universität wien

DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„DNA-based identification of selected medicinal plants and detection of adulterations and admixtures“

verfasst von / submitted by

Mag.rer.nat. Corinna Schmiderer

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 091 438
Dissertationsgebiet lt. Studienblatt / field of study as it appears on the student record sheet:	Botanik
Betreut von / Supervisor:	A.Prof. DI Dr. Johannes Novak

For Werner, Rose
and my parents.

Content

Abbreviations

ARMS	amplification refractory mutation system (allele specific PCR)
BSA	bovine serum albumin
bp	base pair(s)
Cq	quantification cycle (= Ct – threshold cycle, Cp – crossing point)
CTAB	cetyltrimethylammonium bromide
dNTP	2'-deoxynucleotide triphosphate
ddNTP	2',3'-dideoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
HRM, HRMA	high-resolution melting curve analysis
IGS	intergenic spacer
indel	insertion or deletion
ITS	internal transcribed spacer(s) (of nuclear ribosomal DNA)
MCA	melting curve analysis
NGS	next generation sequencing
nrDNA	nuclear ribosomal DNA
nt	nucleotide(s)
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction (real-time PCR)
RAPD	random amplified polymorphic DNA
SCAR	sequence characterized amplified regions
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
Taq	*Thermus aquaticus*

Introduction

Medicinal plants – quality requirements and adulterations

Quality requirements of plant materials used for medicinal purposes are regulated in many countries in national pharmacopoeias. In the European Union, in addition to the national pharmacopoeias the European Pharmacopoeia has become the principle regulatory document in pharmacy. The WHO (2013) lists 46 countries, including most of the European countries, possessing a national pharmacopoeia. In Mexico and Thailand pharmacopoeias dedicated to herbal medicines are published besides their standard national pharmacopoeias, and in Brazil, Germany and Mexico separate homeopathic pharmacopoeias exist containing many herbal drug monographs. Currently the European Pharmacopoeia (8^{th} edition; EDQM, 2015a) contains nearly 290 monographs of herbal drugs and herbal preparations including approx. 40 monographs of plants mainly used in traditional Chinese medicine and eleven homeopathic preparations. Herbal drugs should be free from impurities such as soil, dust, dirt and other biogenic contaminants (e.g. fungi, insects or other animal contaminations) but the European Pharmacopoeia allows a content of foreign matter of ≤2%, unless a specific limit is defined in the monographs (EDQM, 2015b). Each herbal drug monograph contains a definition of the appropriate plant material, particularly the definition of the plant species allowed to be used. The plant materials, including their frequent adulterations and the foreign matter, are commonly identified using macroscopic and microscopic descriptions and by chromatographic methods (primarily thin layer chromatography).

The possibility of adulteration is multifarious and can happen at each stage of the commodity supply chain, accidentally or deliberately. One can assume that a contamination of plant material with other plant species or even a substitution can happen due to several reasons. Initial, the presence of 'weeds' in many plant materials is ubiquitous and not avoidable. This can not only happen with cultivated plants, because the nested growth of many wild growing plants hampers the collection of unadulterated plant materials. Limited expertise may lead to the harvest of incorrect species or a joint harvesting of several morphologically similar species. Mislabelling, confusion or blending of batches may happen at each stage of trade. A confusion of species could also occur due to synonyms or homonyms of common names, e.g. 'black hellebore' is used for *Helleborus niger* and *Veratrum album* (Mader et al., 2011).

Product fraud appears for valuable products, which are adulterated or even substituted with cheaper materials. Fraud starts with increasing the amount of trade goods tolerating quality reduction, like the insufficient cleaning up of the plant materials resulting in i.a. too high proportions of foreign matter, such as undesired or valueless plant parts or species. As reported by e.g. De Mori (2015) two

trade samples of cut 'Salviae officinalis folium' mainly differed in their proportions of sage stems (up to approx. 20%), resulting in a lower essential oil content. Maybe the most profitable target for product fraud is saffron, one of the most expensive spices, where many fraud manners are known in production and trade. Amongst others, florets of several plant species, rhizomes of *Curcuma longa* or even powdered gypsum, chalk or dyes are used as adulterants to maximise profit (Hagh-Nazari and Keifi, 2007; Kanti et al., 2011).

Methods for DNA-based identification of trade samples

The standard analysis procedure of DNA-based identification of trade samples starts with sampling, followed by grinding the plant material and DNA extraction. In most cases, PCR is used to amplify target amplicons, which are either sequenced or visualized by different methods (e.g. gel electrophoresis or melting curve analysis).

In the last years many DNA-based assays were developed in order to authenticate plant samples or to detect their substitutes. Several reviews give an overview of different used identification methods for medicinal plants or herbal drugs (i.a. Ganie et al., 2015; Heubl, 2010; Joshi et al., 2004; Kiran et al., 2010; Sucher and Carles, 2008; Techen et al., 2014). DNA barcoding was applied to authenticate not only raw materials, but also to test the composition of herbal products including tablets and capsules (e.g. Newmaster et al., 2013; Stoeckle et al., 2011; Wallace et al., 2012).

DNA Extraction

The isolation and purification of DNA is the prerequisite for DNA-based techniques. DNA extraction from plants is relatively difficult compared to animals and bacteria, since the rigid cell wall needs to be broken up. Constituents of the primary and secondary cell wall like pectin (a structural heteropolysaccharide), cellulose and hemicelluloses (matrix polysaccharides) and lignin (a cross-linked phenol polymer) necessitate mechanical rupture such as grinding of the sample material or enzymatic cell wall degradation. Secondary metabolites, particularly abundant in medicinal plants and often interfering in subsequent techniques like PCR, additionally complicate the purification of the DNA and request a complete removal of those metabolites. Polysaccharides and polyphenols, e.g., can bind to DNA and interfere with further analysis (Pirttilä et al., 2001).

Many DNA extraction methods for plants were published in the last decades, including either 1) self-prepared extraction buffers and chemicals or 2) commercially available kits. 1) Cetyltrimethylammonium bromide (CTAB) is known to be a strong detergent and useful for DNA extraction. In solutions containing approx. 0.7 M NaCl, CTAB forms insoluble complexes with e.g. proteins and polysaccharides but not with nucleic acids (Murray and Thompson, 1980). Many variants of the CTAB

DNA extraction method exists, which include several additional chemicals like tris-(hydroxymethyl)-aminomethane (TRIS)-HCl (pH8) buffer, ethylenediaminetetraacetic acid (EDTA; deactivation of metal-dependent enzymes to suppress DNA damage), sodium dodecyl sulphate (SDS; cell lysis), *β*-mercaptoethanol (BME; denaturation of proteins) and/or polyvinylpyrrolidone (PVP; absorption of polyphenols and polysaccharides). Several protocols include the addition of proteinase K to digest proteins or RNase to degrade RNA. Phenol, chloroform, isoamyl alcohol and ethanol are often used for further purification. Although CTAB might be the most commonly used agent, also protocols based on e.g. SDS or PVP buffers exist. 2) Commercially available kits apply different basic principles, including silica gel particles or membranes or other DNA binding columns, magnetic separation or the principle of salt precipitation. The composition of included buffers and solutions is generally not proclaimed (compiled by Demeke and Jenkins, 2010).

Polymerase chain reaction (PCR)

PCR was invented in 1983 by Kary Mullis (Bartlett and Stirling, 2003). The principle of the method is the *in vitro* amplification of a defined DNA segment by a DNA polymerase. Primers are short single-stranded DNA (or RNA) molecules complementary to the target DNA. If the primers are annealed to a single-stranded DNA template, the polymerase binds and enzymatically assembles a new DNA strand composed of single nucleotides (deoxynucleoside triphosphates, dNTPs). DNA polymerases can elongate the 3'-OH group of the primer or the nascent DNA strand by condensing the 5'-phosphate group of dNTPs, hence the elongation is unidirectional.

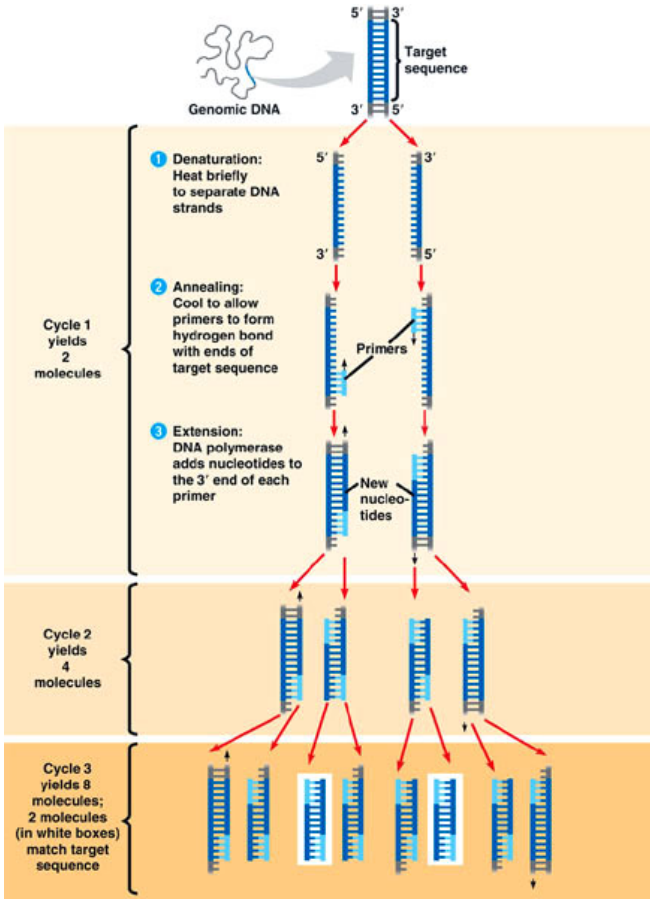

Figure 1: Principle of polymerase chain reaction. [Source: St. Rosemary Educational Institution (http://schoolworkhelper.net/pcr-uses-steps-purpose). Accessed: October 13th, 2015.]

A PCR (Figure 1) starts with an initial denaturation step for several minutes to separate double-stranded DNA to its single strands and to activate the poly-

merase (if applicable, 'hot-start polymerase'). Traditionally one PCR cycle consists of three temperature steps, namely the annealing step (primers bind to the template DNA), the elongation step (the polymerase synthesises the complementary strands), and the denaturation step (the DNA double-strands separate again). The temperature for each step can vary, e.g. the annealing temperature (approx. 37-60 °C) is dependent on the length and GC-content of the used primers. For the elongation commonly a temperature of 72 °C is applied, which is the optimal temperature for *Taq* polymerase activity. The denaturation step is commonly performed at 94-96 °C. For some purposes two-step cycles are performed, then the annealing and elongation take place at the same temperature. After approx. 30-45 cycles, occasionally a final extension step is performed to ensure that all DNA molecules are fully extended. Because each generated DNA strand can act as a template in the following cycles, the reaction is exponential. In the best case the number of DNA strands double in each cycle (Brown, 1999).

The **PCR efficiency** describes the formation rate of new DNA strands in one PCR cycle, 100% efficiency equals a doubling of DNA molecules per cycle. The PCR efficiency is in practice reduced due to several reasons. Some of them are influenced by the primer design (e.g. formation of primer dimers or hairpins), secondary structures of the amplicons, or mismatches in the primer binding sites. Additionally, unsuitable PCR conditions and the presence of inhibitors negatively influence the reaction (Wilson, 1997). Mismatches in the primer binding sites, especially close to the 3' end of the primers (1^{st} or 2^{nd} base), hamper the enzymatic elongation by *Thermus aquaticus* (*Taq*) polymerase in the early cycles of the PCR. Newly synthesized DNA strands contain incorporated primer molecules, thus the transcribed complementary strands have a matching primer binding site. In the following PCR cycles, where perfectly matching opposite strands exist, original primer-template mismatches have less effect on the amplification (Kalle et al., 2014; Lo, 1998; Piñol, 2015). Accordingly, in the amplification plot of a quantitative PCR (qPCR) the hampered elongation in the early cycles is visible as delayed fluorescence signal (a higher Cq value), but it can be assumed that the calculated PCR efficiency is similar to PCR reactions without mismatches. The PCR efficiency can be calculated with several methods, but the probably easiest and most common method is the calculation from the slope of a calibration curve. For this purpose, commonly tenfold dilution series of (pooled) samples are analysed, whereat the correlation between the Cq values (cycle of quantification) and the logarithm of the starting copy number of template DNA should remain linear for five orders of magnitude (Pfaffl, 2004).

PCR inhibition provokes reduced DNA amplification during PCR and can occur in individual samples or in the entire analysis. PCR inhibition can result in delayed amplification, reduced PCR efficiency, decreased PCR product concentration and even a change in the melting behaviour of the PCR products (Opel et al., 2010). Inhibition can be categorised in three essential classes: 1. interference of cell lyses during the DNA extraction, 2. direct inhibition of polymerase enzymes and 3. interference by nucleic acid degradation or capture. Several chemicals (e.g. common DNA extraction ingredients like CTAB, EDTA, SDS, isopropanol, phenol or ethanol), DNA binding or digesting proteins, poly-saccharides or other secondary metabolites are well known to inhibit the PCR, although the mode of action is not known for each substance. Medicinal plants often contain high amounts of secondary metabolites which are desired for the pharmaceutical applications but interfere in molecular approaches. An adaption or change of the DNA extraction protocol depending on the sample matrix may increase the DNA quality and purity and hence reduce the PCR inhibition. E.g. the use of PVP in the extraction buffer reduces polyphenols and polysaccharides, washing steps with 70% ethanol or other solvents remove chemicals of the initially used extraction buffer and e.g. soluble secondary metabolites, and the drying of the DNA pellet removes the solvents, which themselves are inhibiting (Demeke and Jenkins, 2010; Wilson, 1997). An additional purification of DNA extracts with e.g. silica based spin columns causes a loss of DNA templates, but is possible for samples with sufficient DNA content. Samples with high DNA concentrations can also be diluted with water, what dilutes also the concentration of inhibitors. An increased concentration of magnesium, an important co-factor of polymerase enzymes, or additional BSA (bovine serum albumin) in the PCR reactions can improve the enzyme activity in some cases (Opel et al., 2010; Schmiderer et al., 2013).

Amplification Refractory Mutation System (ARMS)

ARMS or allele specific PCR (Lo, 1998; Wu et al., 1989) is a special case of PCR, where primers are designed to discriminate among alleles, differing in only one base. This type of PCR exploits of the inability of *Taq* polymerases to elongate primer 3' ends which are not properly bound to the templates. Hence mutations selected for allele specificity need to be located in the very 3' end of the primer molecules. Anyhow, depending on the base pairing of the mismatch, the polymerase is able to incidentally elongate the primer and assemble a new DNA strand to a certain degree. An arbitrary introduction of a second mismatch within the last four bases of the primer 3' end dramatically decreases the amplification, making the primer much more allele specific (Kwok et al., 1990). As far as the positive amplification indicates the presence of the respective allele, the amplification and diagnostic step are combined (Lo, 1998). If two allele specific primers are designed in this way, that the amplicons differ in their length, the PCR results can be analysed with agarose gels.

RAPD and SCAR Markers

The PCR based randomly amplified polymorphic DNA (RAPD) technique is conceptually very simple and easy to perform. In contrast to a standard PCR, the PCR protocol for RAPD analysis includes one random oligonucleotide primer. Normally short primers with commonly 10 nt in length are used, which bind many times in the whole genome. If two primer binding sites of inverted orientation are close to each other, the amplification of interjacent DNA fragments of variable lengths can take place. The amplification products can be separated and visualised on agarose gels (Hadrys et al., 1992; Williams et al., 1990). Although this method is very fast and cheap, the lack of reproducibility is a huge disadvantage. The applied concentrations of PCR chemicals (e.g. primers, buffers and magnesium chloride), concentration and quality of template DNA and changes in the temperature profile of the PCR (including heating and cooling rates) affect the amplified PCR products and therefore the results of the DNA fingerprints (Power, 1996).

SCAR markers (sequence characterized amplified regions) can be derived from cloned and sequenced RAPD fragments. Based on the RAPD sequence, specific primers with commonly 20-25 nt length are designed to amplify an informative part of the DNA fragment. The SCAR-primers can be used to distinguish a target species from other related species by the amplification of a single, distinct DNA band from the target species only. The specificity of the primers allow a high reproducibility, so – in contrast to RAPD – minor changes in the PCR chemistry or in the PCR conditions should not influence the DNA amplification, and the developed SCAR markers can be easily transferred to other labs (Kiran et al., 2010).

High-resolution melting curve analysis (HRM)

Melting curve analysis (MCA) of PCR products was introduced on the LightCycler® nearly twenty years ago (Ririe et al., 1997; Wittwer et al., 1997). The melting behaviour of PCR products was and still is monitored using either fluorescence labelled probes or intercalating fluorescence dyes. High-resolution melting curve analysis (HRM or HRMA) is an enhancement of this technique using intercalating fluorescence dyes and qPCR machines able to regulate the temperature finely graduated (approx. 0.1 °C/s) and uniform inside the reaction chamber. During a slow temperature increase after the PCR, the melting of PCR products can be observed by the decreasing fluorescence. The melting behaviour of double-stranded DNA into its single strands is dependent on length, GC-content and the base composition of DNA molecules (Hermann et al., 2006; Vossen et al., 2009). For the analysis of single nucleotide polymorphisms (SNPs) generally short amplicons of less than 100 bp are preferred, but HRM analysis of longer fragments is also possible and is used in combination with e.g. DNA barcoding (Jaakola et al., 2010; Liew et al., 2004; Madesis et al., 2012).

Sanger-Sequencing

DNA sequencing is the determination of the order of nucleotides in DNA molecules. Sanger sequencing (Sanger et al., 1977) relies on base-specific chain terminations during DNA synthesis, caused by the incorporation of ddNTPs (2',3'-dideoxynucleotide triphosphates). Originally, four separate reactions were needed, including all four dNTPs (2'-deoxynucleotide triphosphate) and one ddNTP, respectively, in a minute amount. As far as polymerases cannot elongate a newly synthesised DNA strand after an incorporated ddNTP, the extension stops. As the concentration of ddNTPs is much lower than that of the dNTPs, the termination happens rarely and incidentally, resulting in many products of different lengths. A labelling of the ddNTPs or primers with radioactive phosphorus or sulphur isotopes enabled the detection of each product by radiography. The products were separated on polyacrylamide gels, and the sequence could be achieved by combining the results of the four corresponding ddNTP-reactions. Nowadays the four ddNTPs are labelled with different fluorescent dyes and are used in a joint reaction, the DNA fragments are separated in glass capillaries filled with polymers and are detected by computer guided lasers (Men et al., 2008).

The result of a sequencing reaction is depicted as a sequence chromatogram, where each base of the DNA sequence is according to one peak in the chromatogram. Commonly each nucleotide is displayed in a different colour, facilitating the clarity and so the manual data assessment. Disregarding sequencing artefacts and handling mistakes, sequence chromatograms can show polymorphic position. The sporadic appearance of double-peaks can be either caused by heterozygous, diploid or polyploid DNA or by the mixture of samples with slightly different sequences, such as closely related species. The appearance of several to many polymorphic positions can indicate the presence and co-amplification of at least two, more distant related or even unrelated species (personal observation).

