably hampering the access to DNA during the extraction procedure. In order to assess amplifiability, DNA extracts were also tested with universal eukaryotic primers 18SRG-F/18SRG-R as described by Costa, Oliveira, and Mafra (2013), exhibiting positive amplification for all samples (data not shown), thus confirming the absence of any false negative results.

3.1. ITS1 locus

3.1.1. Sequencing

ITS1 locus from five Germplasm seeds (three samples of *H. perforatum* and two samples of *H. androsaemum*) were amplified as voucher species using specific primers targeting the *Hypericum* genus. Each PCR product was amplified with direct sequencing of both strands in opposite directions. Considering that the platform

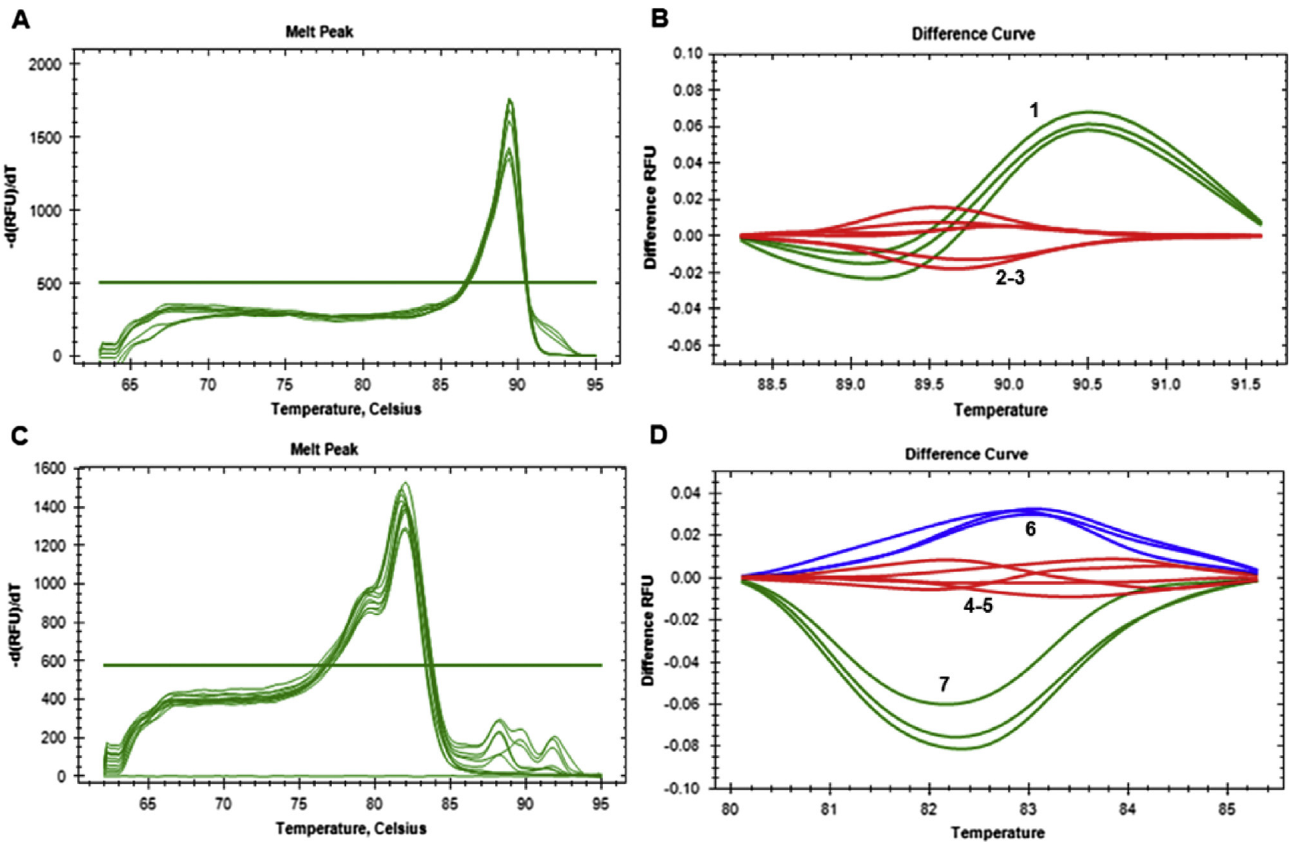

Fig. 3. Conventional melting analysis (A, C) and HRM analysis (B, D) of real-time amplification of ITS1 locus of voucher seeds from *H. androsaemum* (A, B) and *H. perforatum* (C, D). Legend: 1,2, *H. androsaemum* (accessions PI618712 and 0122906 provided by USDA-ARS Germplasm (Beltsville, MD, USA) and by RBG (Kew, Ardingly, UK), respectively); 3, 100% *H. androsaemum* (commercial herbal infusion); 4, 100% *H. perforatum* (commercial herbal infusion); 5–7, *H. perforatum* (accessions PI325351, Ames27495 and PI664858 provided by USDA-ARS Germplasm (Beltsville, MD, USA), respectively).

