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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A B S T R A C T

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, antidepressive and anti-viral properties, as well as a healing agent. In Portuguese folk medicine, Hypericum androsaemum (Hiperício 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 matK) 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 matK 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 phytherapeutic activity demonstrated in different clinical trials, where it was found to be superior than the placebo and as effective as the commonly prescribed anti-depressives 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 Hiperício 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 anti-depressive effect was firstly attributed to its composition in naphthodianthrones, namely hypericins and its derivatives (Butterweck, Peteriet, 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, Msaada, Taarit, 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 (Buter, 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, Technen, Parveen, Pan, & Girardin, 2014). 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 matK) 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, Taxacum 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., Sena 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., Originum vulgare L., Ocinum basilicum L., Coriandrum sativum L., Curcuma longa L., Prunus avium L., Aloysia citrodora Palau, Eucalyptus oblqua L’Her., Ginkgo biloba L., Aesculus hippocastanum L., Vitis vinifera 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 samples, 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-Exutis-Plus™, 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 (Macher, 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 (Macher, 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

### 2.5. Locus selection

For 10 out of the 34 loci, amplification success was achieved for both H. perforatum and H. androsaemum species. For the remaining 24 loci, amplification success was achieved for species identification purposes only. The selected loci were ITS1 (internal transcribed spacer 1) and matK (maternal plastid gene). The ITS1 region is highly conserved among species and has been successfully used for species identification purposes. The matK gene is a chloroplast gene and has been successfully used for species identification purposes.
primer was first 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 (NH4)2SO4, 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 MgCl2 and 100 nM (ITS1-F/ITS1-R and matK-F/matK-R) or 200 nM (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 heterogeneous 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 by electrophoresis in a 1.5% agarose gel containing 1x 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.

Table 1

| Target          | Primers   | Sequence (5’→3’)                  | Amplicons (bp) | Reference or GenBank accession no. |
|-----------------|-----------|-----------------------------------|----------------|-----------------------------------|
| ITS1 sequencing | H. androsaemum | ITS1-F AGATACCTGCGCCAAGGATCATT   | 297/298        | EU796888.1                        |
|                 | H. perforatum | ITS1-R AGATACCTGCGCCAAGGATCATT   |                |                                   |
| ITS1 species-specific | H. androsaemum | HA-ITS1-F/HA-ITS1-R TTATACCCGCTAACAAGGACCC | 116            | This work                        |
|                 | H. perforatum | FO2/HRI-S CATTAAACAGTGTCAGAGGACC |                |                                   |
| matK sequencing | H. androsaemum | matK-F ATTACAAGTTTGGGTACGCC | 358            | HMI05934/1                        |
|                 | H. perforatum | matK-R TCTATCGTGCAAGGATGTA      |                |                                   |
| matK species-specific | H. androsaemum | HA-matK-F/HA-matK-R CATTAAACAGTGTCAGAGGACC | 92             | This work                        |
|                 | H. perforatum | HP-matK-F/HP-matK-R CATTAAACAGTGTCAGAGGACC |                |                                   |

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
(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. matK, rbcL), is considered the best option for the correct differentiation of plant species (Selvaraj et al., 2013).

Among them, two DNA regions, namely the matK and the rbcL 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. 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 P125351, Ames27485 and P9664858, respectively provided by USDA-ARS Germplasm (Beltsville, MD, USA)).
the plastidial gene *matK*, 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

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**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*).

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**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).
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
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.
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 (Hypericum 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 peak of approximately 82.0 °C and a small peak 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 matK 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 matK 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 matK 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 matK 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

| Samples | Species declared in label | H. perforatum | H. androsaemum | H. perforatum | H. androsaemum |
|---------|---------------------------|---------------|----------------|---------------|----------------|
|         |                           | PCR results   | HRM (% of confidence ± SD) | PCR results   | HRM (% of confidence ± SD) |
| 1       | X                         | +             | 99.3 ± 0.2     |               |                |
| 2       | X                         |               | +              | 98.7 ± 0.4    |
| 3       | X                         | +             | 99.9 ± 0.0     |               |                |
| 4       | X                         | +             | 99.5 ± 0.2     |               |                |
| 5       | X                         | +             | 99.9 ± 0.1     |               |                |
| 6       | X                         |               | +              | 99.9 ± 0.1    |
| 7       | X                         |               | +              | 99.8 ± 0.2    |
| 8       | X                         | +             | 98.3 ± 0.1     |               |                |
| 9       | X                         | +             | 99.9 ± 0.1     |               |                |
| 10      | X                         | +             | 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                         | +             | 99.8 ± 0.1     |               |                |
| 16      | X                         | +             | 99.8 ± 0.2     |               |                |
| 17      | X                         | +             | 99.8 ± 0.3     |               |                |
| 18      | X                         | +             | 99.9 ± 0.4     |               |                |

| a Voucher seeds provided by USDA-ARS Germplasm (Beltsville, MD, USA) and by. |
| b RBG (Kew, Arlingly, 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 matK) 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 matK 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, matK 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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