Next Generation Sequencing (NGS)

The Human Genome Project, finished in 2003, needed 13 years and 3 billion USD for sequencing the first whole human genome with the Sanger sequencing method (Grada and Weinbrecht, 2013). In the last two decades, several next generation sequencing (NGS) techniques were developed, which enable a much faster and cheaper data acquisition (Kircher and Kelso, 2010; Metzker, 2010). In 2015, the price for sequencing a human genome, comprising approx. 3 billion bp, dropped to approx. 1.400 USD (NIH, 2015). Illumina Inc. advertised in 2014 the acquisition of up to 600 Gb within 11 days using the HiSeq Sequencing System (Illumina Inc., 2014). Among the NGS techniques, only two concepts have been widely used for scientific publications, namely pyrosequencing and Illumina sequencing[1].

Pyrosequencing,also described as sequencing by synthesis or sequencing during extension, was developed in 1996 and exclusively licensed to 454 Life Sciences (2007 acquired by Roche Diagnostics), which released the first NGS platform to the market in 2005 (Kircher and Kelso, 2010). For sequencing a whole genome a DNA library needs to be prepared before the actual sequencing procedure. The sample DNA is broken in fragments of approx. 400-600 bp and adapters are attached to both ends of these fragments. Then the double-stranded DNA is separated into its single strands. Through the process of emulsion-based clonal amplification, the library DNA binds to capture beads and is amplified by PCR, resulting in thousands of copies fixed on one bead. Most beads contain only one type of DNA fragments, beads with no fragments are sorted out before the sequencing reaction, signals of beads with more fragment types are filtered out during the signal processing. The beads and the enzymes, necessary for the following reactions, are transferred to a PicoTiter^{TM} plate. One plate comprises approx. 1.6 million wells, each with a volume of 75 pL, hence suitable for exactly one single bead. For the pyrosequencing reaction, sequencing primers bind to the adapter sequences and the nucleotides are separately, cyclically washed over the plate. When a complementary nucleotide is incorporated in the newly synthesised DNA strand by the DNA polymerase, one pyrophosphate is released. The ATP sulfurylase converts released pyrophosphate to ATP, which is a substrate for luciferase and cause a detectable light emission. The emitted light of each well is captured by a high-resolution charge-coupled device (CCD) camera. To a certain degree the intensity of the emitted light is correlated with the number of incorporated nucleotides. Homopolymers of more than 10 nt (e.g. a poly-A) cannot be unambiguously resolved with this method. Pyrosequencing delivers sequences with an average length of 300-500 nt, but with maximum lengths of up to 1000 nt. Compared to Sanger sequencing, the average substitution error rate of single nucleotides is approx. ten times higher (1:1,000-1:10,000) in pyrosequencing, but such problems can be resolved by a higher coverage (454 Life Sciences, 2015; Kircher and Kelso, 2010; Roche Diagnostics, 2011).

Illumina sequencing uses a sequencing by synthesis concept combined with a reversible terminator technology. The sample preparation is done in a different way than for pyrosequencing, but a DNA library with two adapters is also required. The templates are fixed on the flow cell surface and a solid phase amplification (‘bridge amplification’) generates approx. 10 million clusters per square centi-metre, each of up to 1000 identical template copies. The sequencing is performed with different fluo-rescent labelled nucleotides jointly provided for the reaction, and the nucleotide label terminates the elongation. In every cycle one complement dNTP is incorporated into a synthesised DNA strand and the fluorescence is imaged in parallel for each cluster to identify the incorporated bases. Afterwards

[1] Scopus (accessed November 23rd, 2015) listed 9.823 documents containing the search item “pyrosequencing” and 1032 documents for “Illumina sequencing”, but only 10 to 94 documents for “Polonator”, “Heliscope”, “SOLiD sequencing” or “Pacific Biosciences” (considered search fields: “article title, abstract, keywords”).

the fluorescent dye is enzymatically cleaved and in the following cycle the next dNTP can be incorporated. The read length increased from initially 26 nt in 2007 to 100 nt in 2014 (Illumina Inc., 2010 and 2014; Kircher and Kelso, 2010). Kircher and Kelso reported in 2010 the average error rate of 1:100-1:1,000 but a higher daily throughput with a lower price per mega base than for pyrosequencing. According to Illumina (2010) the base-by-base sequencing prevents sequence-context specific errors, like in repetitive sequences or in long homopolymers, as they appear in other sequencing techniques.

DNA barcoding

The idea of DNA barcoding arose in the beginning of the last decade and purposed the use of DNA sequences of one or few loci in order to assign unknown individuals to species and to facilitate the discovery of new species (Hebert et al., 2003; Stoeckle, 2003). The term 'molecular barcode' was used in 2002 by Floyd et al. for the identification of soil nematodes by using the DNA sequence of the small subunit ribosomal RNA (SSU) gene (Floyd et al., 2002). Hebert et al. (2003) established the mitochondrial cytochrome *c* oxidase I (COI) gene as universal DNA barcode for animals. Finding such a single barcode for plants proved to be more difficult. Requirements for an optimal barcode are 1) a good PCR amplification with a single primer pair, 2) the possibility of bidirectional sequencing with a minimum need of manual editing and 3) a maximal discriminatory power. So far no plant DNA locus was found which fulfils all three requirements, hence a combination of two or more loci was recommended, e.g. rbcL in combination with matK (Hollingsworth et al., 2009) or rbcL with psbA-trnH (Kress and Erickson, 2007). Li et al. (2011) suggested to additionally utilise the nuclear ITS as barcode for angiosperms. Although the use of ITS has three potential problems (the possibility of co-amplification of fungal DNA, the presence of paralogous gene copies and a relatively low recovery rate of sequences; Hollingsworth, 2011) Li et al. (2011) demonstrated that in plant groups where direct sequencing of ITS is possible, ITS had a higher discriminatory power than the chloroplast markers rbcL, matK or psbA-trnH. In cases where the amplification and sequencing of the whole ITS region is difficult, the analysis of only ITS2 is easier but has still a notable discriminatory power (Chen et al., 2010; Li et al., 2011).

In the last years 'DNA metabarcoding' combined the idea of DNA barcoding with NGS methods, in order to analyse the composition of heterogeneous sample mixtures or environmental samples with complex communities, like soil fungal communities (Schmidt et al., 2013), marine communities (Guardiola et al., 2015; Sun et al., 2015), diet analysis (Lopes et al., 2015) or the floral composition of honey (Hawkins et al., 2015). For such purposes a conventional PCR is performed to amplify the desired DNA barcodes and the amplification products are subsequently used for NGS.

To assign unknown individuals to species, the obtained barcode sequences are compared with corresponding sequences of identified individuals. E.g. the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI; Bethesda, USA) enables an easy comparison of own data with the whole GenBank database and provides simple calculations of sequence similarities (query coverage and sequence identity).

Aim of the work

The studies presented in this thesis took place in the frame of four projects, whereas at least a part of each project was dealing with the DNA-based identification of medicinal plants or plants used as food supplements. In all cases it was requested to find methods in order to identify the species of interest and to detect possible adulterations. Because minor adulterations are in general tolerated, an additional aim was to find quantification methods for possible adulterants.

Prerequisite for DNA analyses is the extraction of DNA with adequate purity, quality and quantity. For the application as standard testing methods, a standardisation of the DNA extraction and of DNA extracts would be preferred to simplify the handling of diverse samples. Hence the first paper includes a comparison of five plant DNA extraction methods and the application of different DNA concentrations introduced to PCR and their effect on the amplification success (Schmiderer et al., 2013).

The papers 2-4 include identification approaches based on HRM analysis for *Helleborus niger* (Ranunculaceae; Schmiderer et al., 2010), *Calendula officinalis* (Asteraceae; Schmiderer et al., 2015a) and *Peucedanum ostruthium* (syn. *Imperatoria ostruthium*, Apiaceae; Schmiderer et al., 2015b). The *Peucedanum* approach was the most challenging one, because some reported adulterants belong to different plant families and are either poisonous (*Aconitum napellus*, Ranunculaceae; *Veratrum album*, Melanthiaceae) or have a noticeable bitter taste (*Gentiana* spp., Gentianaceae). So it was necessary to find a method which is able to detect even traces of this undesired species.

For the identification of *Valeriana officinalis* samples a multiplex-ARMS (developed by my colleague Joana Ruzicka) and a HRM approach were designed, both based on the same SNP located in the atpB-rbcL intergenic spacer. For the detection of *Veratrum album*, which was previously reported as an adulterant of Valerianae radix, a multiplex PCR with HRM was designed (Ruzicka et al., 2016).

Paper 6 (mainly prepared by my former colleague Eduard Mader) includes an attempt to quantify mixtures of one known species with several other ‘unknown’ species and to quantify mixtures of two known species down to very low proportions (Mader et al., 2011).

The last paper deals with DNA metabarcoding of two Salviae officinalis folium (Lamiaceae) trade samples, which were characterised by different essential oil contents (approx. 1.5 and 2.1%, respectively). The initial assumption included the questions, if the unequal essential oil contents were caused by a noticeable admixture of cheaper ‘inconspicuous’ plant material and in general which plants were present in the samples (Lukas et al., submitted).

References

454 Life Sciences (original publication date unknown): How is genome sequencing done? http://www.454.com/downloads/news-events/how-genome-sequencing-is-done_FINAL.pdf. Accessed: November 3rd, 2015.

Bartlett JMS, Stirling D (2003): A Short History of the Polymerase Chain Reaction. PCR Protocols. 2nd Edition. Methods in Molecular Biology 226; pp. 3-6.

Brown TA (1999): Moderne Genetik. 2nd Edition. Heidelberg, Berlin: Spektrum Akademischer Verlag; p. 431.

Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y et al. (2010): Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS ONE* 5 (1): e8613. doi:10.1371/journal.pone.0008613.

Demeke T, Jenkins GR (2010): Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology derived traits. *Anal Bioanal Chem* 396: 1977-90.

De Mori A (2015): Analisi morfologiche, fitochimiche e genetiche di due campioni commerciali di *Salvia officinalis* [Morphological, phytochemical and genetic analyses of two *Salvia officinalis* trade samples]. Diploma thesis, Universitá degli studi di Trieste.

EDQM (European Directorate for the Quality of Medicines and Health Care, 2015a): European Pharmacopoeia 8.5, online version (http://online6.edqm.eu/ep805/). Accessed: July 2nd, 2015.

EDQM (European Directorate for the Quality of Medicines and Health Care; 2015b): Europäisches Arzneibuch 8.0. Amtliche Österreichische Ausgabe. Vol. 1. Pflanzliche Drogen (1433). 8th Edition; Wien: Verlag Österreich; p. 1159.

Floyd R, Abebe E, Papert A, Blaxter M (2002): Molecular barcodes for soil nematode identification. *Mol Ecol* 11 (4): 839-50.

Ganie SH, Upadhyay P, Das S, Prasad Sharma M (2015): Authentication of medicinal plants by DNA markers. *Plant Gene* 4: 83-99.

Grada A, Weinbrecht K (2013): Next-Generation Sequencing: Methodology and Application. *J Invest Dermatol* 133: e11. doi:10.1038/jid.2013.248.

Guardiola M, Uriz MJ, Taberlet P, Coissac E, Wangensteen OS, Turon T (2015): Deep-Sea, Deep-Sequencing: Metabarcoding extracellular DNA from sediments of marine canyons. *PLoS One* 10 (10): e0139633. doi:10.1371/journal.pone.0139633.

Hadrys H, Balick M, Schierwater B (1992): Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol Ecol* 1: 55-62.

Hagh-Nazari S, Keifi N (2007): Saffron and various fraud manners in its production and trades. *Acta Hortic* 739: 411-6.

Hawkins J, de Vere N, Griffith A, Ford CR, Allainguillaume J, Hegarty MJ et al. (2015): Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences. *PLoS One* 10 (8): e0134735. doi:10.1371/journal.pone. 0134735.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003): Biological identifications through DNA barcodes. *Proc R Soc B: Biological Sciences* 270 (1512): 313-21.

Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV (2006): Amplicon DNA melting analysis for mutation scanning and genotyping: Cross-platform comparison of instruments and dyes. *Clin Chem* 52 (3): 494-503.

Heubl G (2010): New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. *Planta Med* 76: 1963-74.

Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, van der Bank M, Chase MW et al. (2009): A DNA barcode for land plants. *Proc Natl Acad Sci USA* 106 (31): 12794-7.

Hollingsworth PM (2011): Refining the DNA barcode for land plants. *Proc Natl Acad Sci USA* 108 (49): 19451-2.

Illumina Inc. (2010): Illumina sequencing technology. Technology spotlight: Illumina® sequencing. https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing. pdf. Illumina Inc., San Diego, CA, USA.

Illumina Inc. (2014): HiSeq™ Sequencing systems. Specification sheet: Illumina® Sequencing. http://www.illumina.com/documents/products/datasheets/datasheet_hiseq_systems.pdf. Illumina Inc., San Diego, CA, USA.

Jaakola L, Suokas M, Häggman H (2010): Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analyses of berry species. *Food Chem* 123: 494-500.

Joshi K, Chavan P, Warude D, Patwardhan B (2004): Molecular markers in herbal drug technology. *Curr Sci* 87 (2): 159-65.

Kalle E, Kubista M, Rensing C (2014): Multi-template polymerase chain reaction. *Biomolecular Detection and Quantification* 2: 11-29.

Kanti R, Sushma K, Gandhi R (2011): Adulteration and marketing of saffron (*Crocus sativus* Linn.). *Journal of Tropical Medicinal Plants* 12 (1): 135-40.

Kiran U, Khan S, Mirza KJ, Rama M, Abdin MZ (2010): SCAR markers: A potential tool for authentication of herbal drugs. *Fitoterapia* 81: 969-76.

Kircher M, Kelso J (2010): High-throughput DNA sequencing – concepts and limitations. *BioEssays* 32 (6): 524-36.

Kress WJ, Erickson DL (2007): A two-locus global DNA barcode for land plants: The coding rbcL gene complements the non-coding trnH-psbA spacer region. *PLoS One* 2 (6): e508. doi:10.1371/journal.pone.0000508.

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky JJ (1990): Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type I model studies. *Nucl Acids Res* 18: 999-1005.

Li D-Z, Gao L-M, Li H-T, Wang H, Ge X-J, Liu J-Q, Chen Z-D et al. (2011): Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proc Natl Acad Sci USA* 108 (49): 19641-6.

Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C (2004): Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem* 50: 1156-64.

Lo YMD (1998): The Amplification Refractory Mutation System. *Methods Mol Med* 16: 61-9.

Lopes CM, De Barba M, Boyer F, Mercier C, da Silva Filho PJS, Heidtmann LM, Galiano D, et al. (2015): DNA metabarcoding diet analysis for species with parapatric *vs.* sympatric distribution: a case study on subterranean rodents. *Heredity* 114: 525-36.

Lukas B, Schmiderer C, Novak J (submitted): DNA-Metabarcoding as a tool for quality control of botanicals. *Food Control*, submitted April 14th, 2015.

Mader E, Ruzicka J, Schmiderer C, Novak J (2011): Quantitative high-resolution melting analysis for detecting adulterations. *Anal Biochem* 409: 153-5.

Madesis P, Ganopoulos I, Anagnostis A, Tsaftaris A (2012): The application of Bar-HRM (Barcode DNA-High Resolution Melting) analysis for authenticity testing and quantitative detection of bean crops (Leguminosae) without prior DNA purification. *Food Control* 25: 576-82.

Men AE, Wilson P, Siemering K, Forrest S (2008): Sanger DNA Sequencing. In: Janitz M (Ed.): Next-generation genome sequencing: Towards personalized medicine. Weinheim: Wiley-VCH; pp. 1-11.

Metzker ML (2010): Sequencing technologies – The next generation. *Nat Rev Genet* 11 (1): 31-46.

Murray MG, Thompson WF (1980): Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8 (19): 4321-5.

Newmaster SG, Grguric M, Shanmughanandhan D, Ramalingam S, Ragupathy S (2013). DNA barcoding detects contamination and substitution in North American herbal products. *BMC Med* 11: 222. doi:10.1186/1741-7015-11-222.

NIH (National Institute of Health) (2015): DNA Sequencing Costs. www.genome.gov/sequencingcosts/. Last Updated: October 2nd, 2015. Accessed: November 23rd, 2015.

Opel KL, Chung D, McCord BR (2010): A study of PCR inhibition mechanisms using real time PCR. *Forensic Sci* 55 (1): 25-33. doi: 10.1111/j.1556-4029.2009.01245.x.

Pfaffl MW (2004): Quantification strategies in real-time PCR. In: Bustin SA (Ed.): A-Z of quantitative PCR. La Jolla (CA, USA): International University Line; pp. 87-112.

Piñol J, Mir G, Gomez-Polo P, Agustí N (2015): Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Mol Ecol Resour* 15: 819-30.

Pirttilä AM, Hirsikorpi M, Kämäräinen T, Jaakola L, Hohtola A (2001): DNA isolation methods for medicinal and aromatic plants. *Plant Mol Bio Rep* 19: 273a–f.

Power EGM (1996): RAPD typing in microbiology – A technical review. *J Hospital Infection* 34: 247-65.

Ririe KM, Rasmussen RP, Wittwer CT (1997): Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* 245: 154-60.

Roche Diagnostics (2011): GS FLX+ System. Sanger-like read lengths – The power of next-gen throughput. http://454.com/downloads/GSFLXApplicationFlyer_FINALv2.pdf. Accessed: November 25th, 2015.

Ruzicka J, Schmiderer C, Novak J (2016): DNA-based identification of *Valeriana officinalis* s.l.: a Multiplexed Amplification-Refractory Mutation System (MARMS) and High Resolution Melting Curve Analysis (HRMA). *Plant Mol Bio Rep*, Online First: DOI 10.1007/s11105-015-0967-6.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463-7.

Schmiderer C, Mader E, Novak, J (2010): DNA-based identification of *Helleborus niger* by high-resolution melting analysis. *Planta Med* 76 (16): 1934-7.

Schmiderer C, Lukas B, Novak, J (2013): Effect of different DNA extraction methods and DNA dilutions on amplification success in the PCR of different medicinal and aromatic plants. *Z Arznei-Gewürzpfla* 18(2):65-72.

Schmiderer C, Lukas B, Ruzicka J, Novak J (2015a): DNA-based identification of *Calendula officinalis* (Asteraceae). *Appl Plant Sci* 3 (11): 1500069. http://dx.doi.org/10.3732/apps.1500069.

Schmiderer C, Ruzicka J, Novak J (2015b): DNA-based identification of *Peucedanum ostruthium* specimens and detection of common adulterants by high-resolution melting curve analysis. *Mol Cell Probes* 29 (6): 343-50.

Schmidt P-A, Bálint M, Greshake B, Bandowa C, Römbke J, Schmitt I (2013): Illumina metabarcoding of a soil fungal community. *Soil Biol Biochem* 65: 128-32.

Stoeckle M (2003): Taxonomy, DNA, and the bar code of life. *BioScience* 53 (9): 796-7.

Stoeckle MY, Gamble CC, Kirpekar R, Young G, Ahmed S, Little DP (2011): Commercial teas highlight plant DNA barcode identification successes and obstacles. *Sci Rep* 1: 42. doi: 10.1038/ srep00042.