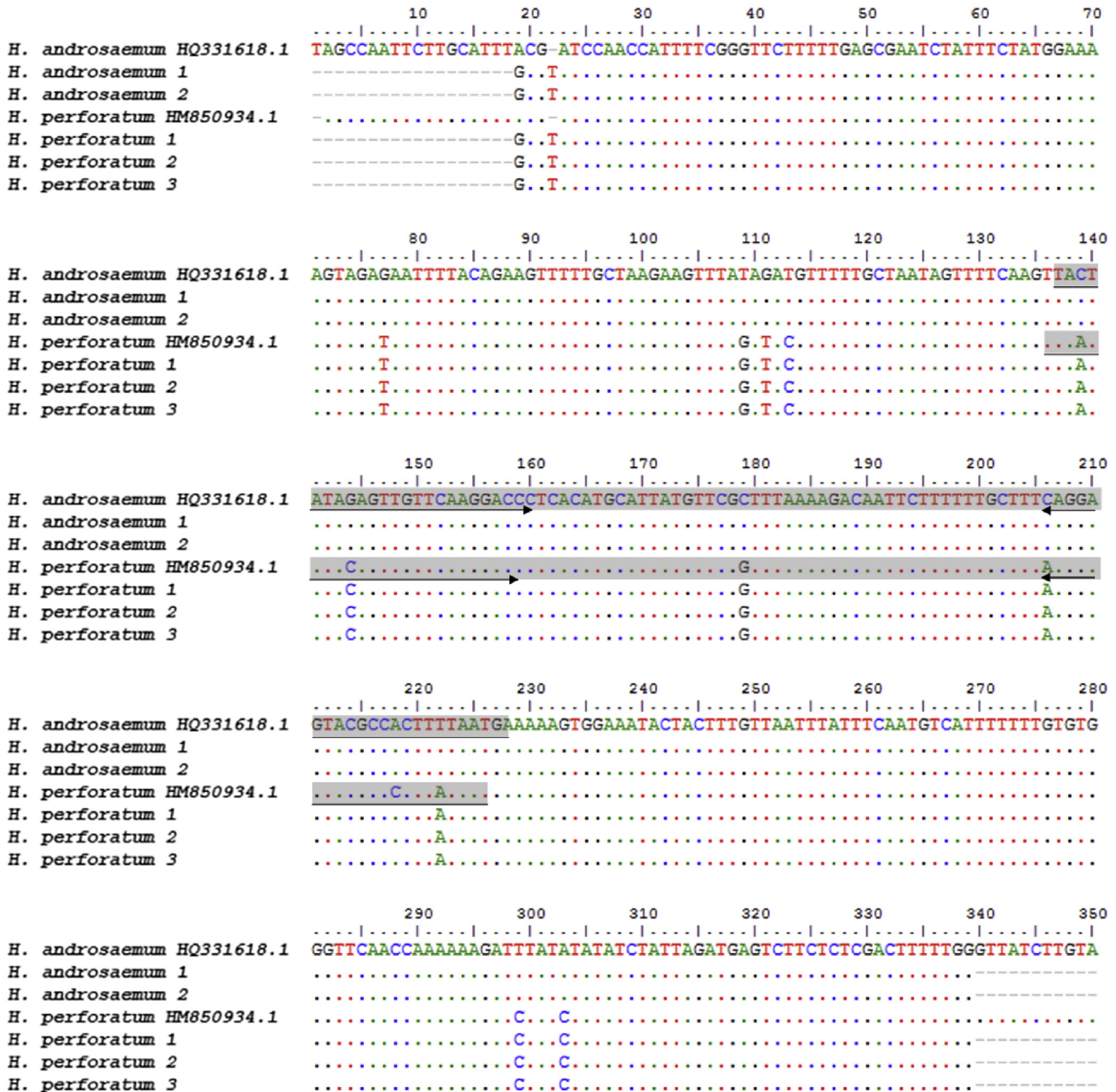

Fig. 4. Alignment of *mat*K locus of sequencing products of *Hypericum* spp with the consensus sequences from NCBI database. Legend: *H. androsaemum* 1–2 (accessions PI618712 and 0122906, provided by USDA-ARS Germplasm (Beltsville, MD, USA) and RBG (Kew, Ardingly, UK), respectively); *H. perforatum* 1–3, (accessions PI325351, Ames27495 and PI664858, respectively provided by USDA-ARS Germplasm (Beltsville, MD, USA)).

often does not allow perfect resolution for the reading of the first ~50 bp at the 5'-end of the sequence, data on Fig. 1 refers to sequencing of 258 bp with double coverage. The sequences revealed some nucleotide variability among voucher seeds from the same species, evidencing high intra-species variability that is often attributed to this noncoding DNA region (internal transcribed spacer). In spite of such variability, *H. androsaemum* exhibited several nucleotide differences from *H. perforatum*, which can be observed in Fig. 1. Using the sequencing data, a new pair of primers was specifically designed for *H. androsaemum* targeting an amplicon of 116 bp. For *H. perforatum*, primers targeting the same region were already available in the literature (Crockett, Douglas, Scheffler, & Khan, 2004), thus being selected for its specific amplification (Table 1, Fig. 1).

3.1.2. Qualitative PCR

Cross-reactivity assays were performed for each set of primers targeting ITS1 locus (HA-ITS1-F2/HA-ITS1-R2 and FO2/HRI-S), using different plant species (medicinal and foods, $n = 37$) and the *Hypericum* species under study (*H. androsaemum* and *H. perforatum*). In both cases, no cross-reactivity was found with any of the medicinal plants, being the reactions specific for each intended target species (data not shown).

Along with voucher species, herbal infusions ($n = 13$) were also

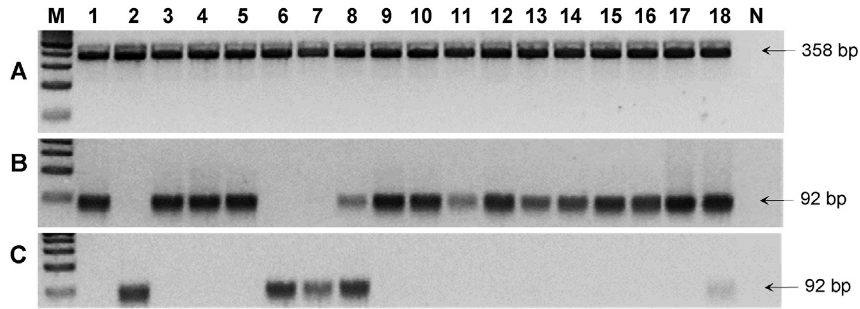

Fig. 5. Agarose gel electrophoresis of PCR products targeting *mat*K region for *Hypericum* spp. (A), *H. androsaemum* (B) and *H. perforatum* (C). Lane 1, 100% *H. perforatum* (commercial herbal infusion); Lane 2, 100% *H. androsaemum* (commercial herbal infusion); Lanes 3–5, *H perforatum* (accessions PI325351, Ames27495 and PI664858, respectively provided by USDA-ARS Germplasm (Beltsville, MD, USA)); Lanes 6,7, *H. androsaemum* (accessions PI618712 and 0122906 provided by USDA-ARS Germplasm (Beltsville, MD, USA) and by RBG (Kew, Ardingly, UK), respectively); Lane 8, 100% *H. androsaemum* (commercial herbal infusion); Lanes 9–18, commercial herbal infusions (plant mixtures labelled as containing *H. perforatum*).