Sucher NJ, Carles MC (2008): Genome-based approaches to the authentication of medicinal plants. *Planta Med* 74 (6): 603-23.

Sun C, Zhao Y, Li H, Dong Y, Maclsaac HJ, Zhan A (2015): Unreliable quantitation of species abundance based on high-throughput sequencing data of zooplankton communities. *Aquat Biol* 24: 9-15. doi: 10.3354/ab00629.

Techen N, Parveen I, Pan Z, Khan IA (2014): DNA barcoding of medicinal plant material for identification. *Curr Opin Biotechnol* 25: 103-10.

Vossen RHAM, Aten E, Roos A, den Dunnen JT (2009): High-Resolution Melting Analysis (HRMA) – More than just sequence variant screening. *Hum Mutat* 30: 860-6.

Wallace LJ, Boilard SMAL, Eagle SHC, Spall JL, Shokralla S, Hajibabaei M (2012): DNA barcodes for everyday life: Routine authentication of Natural Health Products. *Food Res Int* 49: 446-52.

WHO (World Health Organization; 2013): Index of Pharmacopoeias. http://www.who.int/medicines/ publications/pharmacopoeia/pharmacopoeias_index_29082013.pdf. Accessed: September 25th, 2015.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18: 6531-5.

Wilson IG (1997): Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* 63 (10): 3741-51.

Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ (1997): The LightCycler: A microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* 22: 176-81.

Wu DY, Ugozzoli L, Pal BK, Wallace RB (1989): Allele-specific enzymatic amplification of beta-globin genomic DNA for diagnosis of sickle cell anemia. *Proc Natl Acad Sci USA* 86:2757-60.

Paper 1

Schmiderer C, Lukas B, Novak J (2013): Effect of different DNA extraction methods and DNA dilutions on the amplification success in the PCR of different medicinal and aromatic plants. *Zeitschrift für Arznei- und Gewürzpflanzen* 18 (2): 65-72.

Author's contributions:

BL performed the lab work.

CS and JN analysed the data and prepared the manuscript.

C. Schmiderer, B. Lukas and J. Novak

Effect of different DNA extraction methods and DNA dilutions on the amplification success in the PCR of different medicinal and aromatic plants

Introduction

DNA-based identification of biological specimen has become a widely estimated technique in many areas like forensic genetics (special crime scene investigation, disaster victim identification, paternity, relationships) (13), and microbiology (1). In the European Pharmacopoeia, DNA-based methods are already described in the chapter Methods of analysis (19) with their application in microbiology (2).

Due to the multitude of species used as medicinal and aromatic plants and sometimes difficulties to identify processed herbal materials, DNA based methods are becoming increasingly interesting (8, 11). The first prerequisite for a routine identification method is that the method should be applicable to the material in trade, which is in the case of medicinal and aromatic plants mostly dried material of different plant organs and at different processed stages.

The first step in DNA based techniques is the isolation and purification of the DNA. DNA extraction from plants is regarded as difficult since the plant cell wall has to be cracked to isolate the DNA. Furthermore, some plant primary and secondary compounds influence PCR reactions negatively by either interfering with cell lysis, by nucleic acid

Abstract

DNA-based methods to proof identity are becoming increasingly popular. In a routine laboratory it would be desirable to have only as few as possibly different protocols. Therefore we tested the applicability of only one standard polymerase chain reaction (PCR) protocol in nine different plant species, five different DNA extraction methods and three DNA dilutions. DNA was extracted from roots and rhizomes of *Valeriana officinalis*, roots of *Panax ginseng*, leaves of *Mentha* x *piperita* and *Salvia officinalis*, flower heads of *Matricaria chamomilla*, (pseudo-)fruits of *Foeniculum vulgare* and *Juniperus communis* and bark of *Salix* sp. and *Cinnamomum verum*. PCR conditions were kept constant.

Three extraction methods were commercial DNA extraction kits (Nucleospin® Plant (Macherey-Nagel), DNeasy® Plant Mini Kit (Qiagen) and Wizard® Genomic DNA Purification Kit (Promega)), two methods were based on CTAB. The amount of extracted DNA was measured with a fluorimetric method and the applicability was tested by amplifying two DNA loci that became standard in molecular taxonomy with standard primers, the plastid *psbA-trnH* intergenic spacer and the nuclear internal transcribed spacer region (ITS).

The results showed that the DNA concentration used in PCR should be kept variable and that the concentration is in dependency on the DNA amount and quality as well as the amount of secondary compounds remaining in the DNA extract. The quality of the sample material had a higher influence on the PCR amplification success than the choice of the extraction method, which was between 67 % and 100 % depending on the extraction method and the amplified locus. Furthermore, shorter amplification products showed a higher success rate than longer amplification products.

Keywords

Aromatic plants, *Cinnamomum verum*, DNA extraction, DNA locus amplification, *Foeniculum vulgare*, *Juniperus communis*, *Matricaria chamomilla*, medicinal plants, *Mentha* x *piperita*, *Panax ginseng*, *Salix*, *Salix purpurea*, *Salvia officinalis*, *Valeriana officinalis*

degradation or capture or inhibiting polymerase activity for amplification of target DNA (4, 21). Since medicinal and aromatic plants are used because of their abundance of secondary compounds, this group of plants is probably the most difficult to purify DNA from.
In the last decade many protocols for DNA extraction from plants were published and ready-to-use plant DNA extraction kits are available from many suppliers (4). The DNA extraction kits used in this study were restricted to one very fast DNA extraction kit (Wizard® Genomic DNA Purification Kit) and two silica-gel-membrane based systems (Nucleospin® Plant, DNeasy® Plant Mini Kit), because these later two methods are specially adapted to plant material and frequently used. Additionally two inexpensive CTAB-based protocols were included in this study.
The aim of this work was to analyse the applicability of the five selected DNA extraction methods followed by a standard PCR assay on medicinal and aromatic plants representative of the different plant organs in use without any optimisation for any of the samples. The applicability was tested by amplifying for each sample two DNA loci that became standard in molecular taxonomy, one from the plastid genome (*psbA-trnH*) and one from the nuclear genome (ITS) with primers that are supposed to bind to the DNA of all plant species (12). The only variables introduced were five different DNA extraction methods and three different DNA concentrations in the PCR reactions.

Materials and methods

Samples

Single drug preparations of nine different medicinal plant species were chosen for the analysis *(Tab. 1)*. To consider various plant matrices preparations from roots (*Valeriana officinalis*, *Panax ginseng*), leaves (*Mentha* x *piperita*, *Salvia officinalis*), flower heads (*Matricaria chamomilla*), fruits (*Foeniculum vulgare*), berry-like cones (*Juniperus communis*) and bark (*Salix* sp., *Cinnamomum verum*) were included. Each species was represented by one dried sample purchased from a Viennese pharmacy and one (*Valeriana officinalis* and *Salix* sp.) or two commercial samples purchased from local supermarkets and drug stores.
Additionally, fresh material of six species (*V. officinalis*, *M.* x *piperita*, *S. officinalis*, *M. chamomilla*, *F. vulgare* and *Salix* sp.) was collected at the Botanical Garden of the University of Veterinary Medicine Vienna. A part of the fresh sample was used for direct DNA extraction (only with DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany)), the other part of the samples was dried at room temperature to determine the DNA quantity in ng/mg dry mass.

Sample preparation

A scalpel was used to pre-cut fresh samples, large pieces of roots, the flower heads of *M. chamomilla* and the cones of *J. communis*. A representative sample size of each crude drug was ground to fine powder (fresh material resulted in a pulp-like consistency) in a ball mill (Pulverisette, Fritsch, Idar-Oberstein, Germany). For DNA extraction, about 20 mg of dried samples or 100 mg of fresh samples were transferred to sterile 2 mL reaction tubes.

DNA extraction

All samples were extracted in duplicates using five different extraction methods.

Commercial DNA extraction kits

Three methods were based on the commercial DNA extraction kits Nucleospin® Plant (Macherey-Nagel, Düren, Germany), DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) and Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). DNA extraction was performed according to the manufacturers' instructions (for Nucleospin® Plant using lysis buffer C0). The DNA was solved in 100 μL (Nucleospin, Wizard) or 200 μL (DNeasy) supplied buffer, respectively. The Wizard Kit is based on a four-step process, i.e. (1) lysis of cells and nuclei, (2) RNase digestion step, (3) removal of proteins and (4) DNA concentration by precipitation. The Nucleospin and DNeasy kit, respectively, are using silica-gel-membrane and simple spin procedures to isolate DNA.

CTAB method I

The CTAB[I] extraction protocol I was modified from the protocol published by Doyle and Doyle (7). Per sample 1 mL CTAB extraction buffer (containing 27.4 mM CTAB, 0.7 M NaCl, 10 mM TRIS[II]-HCl pH 8, 1 mM EDTA[III] pH 8,

Der Einfluss verschiedener DNS-Extraktionsmethoden und Verdünnungen auf den Amplifikationserfolg in der PCR von verschiedenen Arznei- und Gewürzpflanzen

Zusammenfassung

DNS-basierende Methoden zum Nachweis der Identität gewinnen zunehmend an Bedeutung. Daher untersuchten wir die Anwendbarkeit eines Polymerase-Kettenreaktion (PCR)-Standardprotokolls mit fünf verschiedenen DNS-Extraktionsmethoden und drei DNS-Verdünnungen. DNS wurde aus Wurzeln und Rhizomen von *Valeriana officinalis,* Wurzeln von *Panax ginseng*, Blättern von *Mentha* x *piperita* und *Salvia officinalis*, Blütenköpfchen von *Matricaria chamomilla,* (Pseudo-)Früchten von *Foeniculum vulgare* und *Juniperus communis* und Rinde von *Salix* sp. und *Cinnamomum verum* extrahiert. Die PCR-Bedingungen wurden für alle Arten konstant gehalten.
Drei Extraktionsmethoden basierten auf im Handel erhältlichen Extraktionskits (Nucleospin® Plant von Macherey-Nagel, DNeasy® Plant Mini Kit von Qiagen und Wizard® Genomic DNA Purification Kit von Promega), zwei Extraktionsmethoden basierten auf der CTAB Reagenz. Die Menge der extrahierten DNS wurde fluorimetrisch vermessen und die Verwendbarkeit wurde durch die PCR-Vervielfältigung zweier DNS Loci getestet, die in der molekularen Taxonomie häufig verwendet werden, nämlich dem *psbA-trnH* Intergenic Spacer aus der Plastiden-DNS und der Internal Transcribed Spacer Region (ITS) der Zellkern-DNS.
Die Ergebnisse zeigten, dass die DNS-Konzentration, die in einer PCR eingesetzt werden soll, von der Ausbeute und den verbleibenden Resten an Sekundärstoffen abhängig ist und daher in Prüfvorschriften variabel sein sollte. Die Art und Qualität des Probenmaterials hatte einen höheren Einfluss auf den PCR-Erfolg als die Wahl der Extraktionsmethode. Ausserdem ist der Amplifikationserfolg noch von der Länge des Amplifikationsproduktes abhängig, kürzere Produkte lassen sich besser als längere Produkte amplifizieren. Der PCR-Erfolg lag in Abhängigkeit von der Extraktionsmethode und dem amplifizierten Genlokus zwischen 67 % und 100 %.

Schlagwörter

Arzneipflanzen, *Cinnamomum verum*, DNS-Extraktion, DNS-Lokus-Amplifikation, *Foeniculum vulgare*, Gewürzpflanzen, *Juniperus communis*, *Matricaria chamomilla*, *Mentha* x *piperita*, *Panax ginseng*, *Salix*, *Salix purpurea, Salvia officinalis*, *Valeriana officinalis*

13.5 mM BME[IV], 14.4 mM SDS[V], 4.1 µg Proteinase K and 10 mg PVP40[VI]) was added and the suspension was vortexed carefully. The samples were incubated for 30 min at 65 °C, during incubation they were mixed every 10 min. After cooling down to room temperature two washing steps were performed (addition of 400 µL chloroform/IAA[VII] (24:1), shaking for 10 min, centrifugation and transfer of the upper phase to a new tube). All centrifugation steps were performed at room temperature for 10 min at 11 490 g. Afterwards the upper phase was carefully mixed with 0.6 volume parts of isopropanol, shaken for 30 min and centrifuged. The supernatant was discarded and the remaining pellet was washed with 1 mL of an ethanol/NH_4Ac solution (70 % v/v ethanol, 15 mM NH_4Cl) for 10 min. After centrifugation the supernatant was discarded and the pellet was washed a second time with 1 mL ethanol (70 % v/v). After centrifugation the supernatant was discarded carefully, the pellet was dried at 60 °C and the DNA was dissolved in 30 µL of TRIS-EDTA buffer (10 mM TRIS-HCl pH 8, 1 mM EDTA).

CTAB method II

The CTAB extraction protocol II was performed as described by Pirtillä et al. (14) with some modifications. Per sample 700 µL of CTAB extraction buffer (containing 55 mM CTAB, 1.4 M NaCl, 100 mM TRIS-HCl pH 8, 20 mM EDTA pH 8, 100 mM BME, 2 M LiCl and 17.5 mg PVP40) were added and the suspension was vortexed carefully. The samples were incubated at 65 °C for 15 min with occasionally mixing. After cooling down to room temperature two washing steps with chloroform/IAA (24:1) were performed as described above. 0.5 volume

Tab. 1: List of analysed samples (drug names, source and processing) and obtained DNA amounts (mean values of duplicates) depending on the extraction method. DNA amounts are given in ng/mg dry mass. The mean values of extracted DNA amounts were calculated excluding fresh material.
Tab. 1: Liste der untersuchten Proben (Drogennamen, Herkunft, Verarbeitung) und erhaltenen DNA-Mengen (Mittelwerte der Duplikate) in Abhängigkeit von der Extraktionsmethode. Die DNA-Mengen sind in ng/mg Trockenmasse angegeben. Die Mittelwerte der extrahierten DNA-Mengen wurden ohne das Frischmaterial berechnet.

				DNA amount [ng/mg DM] (DNA-Gehalt [ng/mg TM])				
Drug name (Drogen-bezeichnung)	Denomination on the label (Bezeichnung auf dem Etikett)	Source (Quelle)	Processing (Verarbeitung)	CTAB I	CTAB II	Wizard	Nucleo-spin	DNeasy
Valerianae radix	Valeriana officinalis, radix et rhizoma, fresh	VetMed	coarse cut					105
	Radix Valerianae EAB	pharmacy	coarse cut	65	18	122	<1	47
	Baldrian	Mag. Doskar	capsules/powder	50	<1	102	85	<1
Ginseng radix	Ginseng	pharmacy	coarse cut	70	40	181	3	23
	Lecithin-Ginseng Plus	Aurita	capsules/powder	26	20	99	<1	7
	Ginseng-Plus	Mag. Doskar	capsules/powder	25	5	109	10	<1
Menthae piperitae folium	Mentha x piperita, folium, fresh	VetMed	coarse cut					803
	Folium Menthae piperitae	pharmacy	coarse cut	435	131	351	27	88
	Pfefferminz Tee	Alnatura	fine cut	123	56	241	9	87
	FixMinze	Teekanne	fine cut, pellets	181	95	171	7	120
Salviae officinalis folium	Salvia officinalis, folium, fresh	VetMed	coarse cut					603
	Folium Salviae	pharmacy	coarse cut	204	42	182	11	67
	Salbei Tee	Alnatura	coarse cut	62	27	80	4	22
	FixSalbei	Teekanne	pellets	73	49	155	<1	15
Matricariae flos	Matricaria chamomilla, flos, fresh	VetMed	entire flower heads					1981
	Flos Chamomillae vulgaris	pharmacy	entire flower heads	580	838	1739	91	278
	Kamillen Tee	Alnatura	fine cut	218	151	739	34	138
	FixMille	Teekanne	fine cut	234	197	753	62	110
Foeniculi fructus	Foeniculum vulgaris, fructus, fresh	VetMed	entire fruits					117
	Fructus Foeniculi amari EAB	pharmacy	entire fruits	140	59	313	23	264
	Fenchel Tee	Alnatura	fine cut	95	157	284	23	193
	FixFenchel	Teekanne	fine cut	89	37	271	18	72
Iuniperi pseudo-fructus	Fructus Juniperi	Pharmacy	entire cones	47	14	28	<1	15
	Wacholder Beeren	Fuchs	entire cones	24	11	27	<1	21
	Wacholderbeeren	Kotany	entire cones	11	<1	98	<1	23
Salicis cortex	Salix purpurea, cortex, fresh	VetMed	coarse cut					132
	Salicis cortex	pharmacy	coarse cut	27	22	30	<1	39
Cinnamomi cortex	Cortex Cinnamomi ceylanici	pharmacy	coarse cut	5	5	15	<1	15
	Cinnamon, Ceylon and Cassia mixture	Alnatura	powder	8	2	13	<1	13
	Cinnamon, ground	Kotany	powder	3	13	9	<1	11
mean value [ng/mg DM] (Mittelwert [ng/mg TM])				116	83	255	17	69

VetMed: Botanical Garden of the University of Veterinary Medicine Vienna, Austria
VetMed: Botanischer Garten der Veterinärmedizinischen Universität Wien, Österreich

parts potassium acetate (3 M) were added and the samples were incubated at -20°C for 30 min. The samples were centrifuged (all centrifugation steps were performed at room temperature for 5 min at 11 490 g), in case of visible precipitates the supernatant was transferred into a new tube, otherwise 0.6 volume parts of cold isopropanol were added directly and the DNA was precipitated at -20°C for 30 min. After centrifugation the supernatant was removed, the pellets were dried at 60°C and then re-suspended at room temperature in 300 µL ddH_20^{VIII} until the pellet dissolved completely. 2 volume parts of cold ethanol (96%) were added and the samples were incubated at -20°C for 60 min. After centrifugation the supernatant was discarded and the DNA pellets were washed with ethanol (70% v/v). The tubes were centrifuged, the supernatant was discarded, the DNA pellets were dried and then re-suspended in 50 µL of TRIS-EDTA buffer (10 mM TRIS-HCl pH 8, 1 mM EDTA).

DNA quantification

The concentration of extracted DNA was measured with a Fluorescent DNA Quantitation Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instruction.

PCR amplification

PCR amplification of the nuclear ITS and the chloroplast *psbA-trnH* intergenic spacer was carried out for each sample with the original, undiluted DNA extract and in two dilutions with ddH_2O (1:50 and 1:500). For a 15 µL PCR reaction 0.5 µL of DNA was added to a master mix containing 1.8 U of Taq Polymerase (BioTherm™ DNA Polymerase, GeneCraft, Münster, Germany), 1× PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM TRIS-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.001% Tween 20; GeneCraft), 0.5 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 0.1 mM dNTPs (Solis BioDyne), 0.6 mM forward- and reverse-primers.

ITS (~710–770 bp long) was amplified with the primers Syst-ITS5 (5'-GGAAGGAGAAGTCGTAACAAGG-3'; (6)) and Syst-ITS4 (5'-TCCTTCCGCTTATTGATATGC-3'; (20)), ITS1 (~320–440 bp long) was amplified with Syst-ITS5 and Syst-ITS2 (5'-GCTACGTTCTTCATCGATGC-3'; (6)), *psbA-trnH* was amplified with the primers psbA (5'-GTTATGCATGAACGTAATGCTC-3'; (15)) and $trnH^{GUG}$ (5'-CGCGCATGGTGGATTCACAATCC-3'; (18)). All primers were synthesised by Invitrogen, Lofer, Austria. For the amplification the thermal cycler was programmed with 3 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 53°C (ITS and ITS1) or 60°C (*psbA-trnH*) and 45 s at 72°C and a final extension of 7 min at 72°C.