successfully amplified by qualitative PCR targeting *Hypericum* spp. (Fig. 2A) and each species, *H. androsaemum* (Fig. 2B) and *H. perforatum* (Fig. 2C). From the obtained results, two non-conformities were found in commercial samples after verifying labelling compliance. Sample in lane 8 was labelled as 100% *H. androsaemum*, but it also amplified positively for *H. perforatum* (Fig. 2B, C), which suggests the partial substitution of the first species by the second one in this herbal infusion. Sample in lane 18 is labelled as containing *H. perforatum*, though it also presented a faint band for *H. androsaemum* (Fig. 2B, C), suggesting in this case the eventual cross-contamination during manipulation and/or packaging.

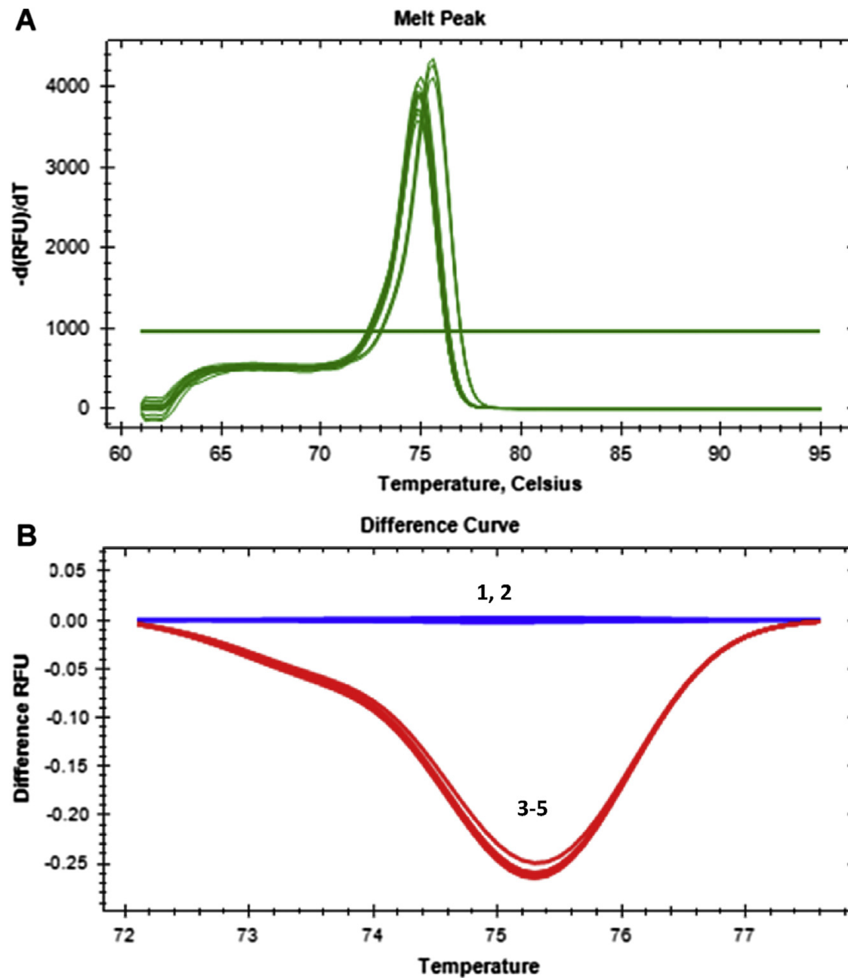

Fig. 6. Conventional melting analysis (A) and HRM analysis (B) of real-time amplification of *mat*K locus of voucher seeds. Legend: 1,2, *H. androsaemum* (accessions PI618712 and 0122906, provided by USDA-ARS Germplasm (Beltsville, MD, USA) and by RBG (Kew, Ardingly, UK), respectively); 3–5, *H. perforatum* (accessions PI325351, Ames27495 and PI664858, respectively provided by USDA-ARS Germplasm (Beltsville, MD, USA)), using specific primers for each species in single assay.