Sequencing

Samples that could not be identified morphologically (cinnamon, 'Baldrian Mag. Doskar', 'Lecithin-Ginseng Plus' and 'Ginseng-Plus') were sequenced to confirm their identity. For each of these samples *psbA-trnH* was amplified with the primers psbA and $trnH^{GUG}$ and the ITS region was amplified with the primers Syst-ITS5 and Syst-ITS4. Sequencing was performed by an external company (Microsynth, Balgach, Switzerland). The resulting sequences were edited with Chromas Version 2.24 (Technelyseum, Tewantin, Australia) and aligned with published reference sequences using MEGA4 (17).

Statistical analysis

Statistical analysis was calculated with SPSS for Windows version 17.0 (SPSS Inc., Chicago, USA).

Results

Drug DNA of different species representing radix, folium, flos, (pseudo-) fructus and cortex and different processing grades (fresh, dried, cut and ground) were amplified using a standard PCR assay and comparing five different extraction systems and three different DNA quantities in the PCR assay (pure DNA extract plus dilutions 1:50 and 1:500).

Comparison of DNA quantity depending on extraction method

DNA was extracted with five extraction methods representing the basic principles most frequently used for DNA extraction (CTAB (two different protocols), desalting (Wizard) and spin column based (DNeasy and Nucleospin)). The obtained DNA concentrations were measured with a fluorimetric method and the extracted DNA amount per dry mass (DM) of extracted plant material was calculated *(Tab. 1)*. The highest mean value of extracted DNA amounts could be obtained with Wizard (255 ng/mg DM), medium amounts were obtained with both CTAB methods and DNeasy (69–116 ng/mg DM) and the lowest amount was obtained with Nucleospin (17 ng/mg DM). The amount of isolated DNA fitted to the expected amount according to the manufacturer's protocol only for Wizard (175–300 ng/mg dry mass). For DNeasy the expected isolated DNA amount was 150–1500 ng/mg dry mass and for Nucleospin 500–1500 ng/mg dry mass.

Comparison of DNA quantity depending on age and processing of plant material

For a subset of samples DNA was additionally extracted from fresh plant material using one method (DNeasy Plant Mini Kit, which showed the best amplification success of the tested kits). DNA extracts from plant material of *Matricaria chamomilla*, *Mentha* x *piperita, Salvia officinalis* and *Salix purpurea*, freshly collected, contained at least four-fold higher DNA amounts than the extracts of the dried and stored material, freshly collected *Valeriana officinalis* contained an approximately two-fold higher DNA amount than the dried and stored material, while the DNA content of freshly collected *Foeniculum vulgare* did not differ from the concentration of the dried and stored correspondent samples *(Tab. 1)*.

Amplification success

PCR was carried out with three primer combinations, which are commonly used for DNA-based plant identification. The first primer pair amplifies the complete nuclear internal transcribed spacer region (ITS) comprising ITS1, 5.8S and ITS2 *(Fig. 1)*, the second primer pair the significantly shorter ITS1 and the third primer pair the chloroplast *psbA-trnH* intergenic spacer. The DNA was added to the PCR reaction in three concentrations (undiluted, 1:50 and 1:500). The reason for adding the DNA in different concentrations is that the DNA extract may still contain impurities that can inhibit PCR amplification. By diluting the DNA, inhibitors are diluted below a limit interfering with PCR.

A PCR was defined as successful if any of the extraction methods resulted in an amplification product in any of the DNA dilutions added. Comparing the two amplification products of the internal transcribed spacer (ITS and ITS1), the longer ITS succeeded in 75% and the shorter

ITS1 in 83%, respectively (calculated on the basis of the values of *Tab. 2* and *Tab. 3*). The amplification of the chloroplast region *psbA-trnH* succeeded in 89%. Only the ITS in one product of juniper berries (Kotany) could not be amplified, all other products resulted in an amplification product in at least one extraction method or dilution *(Tab. 2)*.

Comparing the DNA extraction methods (success, if amplifying in at least one of the dilutions) for the nuclear ITS region, CTAB I succeeded in 71% and 83% (ITS and the shorter ITS1, respectively), CTAB II in 83% and 96%, Wizard and Nucleospin in 67% and 71% and DNeasy in 88% and 92%, respectively. For the chloroplast *psbA-trnH* region CTAB I succeeded in 100%, CTAB II in 92%, Wizard in 88%, Nucleospin in 75% and DNeasy in 92%, respectively. Overall, Wizard and Nucleospin gave a lower success rate than the other three methods, which were very similar in their success. The relatively low success rate of both methods was due to the high failure rates in two specific drugs (see below). Without the two drugs, all DNA extraction methods would be nearly equal in their quality.

Comparing the drugs, Iuniperi pseudofructus and Cinnamomi cortex were the most difficult matrices with success rates of only 38% and 56%, respectively. In these two drugs, Nucleospin failed completely and Wizard had a very high failure rate. These two difficult drugs were followed by Valerianae radix (77% success), Ginseng radix and Salicis cortex (87%, respectively) and Menthae folium (93%). Interestingly PCR worked perfectly well in Ginseng radix in all concentrations and methods in the drug from the pharmacy, while the failures were only in the products from Aurita and Doskar. The product Aurita Lecithin-Ginseng Plus amplified soy bean DNA instead of the target species ginseng as confirmed by DNA sequencing.

In its tendency, DNA of Valerianae radix, Ginseng radix, Iuniperi pseudofructus and Cinnamomi cortex worked better if added to the PCR reaction without dilution. For Matricariae flos, Foeniculi fructus and Salicis cortex, the concentration did not affect the outcome, while Menthae folium and Salviae folium worked better in dilutions, possibly indicating the high amounts of especially polyphenols that inhibit the PCR reaction and could not be removed completely during the DNA extraction.

Discussion

Comparison of DNA quantity depending on the extraction method

In routine analysis it would be favourable if all samples irrespective of their origin could be analysed by one assay. Therefore, a standard PCR assay was used without optimisation of PCR conditions or reagents. The comparison of DNA quantities extracted with different extraction systems is of secondary importance since PCR amplification will overcome these differences easily. The generally lower DNA yield of spin column-based methods is due to the low capacities of the columns. However, if an extracted DNA amount is below the sensitivity of the method used for DNA quantification, sometimes troubles in PCR can be expected. Here, the fluorometric method of DNA quantification was preferred over other methods (especially over the widely used spectrophotometric determination of the DNA concentration) due to its high sensitivity and the low interaction of the fluorescent dye with proteins, urea, ethanol and chloroform (4).

The suitability of the DNA extract for subsequent PCR depends more on the (quality of the) raw material than on the DNA extraction method. Therefore, no general recommendation for a specific extraction method can be given here.

Lower DNA amounts with often bad quality were extracted from roots, barks and cones resulting more often in PCR failures compared to leaves, flowers and fruits. From the latter mentioned plant organs more DNA could be extracted. However, the DNA from these organs worked better in higher dilutions because the samples still contained higher amounts of plant secondary compounds inhibiting the PCR reaction. By simply diluting the DNA extracts the amounts of these interfering compounds were brought to harmless concentrations.

The DNA quality was generally better in drugs sold in pharmacies than those from drug stores. The reason may be connected to the quality of the drug material. Longer storage or high temperature treatments may lead to DNA degradation, i.e. the breaking of the DNA strands into smaller fragments.

Interesting aspects for a routine lab applying such methods are the extraction costs. The costs per sample (without labour costs) are around 0.40 € for the CTAB methods (all reagents prepared in-house; (16)), 0.85 € for Wizard, 1.98 € for Nucleospin Plant II (the follow-up kit of Nucleospin Plant) and 2.83 € for DNeasy (list prices for Austria in cost-saving bulk packs, January 2013). A further interesting aspect is the labour time for the DNA isolation. For the isolation of 24 samples (a standard number of centrifuge rotors) a skilled person needed working time of approximately 2 h for Wizard, 5 h for DNeasy and Nucleospin Plant, 7 h for CTAB I and 8 h for CTAB II.

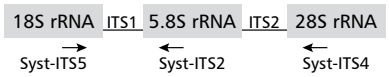

Fig. 1: ITS region with 18S, 5.8S and 28S ribosomal RNA genes and internal transcribed spacers (ITS) 1 and 2. Below the primer binding sites for the used primers are indicated with arrows.
Abb. 1: ITS Region mit den ribosomalen Genen 18S, 5,8S und 28S und den Internal Transcribed Spacers (ITS) 1 und 2. Darunter sind die Primerbindungsstellen mit Pfeilen dargestellt.

Amplification success

The overall amplification success rate of all tested plant species and products was very good. Only ITS in Iuniperi pseudo-fructus could not be successfully amplified. According to published sequences (e.g. GenBank accession numbers GU139567.1, GU139570.1) the primers Syst-ITS2 and Syst-ITS5 do not bind properly to juniper DNA due to several mutations in the primer binding sites. Therefore most of the PCR reactions of Iuniperi pseudo-fructus showed no amplification products. If products were formed, they were supposed to be either fungal DNA or unspecific *Juniperus* DNA products, visible as short DNA bands on an agarose gel (5). Several cinnamon extracts were also problematic. Due to the low amount of non-degraded cinnamon DNA, fungal DNA present in the plant material was amplified. However, fungal DNA can be easily distinguished from plant DNA on an agarose gel because of its smaller length of the internal transcribed spacers.

The presence of soy DNA in the product Aurita Lecithin-Ginseng Plus can be explained by the fact of DNA accumulation in the lecithin fraction during processing of soy beans (3).

Tab. 2: PCR amplification success of analyzed samples depending on extraction method, locus and dilution of applied DNA extract. u: undiluted DNA extract, 50: dilution 1:50, 500: dilution 1:500, "+": positive amplification in at least one duplicate, "-": no amplification in both duplicates. "+" for ITS means a positive amplification of at least ITS1, all samples which are not mentioned in *Tab. 3* showed a positive amplification of the whole ITS region (confer to *Tab. 3*).

Tab. 2: PCR Erfolg der untersuchten Proben in Abhängigkeit von Extraktionsmethode, Lokus und Verdünnung der verwendeten DNA-Extrakte. u: unverdünnter DNA-Extrakt, 50: Verdünnung 1:50, 500: Verdünnung 1:500, „+": positive Vervielfachung in mindestens einem Duplikat, „-: keine Vervielfachung in beiden Duplikaten. Ein „+" für ITS zeigt eine positive Vervielfachung von zumindest ITS1, alle Proben, welche nicht in *Tab. 3* erwähnt werden, zeigten eine positive Vervielfachung der gesamten ITS Region (siehe *Tab. 3*).

		CTAB I						CTAB II						Wizard						Nucleospin						DNeasy					
Drug name	Source	ITS			*psbA-trnH*			ITS			*psbA-trnH*			ITS			*psbA-trnH*			ITS			*psbA-trnH*			ITS			*psbA-trnH*		
(Drogenbezeichnung)	(Quelle)	u	50	500	u	50	500	u	50	500	u	50	500	u	50	500	u	50	500	u	50	500	u	50	500	u	50	500	u	50	500
Valerianae radix	VetMed																									-	+	-	-	+	-
	pharmacy	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+
	Doskar	+	-	-	+	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-
Ginseng radix	pharmacy	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Aurita	+	-	-	+	+	+	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
	Doskar	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	-	-	-
Menthae folium	VetMed																									-	+	+	-	+	+
	pharmacy	-	+	+	-	+	+	-	+	+	+	+	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-
	Alnatura	-	+	+	-	+	+	-	+	+	+	+	-	-	-	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+	-
	Teekanne	-	-	+	-	+	+	-	+	+	+	+	-	-	-	+	-	-	-	-	+	+	-	+	+	-	+	+	-	+	-
Salviae folium	VetMed																									+	+	+	+	+	+
	pharmacy	-	-	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+	+	-	+	-
	Alnatura	-	+	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+
	Teekanne	-	-	+	-	+	+	-	+	+	-	+	-	-	-	+	-	-	+	-	+	+	-	+	+	-	-	+	-	+	-
Matricariae flos	VetMed																									+	+	+	+	+	+
	pharmacy	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alnatura	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-
	Teekanne	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Foeniculi fructus	VetMed																									+	+	+	+	+	-
	pharmacy	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Alnatura	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	Teekanne	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Iuniperi pseudo-fructus	pharmacy	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-
	Fuchs	-	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
	Kotany	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
Salicis cortex	VetMed																									-	+	+	+	-	-
	pharmacy	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	-	+	+	-	+	-	+	+	+	+	+	-
Cinnamomi cortex	pharmacy	+	+	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
	Alnatura	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-	-
	Kotany	-	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-

Tab. 3: PCR amplification success (ITS region only) of selected samples, depending on the extraction method and the PCR-product length. "++": positive amplification of the whole ITS region (ITS1, 5.8S, ITS2) in at least one DNA-dilution, "+": positive amplification of the shorter ITS1 only, "-": no amplification. Samples which are not listed here showed either an amplification product for the whole ITS region or no product (refer to *Tab. 2*).
Tab. 3: PCR Erfolg (ITS Region) von ausgewählten Proben in Abhängigkeit von der Extraktionsmethode und der Länge des PCR-Produkts. „++": positive Vervielfältigung der gesamten ITS Region (ITS1, 5,8S, ITS2) in mindestens einer DNA-Verdünnung, „+": positive Vervielfältigung des kürzeren ITS1-Abschnittes, „-": keine Vervielfältigung. Proben, die nicht in der Liste angeführt sind, zeigten entweder ein Amplifikationsprodukt der gesamten ITS Region oder kein Produkt (wie in *Tab. 2* angegeben)

Drug name (Drogenbezeichnung)	Source (Quelle)	CTAB I	CTAB II	Wizard	Nucleospin	DNeasy
Valerianae radix	Doskar	+	+	-	-	++
Ginseng radix	Aurita	++	+	++	+	+
	Doskar	++	+	++	++	++
Menthae folium	Alnatura	+	++	+	++	++

The difference in the amplification success between the loci has several reasons. The significantly lower success rate of the longer ITS region compared to ITS1 can be explained by DNA fragmentation, that is occurring to a greater extent by processing of plant material (drying, grinding, etc. (3, 10)). The primer pairs amplifying the chloroplast *psbA-trnH* intergenic spacer bind very well to the DNA of all analysed species. Few existing point mutations in the primer binding sites of fennel, valerian, cinnamon and juniper may slightly reduce the primer efficiency in part of the samples, but do not completely inhibit the amplification. The ITS primers bind very well to the DNA of all analysed samples with the exception of juniper, where the amplification is (nearly) impossible with the standard primer pairs because the degree of mutation in the primer binding sites is too high for a successful amplification. Our results confirm that the so-called 'universal' primer sets used by taxonomists are not applicable for the whole plant kingdom (9). Summarizing, a standard approach with 'universal' primers worked well in most of our examples chosen when using an appropriate dilution of DNA extracts. The cases with low PCR success were basically related to mutations in the primer sites (juniper) or DNA degradation by processing (cinnamon and ginseng). The choice of the DNA extraction protocol had only minor influence on the amplification success. However, care should be taken when choosing so-called 'fast' extraction protocols.

Acknowledgement
The authors gratefully thank Mrs. Birgit Grohs for her project management. Furthermore, the authors acknowledge the financial support of the German Federal Ministry of Economics and Technology (IGF project number 15182 N).

Abbreviations

[I] CTAB – cetyltrimethylammoniumbromide
[II] TRIS – tris(hydroxymethyl)aminomethane
[III] EDTA – ethylenediaminetetraacetic acid
[IV] BME – 2-mercaptoethanol
[V] SDS – sodium dodecyl sulphate
[VI] PVP – polyvinylpyrrolidone
[VII] IAA – isoamylalcohol
[VIII] ddH_20 – double-distilled water

References

1. Albuquerque P, Mendes MV, Santos CL, Moradas-Ferreira P, Tavares F. DNA signature-based approaches for bacteria l detection and identification. Sci Total Environ 2009;407:3641-3651.
2. Alternative Methoden zur Kontrolle der mikrobiologischen Qualität (Alternative Methods for Control Microbiological Quality, 7.0/5.1.6) European Pharmacopoeia (Europäisches Arzneibuch). 7th edition. Vienna: Official Austrian Version, Verlag Oesterreich GmbH, pp. 817–834.
3. Bauer T, Weller P, Hammes WP, Hertel C. The effect of processing parameters on DNA degradation in food. Eur Food Res Technol 2003;217(4):338–343.
4. Demeke T, Jenkins GR. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Anal Bioanal Chem 2010;396:1977–1990.
5. de Miranda VFO, Martins VG, Furlan A, Bacci M. Plant or Fungal Sequences? An Alternative Optimized PCR Protocol to Avoid ITS (nrDNA) Misamplification. Braz Arch Biol Technol 2010;53(1):141–152.
6. Downie SR, Katz-Downie DS. A molecular phylogeny of Apiaceae subfamily Apioideae: Evidence from nuclear ribosomal DNA internal transcribed spacer sequences. Am J Bot 1996;83:234–251.
7. Doyle JJ, Doyle JL. A rapid total DNA preparation procedure for fresh plant tissue. Focus 1990;12:13–15.
8. Gnavi G, Bertea CM, Maffei ME. PCR, sequencing and PCR-RFLP of the 5S-rRNA-NTS region as a tool for the DNA fingerprinting of medicinal and aromatic plants. Flavour Fragrance J 2010;25:132–137.
9. Gonzalez MA, Baraloto C, Engel J, Mori SA, Pétronelli P, Riéra B, Roger A, Thébaud C, Chave J. Identification of amazonian trees with DNA barcodes. PLoS ONE 2009;4(10):e7483.
10. Gryson N. Effect of food processing on plant DNA degradation and PCR-based GMO analysis: A review. Anal Bioanal Chem 2010;396(6):2003-2022.
11. Heubl G. New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. Planta Med 2010;76:1963-1974.
12. Hollingsworth PM, Graham SW, Little DP. Choosing and using a plant DNA barcode. PLoS ONE 2011;6:e19254.
13. Morling N. PCR in forensic genetics. Biochem Soc Trans 2009;37:438–440.
14. Pirttilä AM, Hirsikorpi M, Kämäräinen T, Jaakola L, Hohtola A. DNA isolation methods for medicinal and aromatic plants. Plant Mol Bio Rep 2001;19:273a–f.
15. Sang T, Crawford DJ, Stuessy TF. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Am J Bot 1997;84:1120–1136.
16. Smith DS, Maxwell PW, de Boer SH. Comparison of Several Methods for the Extraction of DNA from Potatoes and Potato-Derived Products. J Agric Food Chem 2005;53:9848–9859.
17. Tamura K, Dudley J, Nei M, Kumar S. Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596-1599.
18. Tate JA, Simpson BB. Paraphyly of *Tarasa* (Malvaceae) and Diverse Origins of the Polyploid Species. Syst Bot 2003;28:723–737.
19. Verfahren zur Amplifikation von Nukleinsäuren (Nucleic Acid Amplification Techniques, 7.0/2.6.21) European

Pharmacopoeia (Europäisches Arzneibuch). 7th edition. Vienna: Official Austrian Version, Verlag Oesterreich GmbH, pp. 254–261.

20. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: A guide to methods and applications. San Diego: Academic Press; 1990. pp. 315–322.
21. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 1997;63(10):3741–3751

Anschriften der Verfasser

Mag. Corinna Schmiderer, Dr. Brigitte Lukas, Prof. Dr. Johannes Novak*

Veterinärmedizinische Universität Wien
Institut für Tierernährung und Funktionelle Pflanzenstoffe
Veterinärplatz 1
A-1210 Wien, Austria

*Corresponding author:
Johannes.Novak@vetmeduni.ac.at

Received: 08 August 2012
Accepted: 12 January 2013

Paper 2

Schmiderer C, Mader E, Novak J (2010): DNA-Based Identification of *Helleborus niger* by High-Resolution Melting Analysis. *Planta Medica* 76 (16): 1934-7.

Author's contributions:

CS designed the primers and performed the lab work.

CS and JN prepared the manuscript.

EM revised the manuscript.

DNA-Based Identification of *Helleborus niger* by High-Resolution Melting Analysis

Corinna Schmiderer, Eduard Mader, Johannes Novak
Institute for Applied Botany and Pharmacognosy, University of Veterinary Medicine, Vienna, Austria

Abstract

Hellbori nigri rhizoma is a drug that is difficult to distinguish from other species of the genus *Helleborus*. In this communication we present a DNA-based identification by high-resolution melting analysis (HRM) that is able to differentiate between *Helleborus niger* and other species of the genus. HRM is a very specific, time- and labour-saving method for identifying DNA sequence variations and is ideally suitable for routine PCR analysis. The HRM assay developed is specific for the genus *Helleborus*. This method not only detects the presence of the target species *H. niger* but also, to a certain extent, identifies other *Helleborus* species by their different melting curve shapes. Markers were developed based on the trnL–trnF intergenic spacer and on the matK sequence. For an unambiguous identification of *Helleborus niger*, melting curves of both markers should be used.

Key words
Helleborus niger · Ranunculaceae · high-resolution melting analysis (HRM) · identification

Helleborus niger, commonly known as black hellebore, Christ hellebore, or Christmas rose, belongs to a genus of about 12–16 species and is naturally distributed in Eurasia [1,2]. Many species of this genus, including *H. niger*, are popular ornamental perennial plants.

The underground parts of *Helleborus niger* (rootstock and roots) are used in homeopathy in cases of meningitis, convulsions, hydrocephalus, dropsy, and cachexia due to tumours [3]. The Homöopathisches Arzneibuch [4] demands the strict exclusion of other *Helleborus* species, especially *H. viridis* L., *H. foetidus* L, and *H. odorus* Waldst. et Kit. According to Frohne and Pfänder [5], a clear-cut pharmacognostical identification that excludes *H. viridis* and other *Helleborus* species is not possible.

High-resolution melting analysis (HRM) is a cost- and time-effective, sensitive "post-PCR" method to detect mutations such as indels or single-nucleotide polymorphisms (SNPs). HRM relies on the different melting behaviours of PCR-amplified double-stranded DNA fragments due to their varying GC content and sequence [6–9].

In this paper we report a method to distinguish *Helleborus niger* from other species of the genus *Helleborus*. In the two chloroplast regions matK and the trnL–trnF intergenic spacer, species-specific differences in DNA sequence could be detected, and primers specifically adapted to HRM were developed and tested in an extensive set of species (**Table 1**). The assay was designed to amplify all species from the genus *Helleborus* and to subsequently identify the species by their melting curves. T_m values [melting temperature at the inflection point(s)], inclination of the curves, and number of inflection points were used to characterise the curve types. Different concentrations of PCR ingredients can cause the HRM curves to shift. Therefore, a small set of reference samples is necessary for an unambiguous identification of unknown samples. For the applied primers, a maximum temperature difference of 0.05 °C between reference and unknown samples and the same curve shape (inclination and number of inflection points) are recommended to classify them as one group.

The primer pair based on the trnL–trnF intergenic spacer divided the sample set into three curve types (**Fig. 1**). Group I consisted of *H. argutifolius, H. foetidus, H. lividus*, and *H.* × *sternii*; group II consisted of our target species *H. niger* and one sample of *H. viridis*; and group III consisted of *H. bocconei, H. caucasicus, H. cyclophyllus, H. dumetorum, H. multifidus, H. odorus, H. orientalis, H. purpurascens*, and *H. viridis* ssp. *viridis*.

The primer pair based on matK showed five distinct groups (**Fig. 2**). Group I consisted of *H. cyclophyllus, H. dumetorum, H. multifidus*, some samples of *H. odorus, H. purpurascens*, and both subspecies of *H. viridis*. Group II contained *H. bocconei, H. caucasicus, H. foetidus, H. orientalis*, and the remaining *H. odorus* samples. *H. lividus* formed the monospecific group III, *H. niger* the monospecific target group IV, and *H. argutifolius* group V.

Although it would be possible to identify *H. niger* solely by the matK-based HRM primer pair, it is advisable to add the information from the trnL–trnF intergenic spacer. The melting curves of *H. niger* (group IV) and *H. argutifolius* (group V) are very close in matK but are clearly distant in the trnL–trnF intergenic spacer. The one subspecies of *H. viridis* with a curve type identical to that of *H. niger* in the trnL–trnF intergenic spacer can be clearly distinguished by its distinct and distant curve type in matK. Other species such as *H. argutifolius* and *H. foetidus* also can be distinguished by the combination of the two chloroplast regions.

In the sample set, six species of five different genera from the Ranunculaceae family were included (**Table 1**) to test for cross-amplification in other genera. In the trnL–trnF intergenic spacer, one of the two *Adonis* species (*A. amurensis*), *Delphinum elatum*, and *Eranthis hyemalis* (**Fig. 3**) formed amplification products, while in the matK region, *Delphinium elatum, Eranthis hyemalis, Caltha palustris*, and both *Adonis* species (*A. amurensis* and *A. vernalis*; **Fig. 4**) did. The assay is therefore not completely specific for the *Helleborus* species. However, the melting curves of these species were clearly distinct from the curves of all *Helleborus* species. Additionally, the assay was tested with mixtures of *Helleborus* species, which resulted in new HRM curve shapes due to heteroduplex formation (data in preparation).

In conclusion, *H. niger* can be unambiguously identified by combining the melting curves of the trnL–trnF intergenic spacer and matK regions.

Material and Methods

Ninety-two *Helleborus* and six out-group samples (all Ranunculaceae) were collected from different locations in Austria, the Republic of Kosovo, France (Corse), Italy (Sicily), the herbarium of the University of Vienna (WU), and the Alpengarten in Belvedere (Federal Gardens Vienna-Innsbruck) (**Table 1**). Voucher specimens are stored at the herbarium of the Institute for Applied Botany and Pharmacognosy, University of Veterinary Medicine, Vienna, Austria (registration numbers Hel01-Hel98). The specimens were identified by Avni Hajdari and Corinna Schmiderer.

Genomic DNA was extracted from dried leaf samples using a modified CTAB protocol [10]. This extraction included a mixture of 950 µL CTAB detergents, 0.95 µL beta-mercaptoethanol, 4.1 µL

This is a copy of the author's personal reprint

Table 1 *Helleborus* and out-group samples used for analysis.

Sample	No.	Registration no.	Origin
Helleborus argutifolius Viv.	1	Hel22	AGB, cultivated
Helleborus argutifolius Viv.	1	Hel09	France, Corse, Foret de Bonifatu
Helleborus argutifolius Viv.	2	Hel07-8	France, Corse, Lac de Nino
Helleborus bocconei Ten.	1	Hel23	AGB, cultivated
Helleborus bocconei Ten.	1	Hel11	Italy, Sicily, Melia
Helleborus bocconei Ten.	1	Hel46	WU, Italy, Toscana
Helleborus caucasicus A. Braun	1	Hel48	WU, Georgia, Kartli
Helleborus caucasicus A. Braun	1	Hel47	WU, Georgia, Samtskhe-Javakheti
Helleborus cyclophyllus Boiss.	2	Hel24-25	AGB, cultivated
Helleborus cyclophyllus Boiss.	1	Hel49	WU, Greece, Epirus orientalis
Helleborus dumetorum Waldst. & Kit. ex Willd.	2	Hel68-69	Austria, Styria, Klapping
Helleborus dumetorum Waldst. & Kit. ex Willd.	2	Hel66-67	Austria, Styria, Königsberg
Helleborus dumetorum Waldst. & Kit. ex Willd.	3	Hel63-65	Austria, Styria, Weixelbaum
Helleborus dumetorum Waldst. & Kit. ex Willd.	1	Hel51	WU, Austria, Burgenland, Pinkafeld
Helleborus dumetorum Waldst. & Kit. ex Willd.	1	Hel50	WU, Austria, Styria, Valley of the Mitterbach River
Helleborus foetidus L.	1	Hel26	AGB, cultivated
Helleborus foetidus L.	1	Hel03	Austria, Botanical Garden VetMedUni Vienna, cultivated
Helleborus foetidus L.	2	Hel02, 06	Cultivated
Helleborus foetidus L.	1	Hel53	WU, Austria, Lower Austria, Blumau
Helleborus foetidus L.	1	Hel52	WU, France, Valon de Casterine
Helleborus lividus Ait. f.	1	Hel27	AGB, cultivated
Helleborus multifidus Vis.	1	Hel54	WU, Botanical Garden Vienna, cultivated
Helleborus multifidus Vis.	1	Hel55	WU, Italy, Belluno, Cazzano Valtraminga
Helleborus niger L.	2	Hel28-29	AGB, cultivated
Helleborus niger L.	2	Hel04-5	Austria, Botanical Garden VetMedUni Vienna, cultivated
Helleborus niger L.	6	Hel99-104	Austria, Lower Austria, Lackenhof
Helleborus niger L.	7	Hel71-77	Austria, Lower Austria, Maria Seesal
Helleborus niger L.	12	Hel80-91	Austria, Salzburg, Schafberg
Helleborus niger L.	1	Hel01	Austria, Styria
Helleborus niger L.	1	Hel92	Austria, Styria, Mitterbach
Helleborus niger L.	1	Hel98	Austria, Styria, Seebach Au
Helleborus niger L.	1	Hel93	Austria, Styria, Seebergalm
Helleborus niger L.	4	Hel94-97	Austria, Styria, Seewiesen
Helleborus niger L.	1	Hel56	WU, Austria, Carinthia, Arnoldstein
Helleborus odorus Waldst. & Kit. ex Willd.	1	Hel30	AGB, cultivated
Helleborus odorus Waldst. & Kit. ex Willd.	3	Hel12-14	Kosovo, Germi
Helleborus odorus Waldst. & Kit. ex Willd.	3	Hel18-20	Kosovo, Prizren
Helleborus odorus Waldst. & Kit. ex Willd.	3	Hel15-17	Kosovo, Suhareke
Helleborus orientalis Lam.	3	Hel33-35	AGB, cultivated
Helleborus orientalis ssp. *abchasicus Lam.*	1	Hel57	WU, Austria, Lower Austria, Baden, cultivated
Helleborus orientalis ssp. *guttatus Lam.*	1	Hel58	WU, Austria, Lower Austria, Baden, cultivated
Helleborus purpurascens Waldst. & Kit.	2	Hel59-60	WU, Hungaria
Helleborus sp.	3	Hel40-42	AGB, cultivated
Helleborus viridis Boiss.	1	Hel62	WU, Spain, Navarra, Espinal-Anzperri
Helleborus viridis ssp. *occidentalis Boiss.*	1	Hel61	WU, Spain, Cantrabria, Puerto de Palombera
Helleborus×sternii (H. argutifolius×H. lividus)	2	Hel36-37	AGB, cultivated
Aconitum lycoctonum L. em. Koell.	1	Hel78	Austria, Botanical Garden VetMedUni Vienna, cultivated
Adonis amurensis Regel & Radde	1	Hel43	AGB, cultivated
Adonis vernalis L.	1	Hel45	WU, AGB, cultivated
Caltha palustris L.	1	Hel70	Austria, Styria, Fohnsdorf
Delphinum elatum L.	1	Hel79	Austria, Botanical Garden VetMedUni Vienna, cultivated
Eranthis hyemalis (L.) *Salisb.*	1	Hel44	Cultivated

Note: AGB: Alpengarten in Belvedere (Federal Gardens Vienna-Innsbruck), Vienna, Austria; VedMedUni: University of Veterinary Medicine Vienna, Austria; WU: Herbarium of the University of Vienna, Austria

proteinase K, 10 mg polyvinylpyrrolidone K30 (all reagents from Carl Roth), and 41 µL 10% sodium dodecyl sulphate (Merck) per sample.

For primer design, sequences of the chloroplast matK gene and the trnL–trnF intergenic spacer were taken from GenBank. These sequences were aligned using MEGA4 [11]. Primers with an optimum melting temperature ranging from 56 °C to 60 °C were designed using Primer Express 2.0 (Applied Biosystems) (Hel_matK3-F: ATCCCTTCATGCATTATTTCCG; Hel_matK3-R: TGAGACCAAAAGTAAAAATGATATTCCC; Hel_trnL-F1: GGGCCATACTCCCTAACGAT; Hel_trnL–R1: GAAAGAGTAGAATGCCCGAGAA).

HRM with preamplification was performed with a Rotor-Gene 6000 (Corbett Life Science). For a 10-µL PCR reaction, 1 µL of genomic DNA (1 : 50 dilution of the original DNA extract) was

This is a copy of the author's personal reprint

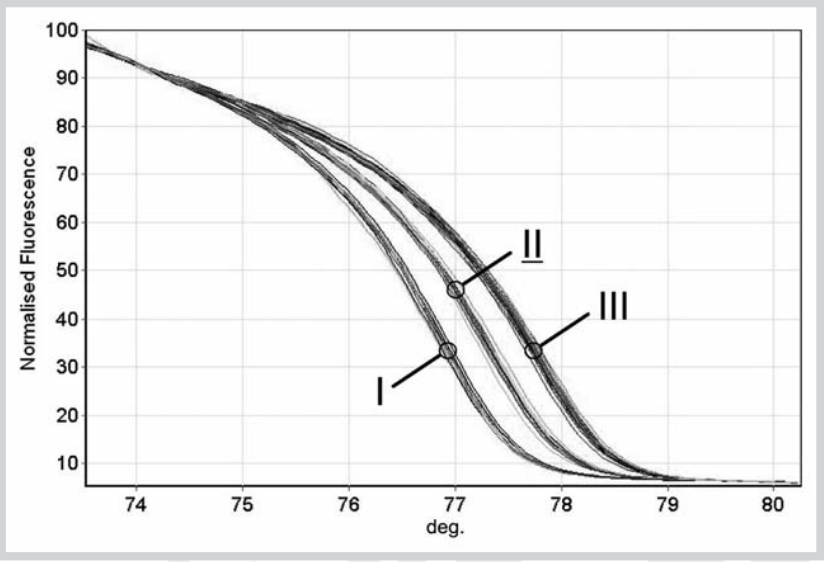

Fig. 1 HRM based on the trnL–trnF region of the chloroplast. Group I: *H. argutifolius*, *H. foetidus*, *H. lividus*, *H.×sternii;* group II: *H. niger*, *H. viridis* ssp. *occidentalis;* group III: *H. bocconei*, *H. caucasicus*, *H. cyclophyllus*, *H. dumetorum*, *H. multifidus*, *H. odorus*, *H. orientalis*, *H. purpurascens*, *H. viridis* ssp. *viridis* (group comprising the target species *H. niger* underlined).

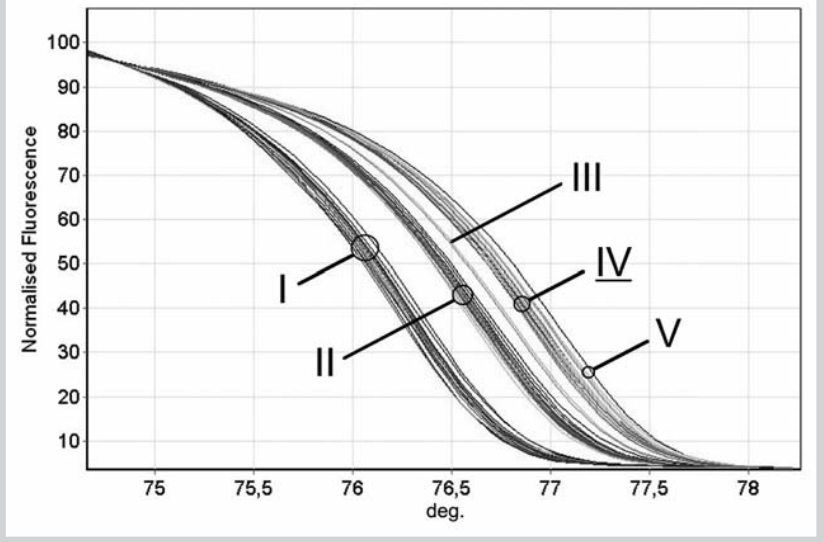

Fig. 2 HRM based on the matK sequence. Group I: *H. cyclophyllus*, *H. dumetorum*, *H. multifidus*, *H. odorus* (p.p.), *H. purpurascens*, *H. viridis;* group II: *H. bocconei*, *H. caucasicus*, *H. foetidus*, *H. odorus* (p.p.), *H. orientalis;* group III: *H. lividus*; group IV: *H. niger*; group V: *H. argutifolius* (group comprising the target species *H. niger* underlined).

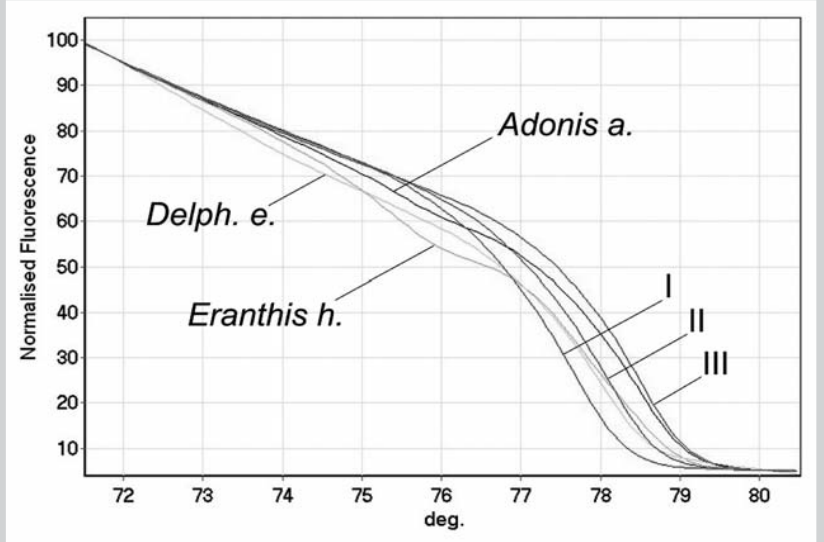

Fig. 3 HRM based on the trnL–trnF region of the chloroplast. The out-groups *Adonis amurensis*, *Delphinum elatum*, and *Eranthis hyemalis* formed amplification products with the *Helleborus*-specific primers. Groups I–III are as in ◉ **Fig. 1**.