3.1.3. Real-time PCR coupled with HRM analysis

Owing to the intra-species variability of the ITS1 region, DNA from voucher species was evaluated by real-time PCR using EvaGreen as a fluorescent dye, targeting each species (*H. androsaemum* or *H. perforatum*). In the case of *H. androsaemum*, real-time PCR assays enabled to confirm the results from qualitative PCR (Fig. 3), evidencing a temperature of melting of approximately 89.6 °C (Fig. 3A). However, when performing HRM analysis, two distinct clusters were defined with high level of confidence (>98.8%). HRM analysis included one voucher species (accession no. 0122906) and the herbal infusion containing 100% *H. androsaemum* in cluster 1 (reference cluster), while the second voucher (accession no. PI618712) was comprised in cluster 2 (Fig. 3B).

Real-time PCR trials targeting *H. perforatum* also confirmed the results obtained by qualitative PCR. Conventional melting allowed the identification of a main melt pick of approximately 82.0 °C and a small pick at 79.6 °C (Fig. 3C). After HRM analysis, PCR products were included in three distinct groups. The voucher seeds ($n = 3$) were comprised in three different clusters (Fig. 3D) with a level of confidence above 98.0%. Since HRM analysis is based on minute differences within amplicon size and/or composition, a simple nucleotide difference could be enough to differentiate two identical sequences. Owing to this principle, different clusters were created by covering products that should present similar size and composition. Although HRM analysis is mainly used as a high-throughput approach for the rapid differentiation of species on the basis of highly similar sequences (Costa, Mafra, & Oliveira, 2012; Osathanunkul, Madesis, & de Boer, 2015), in this work HRM was applied as a confirmatory tool for the evaluation of ITS1 as DNA mini-barcode. For both *Hypericum* species, the results of HRM analysis could be explained by the high intra-species variability of ITS1 region among individuals, which can be highlighted by sequencing (Fig. 1). The results concerning *H. perforatum* are in good agreement with the work described by Howard et al. (2009) since the primers targeting the referred species were designed on a region of considerable sequence diversity.

3.2. matK locus

3.2.1. Sequencing

Following the multi-locus approach, the plastidial *mat*K gene was carefully chosen as a second DNA barcode for the identification of *Hypericum* species. Using voucher seeds, PCR amplification was carried out targeting a 358 bp region of *mat*K gene and the respective sequencing results are presented in Fig. 4. It refers to a complete and correct sequencing of 320 bp products with double coverage. Contrarily to ITS1 locus, sequencing products of *mat*K did not present variability among accessions of the same species. Accordingly, new primers producing amplicons of 92 bp were specifically designed targeting each *Hypericum* species (Table 1, Fig. 4). In both cases, each set of primers included a total of 4 nucleotide mismatches, aiming at conferring high specificity to the PCR systems (Fig. 4).

3.2.2. Qualitative PCR

Assays testing the specificity of each set of designed primers targeting the *mat*K region were also performed (HA-matK-F1/HA-matK-R1 and HP-matK-F1/HP-matK-R1). Each pair of primers was specific for the corresponding *Hypericum* species target and no cross-reactivity was observed with other medicinal plants ($n = 37$) (data not shown).

The application of PCR assays was performed using the voucher species and herbal infusions targeting *Hypericum* spp. (Fig. 5A) and each of the species, *H. androsaemum* (Fig. 5B) and *H. perforatum* (Fig. 5C). From these results, the two previously found non-conformities in commercial samples 8 and 18 were also identified (Fig. 5B, C), thus confirming the results obtained from ITS1 locus amplification.

3.2.3. Real-time PCR coupled with HRM analysis

Results from real-time PCR trials were in good accordance with qualitative PCR results. By conventional melting analysis, amplicons evidenced good resolution with single melt peaks for each target, namely at 75.8 °C or 75.0 °C for *H. androsaemum* or *H. perforatum* (Fig. 6A), respectively. In a single assay, voucher seeds from the two

Table 2
Relevant label information and summarised results of qualitative and real-time PCR coupled with HRM applied to voucher seeds and commercial herbal infusions containing *H. perforatum* or *H. androsaemum*.