This is a copy of the author's personal reprint

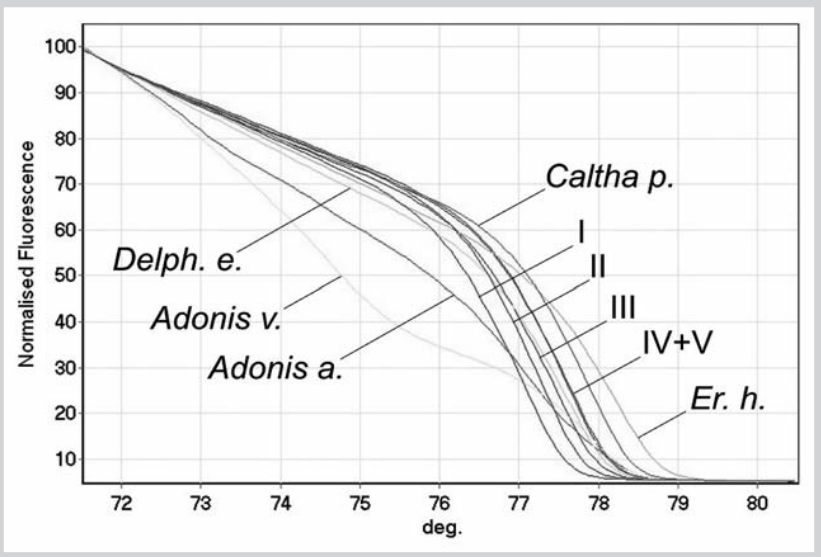

Fig. 4 HRM based on the matK sequence. The outgroups *Adonis amurensis* and *A. vernalis*, *Delphinum elatum*, *Caltha palustris*, and *Eranthis hyemalis* formed amplification products with the *Helleborus*-specific primers. Groups I–V are as in ◻ **Fig. 2**.

added to a master mix containing 1 × PCR buffer B, 3.5 mM $MgCl_2$, 100 µM dNTPs, 0.4 U HotFire Taq polymerase (all reagents from Solis BioDyne), 0.15 µM forward and reverse primer (Invitrogen), and 1.75 µM BEBO DNA-binding dye (Tataa Biocenter).

The PCR cycle profile included a denaturation step at 95 °C for 12 min, followed by 40 cycles at 95 °C for 10 s, at 55 °C for 20 s, and at 72 °C for 20 s. The melting analysis was performed by increasing the temperature from 71 °C to 81 °C by 0.07 °C/s. All reactions were done in duplicate. In each HRM run, reference samples for each curve type expected were included. The melting curves were analysed using Rotor-Gene 6000 Series software (Corbett Life Science).

Acknowledgements

The authors gratefully acknowledge the Herbarium of the University of Vienna and the Federal Gardens Vienna-Innsbruck for making their collection available. Many thanks to Regina and Jutta Sekara, Monika Nell, Avni Hajdari, and Joana Ruzicka for collecting plant material. The authors thank the German Federal Ministry of Economics and Technology (BMWi) and the German Federation of Industrial Research Associations (AiF) for financial support of the study in the context of project no. 15182 N and the Forschungsvereinigung der Arzneimittel Hersteller e.V. (FAH) for collaboration.

References

1 *Sun H, McLewin W, Fay MF.* Molecular phylogeny of *Helleborus* (Ranunculaceae), with an emphasis on the East Asian-Mediterranean disjunction. Taxon 2001; 50: 1001–1018
2 *Tutin T, Heywood V, Burges N, Valentine D, Walters S, Webb D.* Flora Europaea, Vol. 1: Lycopodiaceae to Plantaginaceae. Cambridge: Cambridge University Press; 1964: 207–208
3 *Metzner J.* Gesichtete Homöopathische Arzneimittellehre. Heidelberg: Karl F. Haug; 1985: 710–716
4 Homöopathisches Arzneibuch (HAB). Stuttgart: Deutscher Apotheker Verlag; 2004
5 *Frohne D, Pfänder H.* Giftpflanzen, 5th edition. Stuttgart: Wissenschaftliche Verlagsgesellschaft GmbH; 2004: 316–317
6 *Ririe KM, Rasmussen RP, Wittwer CT.* Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 1997; 245: 154–160
7 *Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C.* Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin Chem 2004; 50: 1156–1164
8 *Erali M, Voelkerding KV, Wittwer CT.* High resolution melting applications for clinical laboratory medicine. Exp Mol Pathol 2008; 85: 50–58
9 *Ruzicka J, Lukas B, Merza L, Göhler I, Abel G, Popp M, Novak J.* Identification of *Verbena officinalis* based on ITS sequence analysis and RAPD-derived molecular markers. Planta Med 2009; 75: 1271–1276
10 *Doyle J.* DNA protocols for plants. In: Hewitt G, Johnston A, Young J, editors. Molecular techniques in taxonomy. Berlin: Springer Verlag; 1991: 283–285
11 *Tamura K, Dudley J, Nei M, Kumar S.* Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24: 1596–1599

received September 28, 2009
revised February 26, 2010
accepted April 9, 2010

Bibliography
DOI http://dx.doi.org/10.1055/s-0030-1249908
Published online May 7, 2010
Planta Med 2010; 76: 1934–1937

ISSN 0032-0943

Correspondence
Ao. Univ.-Prof. Dr. Johannes Novak
Institute for Applied Botany and Pharmacognosy
University of Veterinary Medicine
Veterinärplatz 1
1210 Vienna
Austria
Phone: + 43 1 2 50 77 3 1 02
Fax: + 43 1 2 50 77 3 1 90
Johannes.Novak@vetmeduni.ac.at

This is a copy of the author's personal reprint

Paper 3

Schmiderer C, Lukas B, Ruzicka J, Novak J (2015): DNA-based identification of *Calendula officinalis* (Asteraceae). *Applications in Plant Sciences* 3 (11): 1500069.

Author's contributions:

CS designed the primers, supervised the lab work and performed part of it.

CS and JN prepared the manuscript.

BL and JR revised the manuscript.

Applications in Plant Sciences

Applications in Plant Sciences 2015 3(11): 1500069

Protocol Note

DNA-based identification of *Calendula officinalis* (Asteraceae)[1]

Corinna Schmiderer[2], Brigitte Lukas[2], Joana Ruzicka[2], and Johannes Novak[2,3]

[2]University of Veterinary Medicine, Institute of Animal Nutrition and Functional Plant Compounds, Veterinärplatz 1, 1210 Vienna, Austria

- *Premise of the study:* For the economically important species *Calendula officinalis*, a fast identification assay based on high-resolution melting curve analysis was designed. This assay was developed to distinguish *C. officinalis* from other species of the genus and other Asteraceae genera, and to detect *C. officinalis* as an adulterant of saffron samples.
- *Methods and Results:* For this study, five markers (ITS, *rbcL*, 5′ *trnK-matK*, *psbA-trnH*, *trnL-trnF*) of 10 *Calendula* species were sequenced and analyzed for species-specific mutations. With the application of two developed primer pairs located in the *trnK* 5′ intron and *trnL-trnF*, *C. officinalis* could be distinguished from other species of the genus and all outgroup samples tested. Adulterations of *Calendula* DNA in saffron could be detected down to 0.01%.
- *Conclusions:* With the developed assay, *C. officinalis* can be reliably identified and admixtures of this species as adulterant of saffron can be revealed at low levels.

Key words: Asteraceae; *Calendula*; *Calendula officinalis*; high-resolution melting curve analysis (HRM); molecular phylogeny.

Calendula L. (marigold) is the type genus of the small tribe Calenduleae (Asteraceae). While all other genera of the Calenduleae are native to southern Africa, *Calendula* is distributed in the Northern Hemisphere. *Calendula* species occur mainly in the Mediterranean area, from Morocco and Spain to Iran, southward to the Hoggar Mountains (Algeria) and Yemen (Norlindh, 1946), and northward to Germany and Poland. The center of distribution is northwestern Africa; eight species are listed in the Flora of northern Morocco (Valdés et al., 2002). The genus *Calendula* consists of 12 annual or perennial species, which are regarded as taxonomically complicated due to hybridizations (Norlindh, 1977; Heyn and Joel, 1983). Within the genus, *C. officinalis* L. (common marigold) is of special importance due to its use as an economic crop. *Calendula officinalis* flowers are used for pharmaceutical purposes (EDQM, 2014), in skin care products because of their anti-inflammatory activity (Talhouk et al., 2007), and as feed additives to improve the color of food because of their orange color (carotenoids) (Mukherjee et al., 2011). Florets of orange cultivars are also used as an adulterant of the expensive spice saffron (Marieschi et al., 2012). The fruits of *C. officinalis* are rich in fatty oil that has, because of its unusual composition, numerous technical applications (Zanetti et al., 2013). Common marigold is also an important ornamental plant with many cultivars. The flower heads are up to 5 cm in diameter, which is relatively large compared to other species of the genus. The flower heads vary from pastel yellow to deep orange, and several cultivars are double flowered.

At present, the identification of *C. officinalis* is often performed by (high-performance) thin-layer chromatography (TLC) or by using morphological characters (EDQM, 2014; AHPA, 2015). To the best of our knowledge, DNA-based methods do not yet exist. It can be assumed that TLC is not able to distinguish all *Calendula* species, and that processed plant material (e.g., fine-cut or ground flowers) cannot be identified to species level by morphology. Therefore, a DNA-based method to identify this species has the potential to complement existing methods in quality control. High-resolution melting curve analysis (HRM) is based on the melting behavior of relatively short, double-stranded DNA fragments and is a fast and reliable post-PCR method to detect mutations like single-nucleotide polymorphisms (SNPs) or indels. With a slow, stepwise increase of temperature, a fluorescent dye incorporated between the two DNA strands is released depending on sequence, GC content, and length of PCR products, resulting in a specific melting curve (Ririe et al., 1997; Liew et al., 2004).

Compared to sequencing standard barcode markers, the designed assay is much faster, less labor-intensive, and hence much cheaper. After only 2 h of PCR and subsequent HRM analysis, results are available. Furthermore, the short amplification products facilitate analysis of degraded DNA, as is often present in finely powdered material. Marieschi et al. (2012) developed sequence-characterized amplified region (SCAR) markers for the discrimination of saffron from several adulterants (including *C. officinalis*) and were able to detect adulterations of as little as 1%. Jiang et al. (2014) reported on a barcode melting curve analysis using general *psbA-trnH* primers for the same purpose. According to their methodology and results

[1]Manuscript received 16 June 2015; revision accepted 29 August 2015.

The authors thank the Herbarium of the University of Vienna (Austria) and the IPK Gatersleben (Germany) for making their collections available. The authors thank M. Koch, Z. Aytac, J. Wohlmutter, and A. Gupte for their technical assistance. This report has received funding from the European Community's Seventh Framework Program (FP7/2007–2013) under grant agreement n_245199. It has been carried out within the PlantLIBRA project (http://www.plantlibra.eu).

[3]Author for correspondence: Johannes.Novak@vetmeduni.ac.at

doi:10.3732/apps.1500069

(extensively overlapping peaks of *Calendula* and saffron), we would suppose that the detection limit of *Calendula* adulterations is considerably higher than 1%. Both assays were not tested for the species-specificity of *C. officinalis*.

The aim of this study was to develop a DNA-based assay to identify the economically important plant *C. officinalis* and to distinguish it from other species of the genus. The analysis of outgroup samples should demonstrate the specificity of the assay and improve the reliability of the results. Several outgroup species grow wild in Central Europe and are therefore potential contaminants as "weeds," but frequent adulterations are not reported. Additionally, we tested whether the assay is able to detect *C. officinalis* as an adulterant in saffron samples.

METHODS AND RESULTS

***DNA extraction*—**The sample set included dried leaves of 225 *Calendula* samples of 10 species, 63 outgroup samples of 14 genera (all Asteraceae), and three samples of saffron stigmata (*Crocus sativus* L., Iridaceae) (Appendix 1). Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol ("CTAB method 1"; Schmiderer et al., 2013, based on Doyle and Doyle, 1990). This extraction included a mixture of 1 mL CTAB extraction buffer containing 27.4 mM CTAB, 0.7 M NaCl, 13.5 mM β-mercaptoethanol, 14.4 mM sodium dodecyl sulphate, 4.1 µg Proteinase K, 10 mg polyvinylpyrrolidone K30 (all reagents from Carl Roth GmbH, Karlsruhe, Germany), 1 mM EDTA (pH 8), and 10 mM Tris-HCl (pH 8) (Sigma-Aldrich, Vienna, Austria) per sample. For the DNA extraction of saffron samples, an additional washing step with 70% ethanol was performed.

***Sequencing and sequence analysis*—**The nuclear internal transcribed spacer region (ITS), the chloroplast *rbcL* gene, and part of the *matK* gene, all commonly used DNA barcoding regions (Fazekas et al., 2012), and the *trnK* 5′ intron, *trnL-trnF* intergenic spacer, and the *psbA-trnH* intergenic spacer were sequenced from 22 samples of 10 *Calendula* species and two *Dimorphotheca pluvialis* (L.) Moench samples (GenBank accession no.: KM356075–KM356196, KM668487). For a 15-µL PCR reaction, 1 µL of genomic DNA (1 : 50 dilution of the original DNA extract, equivalent to approx. 1–50 ng) was added to a master mix containing 1× PCR buffer B, 2.5 mM $MgCl_2$, 133 µM dNTP mix, 0.6 units *Taq* HOT FIREPol DNA Polymerase (all reagents from Solis BioDyne, Tartu, Estonia), and 0.6 µM forward and reverse primer (Life Technologies, Vienna, Austria). The PCR cycle profile included a denaturation step at 95°C for 15 min, followed by 45 cycles at 95/55/72°C for 45/45/90 s, with a final elongation step of 9 min at 72°C. PCR products were checked on 1.4% agarose gels and purified with ExoI and SAP (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. Sequencing was performed by Microsynth (Vienna, Austria) using the same primers as for the original amplification (Table 1). The obtained sequences were edited using Chromas version 2.24 (Technelysium, Tewantin, Australia) and aligned with MEGA6 (Tamura et al., 2013). The sequence analysis involved an alignment of 37 ITS sequences with a total of 641 positions (Appendix S1) and an alignment of 23 chloroplast sequences with a total of 2413 positions (Appendix S2). Each chloroplast sequence was a combination of the *trnK* 5′ intron, part of *matK*, *trnL-trnF*, *psbA-trnH*, and *rbcL* sequences of one sample. Candidate diagnostic nucleotides were identified using nucDiag from the R package Spider 1.3-0 (Brown et al., 2012).

***Primer design and HRM*—**HRM-suitable primers were designed based on the chloroplast *trnK* 5′ intron and *trnL-trnF* intergenic spacer alignments. Primers with an optimum melting temperature ranging from 56°C to 58°C were designed using Primer Express 2.0 (Applied Biosystems, Foster City, California, USA) (Table 1). HRM with preamplification was performed with a Rotor-Gene 6000 (QIAGEN, Hilden, Germany). For a 10-µL PCR reaction, 1 µL of genomic DNA (1 : 50 dilution of the original DNA extract, equivalent to approx. 1–50 ng) was added to a master mix containing 1× HOT FIREPol EvaGreen HRM Mix (no ROX) (Solis BioDyne) and 0.15 µM forward and reverse primers (Life Technologies). The PCR cycle profile included a denaturation step at 95°C for 15 min, followed by 45 cycles at 95/58/72°C for 10/20/20 s. The melting analysis was performed by increasing the temperature from 68°C to 82°C by 0.1°C/s. All reactions were done in duplicates. In each HRM run, reference samples for each expected curve type were included. The melting curves were analyzed using Rotor-Gene 6000 Series software (QIAGEN). The PCR efficiency (E) was calculated with a 10-fold dilution series following the formula $E = 10^{(-1/slope)} - 1$. The straight calibration line included five measuring points for each primer combination. The efficiency of the *trnK* primers was 93.0% ($R^2 = 0.9994$), and the efficiency of the *trnL-trnF* primers was 78.5% ($R^2 = 0.9981$).

***Identification of* C. officinalis—**For *C. officinalis*, only one species-specific mutation could be found in all sequenced loci, located at position 211 of the *trnK-matK* alignment (Table 2). The confirmation of this diagnostic nucleotide was performed by developing HRM-suitable primers and testing an extensive sample set (Appendix 1). The primer pair Cal_trnK_2F&R was designed to amplify 71 bp of the *trnK* 5′ intron including this SNP (A/C transversion), which divided all *Calendula* samples into two groups. Group 1 consisted only of *C. officinalis* samples, and group 2 consisted of samples of all other *Calendula* species (Fig. 1A). One outgroup sample of *Senecio* L. sp. grouped with *C. officinalis*, whereas *Tagetes patula* L. and a part of the *Anthemis tinctoria* L. samples showed melting curves of group 2. The other outgroup samples formed three further groups with higher melting temperatures (Fig. 1B). The *Helianthus* L. samples showed poor amplification due to an indel in the primer-binding site and unspecific HRM curves. The primer pair Cal_trnL-F_1F&R amplifies 126 bp of the *trnL-trnF* intergenic spacer. Several SNPs divided the *Calendula* samples in three groups. Group I consisted of samples of *C. maroccana* (Ball) B. D. Jacks. and *C. lanzae* Maire, group II consisted of samples of *C. eckerleinii* Ohle and *C. meuselii* Ohle, and group III consisted of samples of *C. officinalis* and all other *Calendula* species (Fig. 1C). The tested outgroup samples showed many different melting curves, but all of them with higher melting temperatures than the *Calendula* samples, except *Petasites* Mill. spp. The latter showed melting curves very similar to *C. officinalis* but distinguishable from our target species by the *trnK* primers (Fig. 1D). The *Tagetes* L. samples showed an

TABLE 1. Base composition of PCR, sequencing(*), and HRM primers used in this study.

Forward primer	Sequence (5′–3′)	Reverse primer	Sequence (5′–3′)	References
PCR and Sequencing				
ITS5*	GGAAGGAGAAGTCGTAACAAGG	ITS4*	TCCTTCCGCTTATTGATATGC	White et al., 1990
Cal_trnK_2F*	CCCCCAAATCCTCTACCTTTC	12 matK-1506R	TTCCATAGAAATATATTCG	Johnson and Soltis, 1994
Cal_trnK_2F*	CCCCCAAATCCTCTACCTTTC	13 matK-1848R	TATCGAACTTCTTAATAGC	Johnson and Soltis, 1994
matKf1	ATACTCCTGAAAGATAAGTGG	ccmp1r*	CCGAAGTCAAAAGAGCGATT	Heinze, 2007 (matKf1); Weising and Gardner, 1999 (ccmp1r)
trnL-trnF e*	GGTTCAAGTCCCTCTATCCC	trnL-trnF f	ATTTGAACTGGTGACACGAG	Taberlet et al., 1991
psbA3′f*	GTTATGCATGAACGTAATGCTC	trnHf	CGCGCATGGTGGATTCACAATCC	Sang et al., 1997 (psbA3′f); Tate and Simpson, 2003 (trnHf)
rbcLa_F	ATGTCACCACAAACAGAGACTAAAGC	rbcL_ajf634R*	GAAACGGTCTCTCCAACGCAT	Levin et al., 2003 (rbcLa_F); Fazekas et al., 2008 (rbcL_aj634R)
HRM Analysis				
Cal_trnK_2F[a]	CCCCCAAATCCTCTACCTTTC	Cal_trnK_2R	TCTAGCCCTAAATAGCTTTGGAATT	This study
Cal_trnL-F_1F[a]	TAAAAATGAACATCTTTGAGCAAGAA	Cal_trnL-F_1R	GAACGTGGGTCTATGTCAATTG	This study

[a]Amplicon size: Cal_trnK_2F&R = 71 bp; Cal_trnL-F_1F&R = 126 bp.

TABLE 2. Diagnostic nucleotide candidates to distinguish individual species.[a]

Species	*n*	ITS	*trnK-matK*	*psbA-trnH*	*rbcL*	*trnL-trnF*
Calendula arvensis	7	0	0	0	0	0
C. eckerleinii	1	0	0	1149 (C/A) 1166 (T/C)	0	0
C. incana subsp. *microphylla*	1	513 (T/C)	254 (C/A)	1199 (T/G)	0	0
C. lanzae	1	104 (T/C)	0	0	0	0
C. maroccana	1	0	0	0	0	2260 (A/C)
C. meuselii	1	0	855 (C/T)	0	0	2327 (A/G)
C. officinalis	2	0	211 (A/C)	0	0	0
C. stellata	2	0	0	0	0	0
C. suffruticosa	3	0	0	0	0	0
C. tripterocarpa	2	0	0	0	0	0

Note: *n* = number of individuals.

[a] Nucleotide position is given, with diagnostic nucleotides in parentheses; the first is the species-specific nucleotide.

insufficient amplification resulting in unspecific HRM curves. With the application of both primer pairs, all samples of *C. officinalis* were reliably identified.

***Detection of* C. officinalis *as an adulterant of saffron*—**For the detection of *Calendula* in saffron, artificial DNA admixture series of 0%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, 10%, and 100% *C. officinalis* DNA in *Crocus sativus* DNA were prepared and standardized to 10 ng/µL. Concentrations of the DNA extracts were determined using a NanoDrop ND-2000c (Peqlab Biotechnologie GmbH, Erlagen, Germany). For the mixture series, two different samples of saffron (Cal139 and Cal142) were used; each mixture series was prepared and tested twice. The amplification ability of the admixture series and pure saffron DNA was tested with both primer combinations. The homology of primer-binding sites in saffron was tested in silico with the most closely related, published sequences (*trnK*: *Crocus banaticus* Heuff. [GenBank accession no. JX903623.1], *Crocus cartwrightianus* Herb. [JX903624.1], *Iris pseudacorus* L. [KC118962.1]; *trnL-trnF*: *Iris laevigata* Fisch. [DQ286792.1]). Several mismatches in the primer-binding sites led to no or very poor, unspecific amplification products of saffron DNA. The analysis of the admixture series revealed that with both primer combinations, admixtures of above 0.01% *C. officinalis* (equivalent to 1 pg DNA, = limit of detection) were consistently identified as *C. officinalis* (Fig. 2A, C). In the qPCR, the admixtures showed an increase of the *Cq* value according to the decrease of the *Calendula* DNA concentration (Fig. 2B, D), while the HRM curves of samples containing between 1 pg and 100 ng DNA (introduced to PCR) were equal. Lower admixtures were amplified only randomly but showed, if properly amplified, in most cases an HRM curve like that of higher admixtures.

DISCUSSION

DNA barcoding has become an important technique for taxonomy, as well as in applications like quality (i.e., identity) control of food or herbal raw materials. Although genetic differences in the chloroplast set as well as in ITS were relatively small, one SNP was detected that distinguished the economically important target species *C. officinalis* from all other *Calendula* species. Testing our HRM assay with an extensive set of Asteraceae species revealed that one sample of *Senecio* sp. gave the same result as *C. officinalis* in the *trnK* primer combination. Therefore, a second assay in the *trnL-trnF* intergenic spacer was applied, to

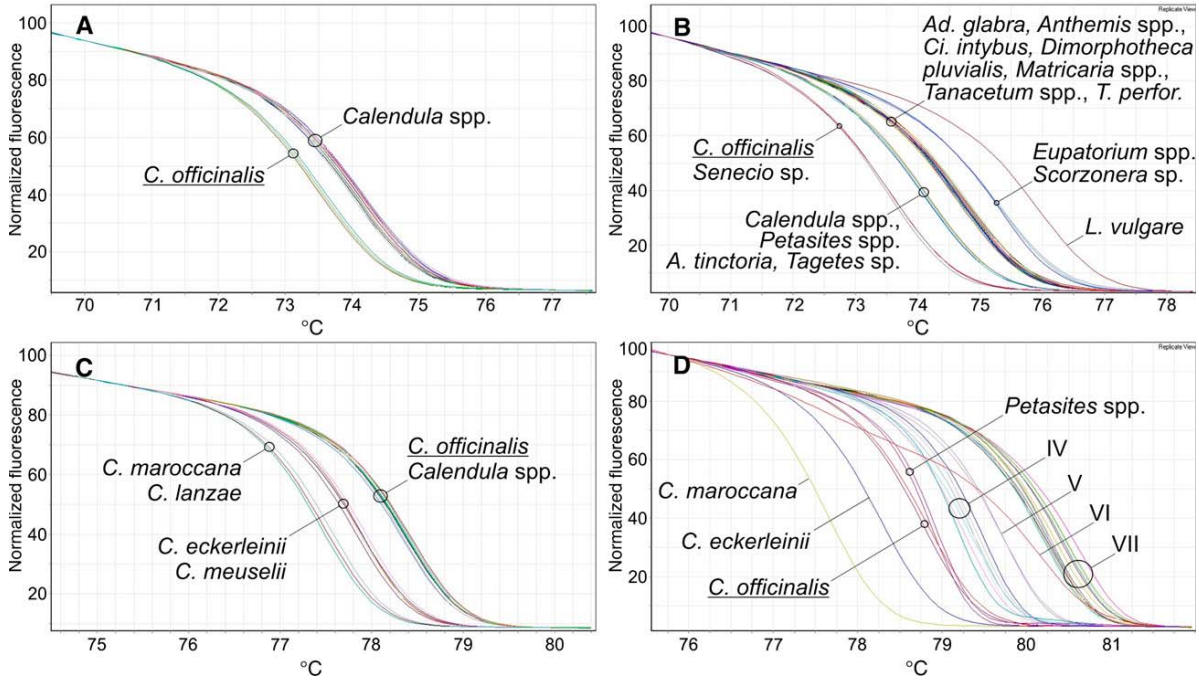

Fig. 1. HRM analysis based on two chloroplast markers. *A.* = *Anthemis*, *Ad.* = *Adenostyles*, *C.* = *Calendula*, *Ci.* = *Cichorium*, *L.* = *Leucanthemum*, *T. perfor.* = *Tripleurospermum perforatum*. (A) HRM analysis with the primer pair Cal_trnK_2F&R amplifying one species-specific SNP (A/C) located in the *trnK* 5′ intron, distinguishing *Calendula officinalis* samples from all other analyzed samples of the genus. (B) HRM analysis of outgroup samples with the primers Cal_trnK_2F&R. (C) HRM analysis with the primer pair Cal_trnL-F_1F&R of a 126-bp part of the *trnL-trnF* intergenic spacer including several SNPs. The *Calendula* samples were divided in three groups. Group I: *C. maroccana* and *C. lanzae*, group II: *C. eckerleinii* and *C. meuselii*, group III: *C. officinalis* and all other *Calendula* samples. (D) HRM analysis of outgroup samples with the primers Cal_trnL-F_1F&R. Group IV: *Adenostyles glabra*, *Eupatorium cannabinum*, *E. perfoliatum*, *Matricaria nigellifolia*, *Scorzonera* sp., *Senecio* sp. Group V: *E. purpureum*, *Helianthus annuus*. Group VI: *Tanacetum vulgare*. Group VII: *Anthemis* spp., *Ci. intybus*, *Dimorphotheca pluvialis*, *Helianthus tuberosus*, *Leucanthemum vulgare*, *Matricaria* spp., *Tanacetum parthenium*, *Tripleurospermum perforatum*. HRM curves of other *Tanacetum* samples appeared between V and VI (data not shown).

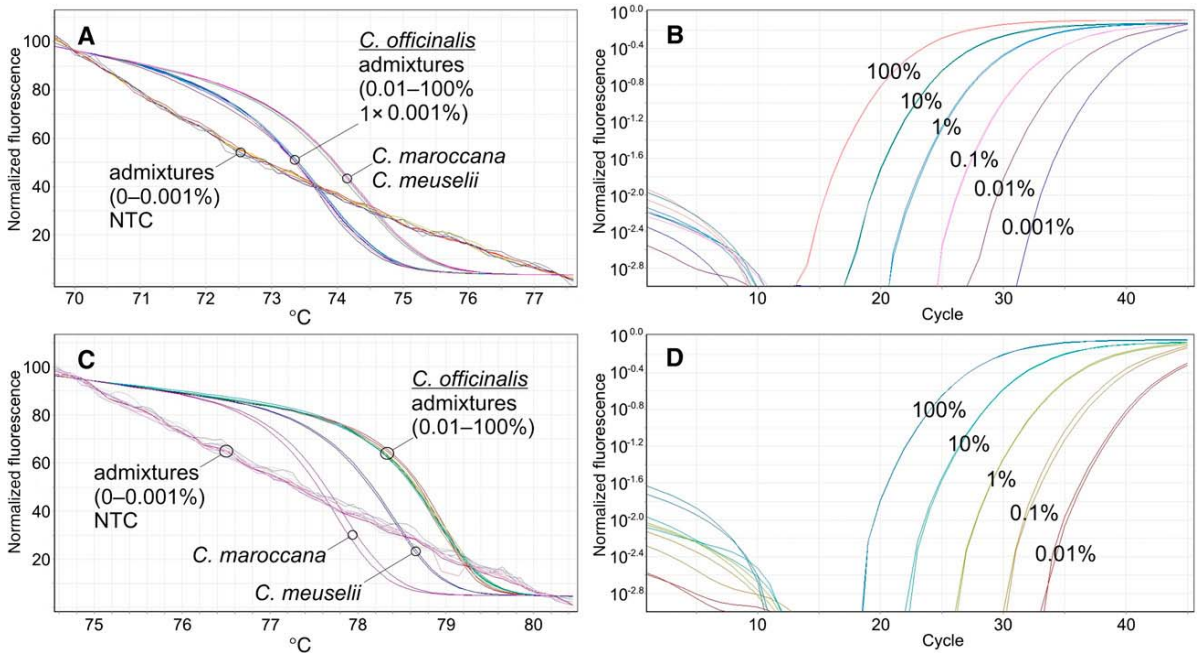

Fig. 2. Analysis of artificial admixtures of *Calendula officinalis* in saffron. All properly amplified admixture samples showed an equivalent HRM curve like the *C. officinalis* references. (A) HRM analysis with the primer pair Cal_trnK_2F&R. (B) Amplification plot of the qPCR corresponding to A. (C) HRM analysis with the primer pair Cal_trnL-F_1F&R. (D) Amplification plot of the qPCR corresponding to B. %-Values mean proportion of *C. officinalis* DNA in saffron DNA of each sample. NTC = no template control.

distinguish this *Senecio* sample from *C. officinalis*. The combination of both analyses had greater discriminatory power than just the *trnK* assay, although all closely related species could be distinguished with the *trnK* primers only. Additionally, this assay can be used to detect adulterations of saffron with *Calendula* flowers. Due to the high specificity of the used *Calendula* primers, even traces of marigold would be detected.

LITERATURE CITED

AHPA (American Herbal Products Association). 2015 onward [last modification 5 May 2015]. Website http://www.botanicalauthentication.org/index.php/Calendula_officinalis_(flower) [accessed 8 August 2015].

Brown, S. D. J., R. A. Collins, S. Boyer, M. C. Lefort, J. Malumbres-Olarte, and C. J. Vink. 2012. Spider: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. *Molecular Ecology Resources* 12: 562–565.

Doyle, J. J., and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus (San Francisco, Calif.)* 12: 13–15.

EDQM (European Directorate for the Quality of Medicines and Health Care). 2014. Europäisches Arzneibuch, 8th ed., Vol. 2. Amtliche österreichische Ausgabe. Verlag Österreich, Vienna, Austria.

Fazekas, A. J., K. S. Burgess, P. R. Kesanakurti, S. W. Graham, S. G. Newmaster, B. C. Husband, S. C. Barrett, et al. 2008. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS One* 3: e2802.

Fazekas, A. J., M. L. Kuzmina, S. G. Newmaster, and P. M. Hollingsworth. 2012. DNA barcoding methods for land plants. In W. J. Kress and D. L. Erickson [eds.], Methods in molecular biology, vol. 858: DNA barcodes: Methods and protocols, 223–252. Humana Press, Totowa, New Jersey, USA.

Heinze, B. 2007. A database of PCR primers for the chloroplast genomes of higher plants. *Plant Methods* 3: 4.

Heyn, C. C., and A. Joel. 1983. Reproductive relationships between annual species of *Calendula* (Compositae). *Plant Systematics and Evolution* 143: 311–329.

Jiang, C., L. Cao, Y. Yuan, M. Chen, Y. Jin, and L. Huang. 2014. Barcoding melting curve analysis for rapid, sensitive, and discriminating authentication of saffron (*Crocus sativus* L.) from its adulterants. *BioMed Research International* 2014: 809037.

Johnson, L. A., and D. E. Soltis. 1994. *mat*K DNA sequences and phylogenetic reconstruction in Saxifragaceae s. str. *Systematic Botany* 19: 143–156.

Levin, R. A., W. L. Wagner, P. C. Hoch, M. Nepokroeff, J. C. Pires, E. A. Zimmer, and K. J. Sytsma. 2003. Family-level relationships of Onagraceae based on chloroplast *rbcL* and *ndhF* data. *American Journal of Botany* 90: 107–115.

Liew, M., R. Pryor, R. Palais, C. Meadows, M. Erali, E. Lyon, and C. Wittwer. 2004. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clinical Chemistry* 50: 1156–1164.

Marieschi, M., A. Torelli, and R. Bruni. 2012. Quality control of saffron (*Crocus sativus* L.): Development of SCAR markers for the detection of plant adulterants used as bulking agents. *Journal of Agricultural and Food Chemistry* 60: 10998–11004.

Mukherjee, A., B. Mandal, and S. Banerjee. 2011. Role of plant based natural carotenoids on feeding, growth and colouration of fish: A case study. *In* M. Yamaguchi [ed.], Carotenoids: Properties, effects and diseases, 179–188. Nova Science Publishers, Hauppauge, New York, USA.

Norlindh, T. 1946. Studies in the Calenduleae. II. Phytogeography and interrelation. *Botaniska Notiser* 4: 471–506.

Norlindh, T. 1977. Calenduleae—Systematic review. *In* V. H. Heywood, J. B. Harborne, and B. L. Turner [eds.], Biology and chemistry of the Compositae, 961–987. Academic Press, London, United Kingdom.

Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* 245: 154–160.

Sang, T., D. J. Crawford, and T. F. Stuessy. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84: 1120–1136.

Schmiderer, C., B. Lukas, and J. Novak. 2013. Effect of different DNA extraction methods and DNA dilutions on the amplification success in the PCR of different medicinal and aromatic plants. *Zeitschrift für Arznei- und Gewürzpflanzen* 18: 65–72.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

Talhouk, R. S., C. Karam, S. Fostok, W. El-Jouni, and E. K. Barbour. 2007. Anti-inflammatory bioactivities in plant extracts. *Journal of Medicinal Food* 10: 1–10.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30: 2725–2729.

Tate, J. A., and B. B. Simpson. 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany* 28: 723–737.

Valdés, B., M. Rejdali, A. Achhal el Kadmiri, S. L. Jury, and J. M. Montserrat [eds.]. 2002. Catalogue des plantes vasculaires du nord du Maroc, incluant des clés d'identification / Checklist of vascular plants of N Morocco with identification keys, vol. 2, 670–672. Consejo Superior de Investigaciones Científicas, Madrid, Spain.

Weising, K., and R. C. Gardner. 1999. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome* 42: 9–19.

White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White [eds.], PCR protocols: A guide to methods and applications, 315–322. Academic Press, San Diego, California, USA.

Zanetti, F., A. Monti, and M. T. Berti. 2013. Challenges and opportunities for new industrial oilseed crops in EU-27: A review. *Industrial Crops and Products* 50: 580–595.

Appendix 1. Locality and specimen information of reference samples used in this study.[a]

Species	*n*	Herbarium ID no. (Laboratory code)[b]	Collection locality (Collection date)[c]
Calendula arvensis L.	1	Cal104	Cultivated
C. arvensis	1	WU082667 (Cal119)	WU: Turkey (5.4.2002)
C. arvensis	1	WU082668 (Cal120)	WU: Jordan (9.3.1992)
C. arvensis	1	WU082669 (Cal121)	WU: Italy (14.4.2004)
C. arvensis (*C. micrantha*)	1	WU082670 (Cal125)	WU: Greece, Crete (24.4.1914)
C. arvensis (*C. micrantha*)	1	WU082671 (Cal126)	WU: Greece, Crete (24.4.1914)
C. arvensis (*C. persica*)	1	WU082672 (Cal128)	WU: Iran (24.4.1885)
C. arvensis	3	IPK-CAL 38	Morocco, ACCID: 50036
C. arvensis	6	IPK-CAL 75	Spain, ACCID: 98773
C. arvensis	7	IPK-CAL 82	Egypt, ACCID: 247372
C. arvensis	9	IPK-CAL 27	Italy, ACCID: 80458
C. arvensis	10	IPK-CAL 13	Spain, ACCID: 77842
C. arvensis	10	IPK-CAL 40	Morocco, ACCID: 50038
C. arvensis	10	IPK-CAL 42	Greece, ACCID: 50040
C. arvensis	12	IPK-CAL 17	Libya, ACCID: 82082
C. eckerleinii Ohle	12	IPK-CAL 9	Morocco, ACCID: 49196
C. incana Willd. (*C. tomentosa*)	1	WU082676 (Cal132)	WU: Tunisia (12.4.1913)
C. incana (*C. tomentosa*)	1	WU082677 (Cal133)	WU: Tunisia (12.4.1913)
C. incana subsp. *algarbiensis* (Boiss.) Ohle	1	WU082673 (Cal122)	WU: Portugal (12.8.1968)
C. incana subsp. *microphylla* (Lange) Ohle	2	WU082674 (Cal123), WU082675 (Cal124)	WU: Portugal (8.4.1971)
C. lanzae Maire	1	IPK-CAL 41	Morocco, ACCID: 50039
C. maroccana (Ball) B. D. Jacks.	4	IPK-CAL 95	Morocco, ACCID: 236458
C. maroccana	10	IPK-CAL 29	Cultivated, ACCID: 49214
C. meuselii Ohle	9	IPK-CAL 8	Morocco, ACCID: 49195
C. officinalis L.	1	Cal101	Cultivated at VMU
C. officinalis	1	Cal102	Cultivated at VMU
C. officinalis	1	Cal103	Cultivated
C. officinalis	1	WU08267 (Cal127)	WU: cultivated at HBV
C. officinalis	5	Cal105-9	Cultivated
C. officinalis	12	IPK-CAL 16	Libya, ACCID: 81928
C. officinalis 'Bico'	1	Cal118	Cultivated at VMU
Calendula L. sp.	5	IPK-CAL 54	Morocco, ACCID: 50052
Calendula sp.	6	IPK-CAL 53	Morocco, ACCID: 50051
C. stellata Cav.	1	WU082679 (Cal129)	WU: Morocco (17.4.2003)
C. stellata	5	IPK-CAL 45	Morocco, ACCID: 50043
C. stellata	5	IPK-CAL 51	Morocco, ACCID: 50049
C. stellata	7	IPK-CAL 98	Morocco, ACCID: 236450
C. suffruticosa Vahl	6	IPK-CAL 63	Tunisia, ACCID: 59220
C. suffruticosa	6	IPK-CAL 94	Portugal, ACCID: 259716
C. suffruticosa	6	IPK-CAL 96	Italy, ACCID: 259717
C. suffruticosa	7	IPK-CAL 44	Algeria, ACCID: 50042
C. suffruticosa	8	IPK-CAL 22	Italy, ACCID: 80066
C. suffruticosa	9	IPK-CAL 33	Cultivated, ACCID: 50034
C. suffruticosa	12	IPK-CAL 15	Algeria, ACCID: 49202
C. suffruticosa	1	WU027733 (Cal131)	WU: Spain (9.3.2002)
C. suffruticosa	1	WU082680 (Cal130)	WU: Morocco (21.4.2003)

Appendix 1. Continued.