Samples	Species declared in label		*H. perforatum*		*H. androsaemum*	
	H. perforatum	*H. androsaemum*	PCR results	HRM (% of confidence ± SD[d])	PCR results	HRM (% of confidence ± SD[d])
1	X		+	99.3 ± 0.2	−	
2		X	−		+	98.7 ± 0.4
3	X[a]		+	99.9 ± 0.0	−	
4	X[b]		+	99.5 ± 0.2	−	
5	X[a]		+	99.9 ± 0.1	−	
6		X[a]	−		+	99.9 ± 0.1
7		X[a]	−		+	99.8 ± 0.2
8	X		+	98.5 ± 0.1	+	99.1 ± 0.5
9	X		+	99.8 ± 0.1	−	
10	X[c]		+	99.7 ± 0.1	−	
11	X		+	98.9 ± 0.2	−	
12	X		+	99.7 ± 0.1	−	
13	X		+	99.7 ± 0.3	−	
14	X		+	99.8 ± 0.1	−	
15	X		+	99.9 ± 0.1	−	
16	X		+	99.8 ± 0.2	−	
17	X[c]		+	99.5 ± 0.3	−	
18	X		+	99.5 ± 0.4	+	98.9 ± 0.1

[a] Voucher seeds provided by USDA-ARS Germplasm (Beltsville, MD, USA) and by.
[b] RBG (Kew, Ardingly, UK).
[c] Labels state the presence of "Hipericão" without specifying *Hypericum* species.
[d] Mean of % of confidence ± standard deviation.

Hypericum species were amplified using the correspondent set of primers. In spite of presenting very similar melt peaks, HRM analysis allowed discriminating *H. perforatum* and *H. androsaemum* in clusters 1 and 2 (Fig. 6B), respectively, with high level of confidence (>99.5%).

HRM analysis was further applied to commercial herbal infusions, enabling their authentication regarding the presence of *Hypericum* species. Table 2 presents the summary of relevant information concerning qualitative and real-time PCR coupled with HRM analysis. The obtained results were then compared with information on the labels and the two non-conformities observed by qualitative PCR were confirmed. HRM analysis allowed identifying *H. androsaemum* in sample 8 with 99.1% of confidence, which is in accordance with the labelled information of 100% *H. androsaemum* (Table 2). However, in the same sample, HRM analysis also detected *H. perforatum* with a level of confidence of 98.5%, thus confirming previous findings. The same pattern was observed for sample 18, in which both *Hypericum* species (*H. androsaemum* and *H. perforatum*) were identified with high level of confidence (98.9% and 99.5%, respectively).

4. Conclusion

In this work, two DNA mini-barcode regions (ITS1 and *mat*K) were proposed for the differentiation of *Hypericum* species, namely *H. androsaemum* or *H. perforatum*. Species-specific PCR assays were successfully developed for both ITS1 and *mat*K regions, revealing its adequacy as simple and fast approaches for species identification. Both regions were also proposed for the development of real-time PCR assays coupled to HRM analysis. However, ITS1 region revealed some intra-species variability from sequencing results, which compromises HRM analysis. Conversely, *mat*K showed to be an adequate mini-barcode region for the differentiation of both species by real-time PCR coupled to HRM analysis. All PCR systems were effectively applied to commercial herbal infusions, enabling the consistent identification of two labels with non-declared *Hypericum* species. Herein, simple, fast, reliable, cost-effective and high throughput approaches have been proposed for the authentication of herbal infusions.

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References

Almeida, I. F., Fernandes, E., Lima, J. L. F. C., Costa, P. C., & Bahia, M. F. (2009). In vitro protective effect of *Hypericum androsaemum* extract against oxygen and nitrogen reactive species. *Basic & Clinical Pharmacology & Toxicology, 105*, 222–227.

Büter, B., Orlacchio, C., Soldati, A., & Berger, K. (1998). Significance of genetic and environmental aspects in the field cultivation of *Hypericum perforatum*. *Planta Medica, 64*, 431–437.

Butterweck, V., Petereit, F., Winterhoff, H., & Nahrstedt, A. (1998). Solubilized hypericin and pseudohypericin from *Hypericum perforatum* exert antidepressant activity in the forced swimming test. *Planta Medica, 64*, 291–294.

Butterweck, V., & Schmidt, M. (2007). St. John's wort: role of active compounds for its mechanism of action and efficacy. *Wiener Medizinische Wochenschrift, 157*, 356–361.

Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., et al. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One, 5*(1), e8613.

Conforti, F., Statti, G. A., Tundis, R., Bianchi, A., Agrimonti, C., Sacchetti, G., et al. (2005). Comparative chemical composition and variability of biological activity of methanolic extracts from *Hypericum perforatum* L. *Natural Product Research, 19*(3), 295–303.

Costa, J., Mafra, I., & Oliveira, M. B. P. P. (2012). High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. *Food Chemistry, 133*, 1062–1069.

Costa, J., Melo, V. S., Santos, C. G., Oliveira, M. B. P. P., & Mafra, I. (2015). Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols. *Food Chemistry, 187*, 469–476.

Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Research International, 54*, 1722–1729.

Crockett, S. L., Douglas, A. W., Scheffler, B. E., & Khan, I. A. (2004). Genetic profiling of Hypericum (St. John's Wort) species by nuclear ribosomal ITS sequence analysis. *Planta Medica, 70*, 929–935.

Egan, B., Hodgkins, C., Shepherd, R., Timotijevic, L., & Raats, M. (2011). An overview of consumer attitudes and beliefs about plant food supplements. *Food and Function, 2*, 747–752.

Ernst, E., & Izzo, A. A. (2003). The clinical pharmacology of *Hypericum perforatum*. In E. Ernst (Ed.), *Hypericum: The Genus Hypericum* (Vol. 31, pp. 155–172). New York: Taylor's and Francis.

European Medicines Agency. (2009). *Community herbal monograph on Hypericum perforatum L., Herba (well-established medicinal use)*. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_Community_herbal_monograph/2010/01/WC500059149.pdf.

Ferri, G., Corradini, B., Ferrari, F., Santunione, A. L., Palazzoli, F., & Alu', M. (2015). Forensic botany II, DNA barcode for land plants: which markers after the international agreement? *Forensic Science International: Genetics, 15*, 131–136.

Fields, A. T., Abercrombie, D. L., Eng, R., Feldheim, K., & Chapman, D. D. (2015). A novel mini-DNA barcoding assay to identify processed fins from internationally protected shark species. *PLoS One, 10*(2), 1–10.

Franz, C., Chizzola, R., Novak, J., & Sponza, S. (2011). Botanical species being used for manufacturing plant food supplements (PFS) and related products in the EU member states and selected third countries. *Food & Function, 2*, 720–730.

Guedes, A. P., Franklin, G., & Fernandes-Ferreira, M. (2012). *Hypericum* sp.: essential oil composition and biological activities. *Phytochemistry Reviews, 11*, 127–152.

Hajibabaei, M., & McKenna, C. (2012). DNA mini-barcodes. In W. J. Kress, & D. L. Erickson (Eds.), *DNA barcodes* (Vol. 858, pp. 339–353). New York: Humana Press.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identification through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences, 270*(1512), 313–321.

Hosni, K., Msaâda, K., Taârit, M. B., Hammami, M., & Marzouk, B. (2010). Bioactive components of three *Hypericum* species from Tunisia: a comparative study. *Industrial Crops and Products, 31*, 158–163.

Howard, C., Bremner, P. D., Fowler, M. R., Isodo, B., Scott, N. W., & Slater, A. (2009). Molecular identification of *Hypericum perforatum* by PCR amplification of the ITS and 5.8S rDNA region. *Planta Medica, 75*, 864–869.

Kitanov, G. M. (2001). Hypericin and pseudohypericin in some *Hypericum* species. *Biochemical Systematics and Ecology, 29*, 171–178.

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 8369–8374.

Linde, K., Ramirez, G., Mulrow, C. D., Pauls, A., Weidenhammer, W., & Melchart, D. (1996). St John's wort for depression–an overview and meta-analysis of randomised clinical trials. *British Medicine Journal, 313*, 253–258.

Little, D. P. (2014). A DNA mini-barcode for land plants. *Molecular Ecology Resources, 14*, 437–446.

Li, X., Yang, Y., Henry, R. J., Rossetto, M., Wang, Y., & Chen, S. (2015). Plant DNA barcoding: from gene to genome. *Biological Reviews, 90*, 157–166.

Nahrstedt, A., & Butterweck, V. (1997). Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. *Pharmacopsychiatry, 30*, 129–134.

Newmaster, S., Grguric, M., Shanmughanandhan, D., Ramalingam, S., & Ragupathy, S. (2013). DNA barcoding detects contamination and substitution in North American herbal products. *BMC Medicine, 11*(1), 222.

Osathanunkul, M., Madesis, P., & de Boer, H. (2015). Bar-HRM for authentication of plant-based medicines: evaluation of three medicinal products derived from Acanthaceae species. *PLoS one, 10*(5), e0128476.

Penso, G. (1983). *Index plantarum medicinalium totius mundi eorumque synonymorum: INPLAMED*. Milan: O. E. M. F.

Poutaraud, A., & Girardin, P. (2004). Agronomic and chemical characterization of 39 *Hypericum perforatum* accessions between 1998 and 2000. *Plant Breeding, 123*, 480–484.

Russo, E., Scicchitano, F., Whalley, B. J., Mazzitello, C., Ciriaco, M., Esposito, S., et al. (2014). *Hypericum perforatum*: pharmacokinetic, mechanism of action, tolerability, and clinical drug–drug interactions. *Phytotherapy Research, 28*, 643–655.

Sarris, J. (2007). Herbal medicines in the treatment of psychiatric disorders: a systematic review. *Phytotherapy Research, 21*, 557–559.

Schrader, E., Meier, B., & Brattstrom, A. (1998). *Hypericum* treatment of mild-moderate depression in a placebo controlled study: a prospective, double-blind, randomized, placebo-controlled, multicentre study. *Human*

Psychopharmacology, 13, 163–169.

Selvaraj, D., Park, J.-I., Chung, M.-Y., Cho, Y.-G., Ramalingam, S., & Nou, I.-S. (2013). Utility of DNA barcoding for plant biodiversity conservation. *Plant Breeding and Biotechnology, 1*, 320–332.

Sucher, N. J., & Carles, M. C. (2008). Genome-Based approaches to the authentication of medicinal plants. *Planta Medica, 74*, 603–623.

Techen, N., Parveen, I., Pan, Z., & Khan, I. A. (2014). DNA barcoding of medicinal plant material for identification. *Current Opinion in Biotechnology, 25*, 103–110.

Valentão, P., Carvalho, M., Fernandes, E., Carvalho, F., Andrade, P. B., Seabra, R. M., et al. (2004). Protective activity of *Hypericum androsaemum* infusion against tert-butyl hydroperoxide-induced oxidative damage in isolated rat hepatocytes. *Journal of Ethnopharmacology, 92*, 79–84.

Valentão, P., Dias, A., Ferreira, M., Silva, B., Andrade, P. B., Bastos, M. L., et al. (2003). Variability in phenolic composition of *Hypericum androsaemum. Natural Product Research, 17*, 135–140.

Woelk, H. (2000). Comparison of St. John's wort and imipramine for treating depression: randomised controlled trial. *British Medical Journal, 321*, 536–539.

Xanthopoulou, A., Ganopoulos, I., Kalivas, A., Osathanunkul, M., Chatzopoulou, P., Tsaftaris, A., et al. (2016). Multiplex HRM analysis as a tool for rapid molecular authentication of nine herbal teas. *Food Control, 60*, 113–116.

Zhang, D., Yang, Y., Jiang, B., Lizhen, D., & Zhou, N. (2014). How to correctly identify herbal materials in market: a case study based on DNA barcodes. *African Journal of Traditional, Complementary and Alternative Medicines, 11*, 66–76.