Species	*n*	Herbarium ID no. (Laboratory code)[b]	Collection locality (Collection date)[c]
C. tripterocarpa Rupr.	1	IPK-CAL 49	Morocco, ACCID: 50047
C. tripterocarpa	2	WU082681 (Cal134-5)	WU: Morocco (22.4.2003)
Adenostyles glabra DC.	1	Ast 06	Austria, LA, Hohe Wand; 47°51′07″N, 16°02′31″E (5.5.2011)
Anthemis altissima L.	1	IPK-ANTHE 18	Cultivated, ACCID: 49159
A. arvensis L.	1	IPK-ANTHE 7	Cultivated, ACCID: 49154
A. austriaca Jaq.	1	Anth 01	Austria, LA, Bisamberg; 48°19′00″N, 16°21′40″E (11.5.2015)
A. austriaca	1	IPK-ANTHE 17	Cultivated, ACCID: 49158
A. cotula L.	1	IPK-ANTHE 10	Cultivated, ACCID: 49156
A. tinctoria L.	1	IPK-ANTHE 25	Armenia, ACCID: 57847
A. tinctoria	1	IPK-ANTHE 33	Cultivated, ACCID: 236444
A. tinctoria	1	Rühl-Ant05x	Trade sample
A. tinctoria	2	Anth 14	Austria, LA, Kamptal; 48°37′51″N, 15°36′51″E (6.8.2011)
Cichorium intybus L.	3	Ast 03-5	Austria, V, J. Baumann Gasse; 48°15′15″N, 16°25′54″E (23.6.2011)
Crocus sativus L.	1	Cal138	Trade sample (Kotany)
C. sativus	1	Cal139	Trade sample (Iran)
C. sativus	1	Cal142	Trade sample (Greece)
Dimorphotheca pluvialis (L.) Moench	1	IPK-DIM 3	Cultivated, ACCID: 86120
D. pluvialis	7	IPK-DIM 17	Cultivated, ACCID: 258980
Eupatorium cannabinum L.	1	Ast 01	Austria, V, Lainzer Tiergarten; 48°10′01″N, 16°15′15″E (5.5.2011)
E. cannabinum	1	Ast 02	Austria, V, Wienerwald; 48°14′00″N, 16°16′16″E (7.5.2011)
E. cannabinum	1	Ast 07	Austria, LA, Hohe Wand; 47°51′07″N, 16°02′31″E (21.6.2011)
E. cannabinum	1	Ast 08	Austria, ST, Spielberg; 47°14′18″N, 14°47′06″E (10.7.2011)
E. cannabinum	1	Ast 15	Austria, LA, Kamptal; 48°37′55″N, 15°36′49″E (6.8.2011)
E. perfoliatum L.	1	Rühl-Eup02	Trade sample
E. purpureum L.	1	Rühl-Eup03	Trade sample
Helianthus annuus L.	1	Cal111	Cultivated, V, Siebensterngasse
H. tuberosus L.	1	Cal110	Cultivated
Leucanthemum vulgare Lam. agg.	1	Anth 05	Austria, LA, Hohe Wand; 47°50′08″N, 16°03′26″E (21.6.2011)
Matricaria disciformis (C. A. Mey.) DC.	1	IPK-TRIP 7	Cultivated, ACCID: 49972
M. discoidea DC.	1	Anth 09-10	Austria, ST, Spielberg; 47°13′10″N, 14°47′20″E (10.7.2011)
M. nigellifolia DC.	1	IPK-MAT 13	Cultivated, ACCID: 49705
M. perforata Mérat	1	IPK-MAT 30	Cultivated, ACCID: 87870
M. recutita L.	1	IPK-MAT 10	Cultivated, ACCID: 49703
M. recutita	1	IPK-MAT 16	Cultivated, ACCID: 49707
M. recutita	1	IPK-MAT 17	Germany, ACCID: 49708
M. recutita	1	IPK-MAT 20	Italy, ACCID: 81538
M. recutita	1	IPK-TRIP 8	Bulgaria, ACCID: 50939
Petasites albus (L.) Gaertn.	2	Ast 11-2	Austria, ST, Spielberg; 47°13′50″N, 14°46′39″E (24.4.2011)
P. hybridus (L.) G. Gaertn., B. Mey. & Scherb.	1	Ast 13	Austria, ST, Spielberg; 47°14′05″N, 14°46′35″E (24.4.2011)
Scorzonera L. sp.	1	Ast 14	Austria, LA, Groß Enzersdorf; 48°11′57″N, 16°33′45″E (15.5.2011)
Senecio L. sp.	1	Sen 01	Austria, V, Baumgartner Höhe; 48°12′24″N, 16°16′50″E (7.5.2011)
Tagetes L. sp.	6	Cal112-7	Cultivated, V, Siebensterngasse
Tanacetum balsamita L.	1	Rühl-Bal01	Trade sample
T. balsamita	1	Rühl-Bal02	Trade sample
T. corymbosum (L.) Sch. Bip.	1	Anth 02	Austria, ST, Spielberg; 47°14′18″N, 14°47′6″E (10.7.2011)
T. corymbosum	1	Anth 03	Austria, LA, Würnitz; 48°25′25″N, 16°26′18″E (22.6.2011)
T. corymbosum	1	Anth 11	Austria, LA, Hollabrunn; 48°32′40″N, 16°06′11″E (12.7.2011)
T. parthenium (L.) Sch. Bip.	1	Rühl-Chr02	Trade sample
T. vulgare L.	1	Anth 12	Austria, LA, Kaltenleutgeben; 48°06′51″N, 16°12′50″E (16.7.2011)
T. vulgare	1	Anth 13	Austria, LA, Kamptal; 48°37′51″N, 15°36′51″E (6.8.2011)
Tripleurospermum perforatum (Mérat) M. Laínz	1	Anth 04	Austria, LA, Hollabrunn; 48°35′05″N, 16°03′55″E (25.6.2011)
T. perforatum	2	Anth 16-7	Austria, LA, Kamptal; 48°37′51″N, 15°36′51″E (6.8.2011)

Note: *n* = number of individuals.

[a] Voucher specimens (excluding those from WU) are stored at the herbarium of the Institute for Animal Nutrition and Functional Plant Compounds under the given herbarium ID numbers.

[b] HBV = Botanical Garden of the University of Vienna, Austria; IPK = Leibniz Institute of Plant Genetics and Crop Plant Research Gatersleben, Germany. Accessions were received as seeds, which were raised in the University's greenhouse in 2012. GPS coordinates of the specimen origins are not known.

[c] ACCID = accession identification number (assigned by IPK); LA = Province Lower Austria; Rühl = Rühlemann's Kräuter und Duftpflanzen, Horstedt, Germany; ST = Province Styria; V = Province Vienna; VMU = University of Veterinary Medicine, Vienna, Austria; WU = Herbarium of the University of Vienna, Austria. Collection dates are presented in the format: day.month.year. GPS coordinates of the specimen origins are not known.

Paper 4

Schmiderer C, Ruzicka J, Novak J (2015): DNA-based identification of *Peucedanum ostruthium* specimens and detection of common adulterants by high-resolution melting curve analysis. *Molecular and Cellular Probes* 29 (6): 343-50.

Author's contributions:

CS designed the *Peucedanum* and *Aconitum* primers, performed the lab work and prepared the manuscript.

JR designed and tested the *Gentiana* and *Veratrum* primers.

JN revised the manuscript.

Molecular and Cellular Probes 29 (2015) 343–350

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Original research article

DNA-based identification of *Peucedanum ostruthium* specimens and detection of common adulterants by high-resolution melting curve analysis

Corinna Schmiderer, Joana Ruzicka, Johannes Novak*

Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

ARTICLE INFO

Article history:
Received 1 June 2015
Received in revised form
15 October 2015
Accepted 15 October 2015
Available online 19 October 2015

Keywords:
Apiaceae
DNA-based authentication
HRM
Identification
Imperatoria ostruthium
Peucedanum ostruthium

ABSTRACT

Masterwort (*Peucedanum ostruthium*, syn. *Imperatoria ostruthium*, Apiaceae) is an old economic plant in Alpine countries cultivated as ornamental plant and used for spirits and in folk medicine. *P. ostruthium* is a species that has often been confused with related Apiaceae species or morphologically similar roots or tubers resulting in products of minor quality. Masterwort can be distinguished from other Apiaceae species by nrDNA (ITS1 and ITS2). The analysed chloroplast markers (trnK 5′ intron, trnT-trnL, and psbA-trnH), however, showed no species-specific mutations. With the application of two primer pairs amplifying parts of ITS and developed for high-resolution melting curve analysis (HRM) the target species was distinguishable from the other *Peucedanum* and Apiaceae species of our reference set. A multiplex PCR/HRM was developed to detect adulterations with *Gentiana* spp., *Aconitum napellus* and *Veratrum album*.

1. Introduction

Masterwort (*Peucedanum ostruthium* (L.) Koch, syn. *Imperatoria ostruthium* L., Apiaceae) has been used in Austrian folk medicine since ancient times against various human diseases like common cold, inflammatory diseases, stomach pain and ulcer, dorsalgia and for treatment of cattle diseases like claw disorders, gastrointestinal diseases or ulcerous wounds [1] and [2]. The rhizomes and leaves contain essential oil and coumarins and possess antiphlogistic and antipyretic properties [3]. Masterwort is also traded as ornamental plant, wild types and varieties with variegated leaves are available.

Peucedanum is often considered as a relatively large genus with more than 100 species distributed in Europe, Asia and Africa [4] and [5]. The Flora Europaea [6] lists 29 species and the flora of Austria, South Tyrol and Liechtenstein [7] lists 10 species. The genus is taxonomically complex and several phylogenetic studies of *Peucedanum* and related genera were published in the last decades. Analysis of nrDNA revealed that *Peucedanum* s. l. is polyphyletic and that the African and several Eurasian species would need to be shifted to other genera [8] [9], and [10]. According to Spalik et al. [9] the segregation of *Peucedanum* s. l. into *Cervaria*, *Holandrea*, *Imperatoria*, *Oreoselinum*, *Peucedanum* s. str., *Pteroselinum*, *Thomasinia*, *Thysselinum* and *Xanthoselinum* results with the exception of *Cervaria* and *Holandrea* in a group of monophyletic genera and retaining such a delimitation of the genus would be advantageous from the point of view of nomenclatural stability. For this reason and according to The Plant List [11], several investigated species are treated in this paper as *Peucedanum* but common or suggested synonyms [9] and [11] are given in Table 1.

Besides the problems of taxonomy, the correct identification of plant material used in trade, food or medicine is of utmost importance. Nowadays existing methods for identification are increasingly complemented by molecular approaches. DNA based methods can get very helpful where morphological features are limited, like in roots, seeds or in cut or ground plant material, or where phytochemical analysis show only limited differentiation. *P. ostruthium* is a species that has often been confused in the past with similar looking roots or tubers that led to products of minor quality. According to Berger [12] and List and Hörhammer [13] confusions or admixtures of masterwort roots (Radix Imperatoriae) with *Peucedanum officinale* L., *Angelica archangelica* L., *Gentiana lutea* L., *Gentiana purpurea* L., *Gentiana punctata* L. or *Gentiana*

* Corresponding author.
E-mail addresses: Corinna.Schmiderer@vetmeduni.ac.at (C. Schmiderer), Joana.Ruzicka@vetmeduni.ac.at (J. Ruzicka), Johannes.Novak@vetmeduni.ac.at (J. Novak).

http://dx.doi.org/10.1016/j.mcp.2015.10.002
0890-8508/

Table 1
Peucedanum and out-group samples and their origin used for analysis.

Sample (synonyms)	Reference no	Origin
Peucedanum ostruthium (L.) W.D.J.Koch (*Imperatoria ostruthium* L.)	Peu17	WU: Austria, C, Hafnergruppe
	Peu49	Austria, LA, Wechsel
	Peu54, 130-4	trade samples (seeds)
	Peu58-63, 148-52	trade samples (nursery/roots and rhizomes)
	Peu66-7, 69	Austria, St, Rosenkogel
	Peu73-7	Austria, N-T, Kühtai
	Peu78-81	Austria, N-T, Fotscher Bach
	Peu84-6, 116-20	Austria, Va, Zug im Lechtal
	Peu88-9	Austria, C, Kleiner Pal
	Peu92-5	Austria, C, Turracher Höhe
	Peu99-101	Austria, C, Nockberge
	Peu102-5	Austria, St, Gaberl
	Peu107-9	Austria, N-T, Fotscher Bach
	Peu112-5	Austria, Va, Lech am Arlberg
	Peu146	Austria, S, Katschberg
	Peu147	Austria, S, Weißpriachtal
	Peu155-60	Italy, Friuli, Laghi di Fusine
	Peu177	RBGE (E00043120): Andorra, El Serrat
Peucedanum aegopodioides (Boiss.) Vandas (*Aegopodium involucratum* Orph. Ex Boiss.)	Peu164-5	RBGE (E00253236, E00253239): Serbia, Uzice
Peucedanum alsaticum L. *(Xanthoselinum alsaticum* (L.) Schur)	Peu46	Austria, V, Stammersdorf
Peucedanum austriacum (Jacq.) W. D. J. Koch *(Pteroselinum austriacum* (Jacq.) Rchb.)	Peu01-3	WU: Austria, LA, Wildendürnbach/Baden/ Gutenstein
Peucedanum carvifolia (*Holandrea carvifolia* (Vill.) Reduron, Charpin et Pimenov, *Dichoropetalum carvifolia* (Vill.) Pimenov & Kljuykov)	Peu04	WU: Bulgaria
	Peu167	RBGE (E00265976): Serbia
Peucedanum cf. *carvifolia*	Peu166	RBGE (E00043050): France, Haut-Rhin
Peucedanum cervaria (L.) Lapeyr. (*Cervaria rivini* Gaertn.)	Peu05-7	WU: cultivated/Austria, LA, Großes Erlauftal/ Eichkogel
	Peu08	WU: Italy, Toscana, Ortaglia
	Peu18, 47	WU: Austria, LA, Pottenstein/Bisamberg
	Peu71-2	Austria, LA, Gainfarn
Peucedanum formosanum Hayata (*Peucedanum terebinthaceum* subsp. *Formosanum* (Hayata) Kitag)	Peu168	RBGE (E00328523): Taiwan, Hsiulin Hsiang
Peucedanum gallicum Latourr.	Peu169	RBGE (E00043049): Portugal, Minho
Peucedanum hispanicum (Boiss.) Endl. (Imperatoria hispanica Boiss)	Peu170	RBGE (E00134254): Spain, Granada
Peucedanum japonicum Thunb.	Peu171	RBGE (E00019242): Taiwan, Chimeiyu
Peucedanum longifolium Waldst. & Kit.	Peu09, 172	WU: cultivated/RBGE (E00043048): Yugoslavia, Crna Gora
Peucedanum obtusifolium Sm. (*Ferula obtusifolia* (Sm.) Spreng.)	Peu173	RBGE (E00043100): Bulgaria, Burgas
Peucedanum officinale L.	Peu10	WU: Austria, B, Götschlacke
	Peu11-3	WU: France, Villeneuves/Bulgaria, Sophia/ cultivated
	Peu178	Kosovo, Prizren, Mirusha
Peucedanum oligophyllum (Griseb.) Vandas (*Dichoropetalum oligophyllum* (Griseb.) Pimenov & Kljuykov, *Seseli oligophyllum* Griseb.)	Peu174	RBGE (E00043066): Macedonia, Bitola
Peucedanum oreoselinum (L.) Moench (*Oreoselinum nigrum* Delarbre)	Peu14	WU: Austria, B, Hirmer Wald
	Peu15	WU: Austria, LA, Pfaffenberg
	Peu175-6	RBGE (E00043140): Italy, Bergamo/ (E00249801): Spain, Cataluña
Peucedanum palustre (L.) Moench) (*Thysselinum palustre* (L.) Hoffm.)	Peu16	WU: Austria, LA, Göpfritz a.d. Wild
Peucedanum praeruptorum Dunn	Peu162-3	Kew Gardens (TCMK 546, 589): China
Peucedanum sp.	Peu110	Austria, T, Nassereith
Peucedanum terebinthaceum (Fisch. ex Trevir.) Ledeb. (*Kitagawia terebinthacea* (Fisch. ex Trevir.) Pimenov, *Selinum terebinthaceum* Fisch. ex Trevir.)	Peu19	WU: Korea
Peucedanum verticillare (L.) W. D. J. Koch ex DC.) (*Tommasinia verticillaris* (L.) Bertol.)	Peu20-1	WU: Austria, LA, Pernitz/Haselrast
Aegopodium podagraria L.	Peu51	Austria, LA, Ybbsitz
Aethusa cynapium L.	Peu33	Austria, LA, Heiligenkreuz
Angelica archangelica L.	Peu22-3, 136, 153-4	trade samples
	Peu34	Austria, V, Anton-Schmid-Promenade
Angelica decursiva (Miq.) Franch. et Sav. *(Peucedanum decursivum* (Miq.) Maxim.)	Peu161	Kew Gardens (TCMK 483): China
Angelica sylvestris L. (*Peucedanum angelicifolium* Turcz.)	Peu35	Germany, Baden-Württemberg, Memmingen
	Peu37, 53	Austria, V, Sulzwiese/LA, Semmering
Anthriscus cerefolium (L.) Hoffm.	Peu36	Austria, V, Süttelstraße
Apium graveolens L.	Peu140	trade sample
Berula erecta (Huds.) Coville	Peu38	cultivated
Carum carvi L.	Peu24, 142	cultivated
Chaerophyllum bulbosum L.	Peu39	Austria, LA, Bisamberg
Chaerophyllum tremulum L.	Peu40	Austria, V, Prater
Conium maculatum L.	Peu143	cultivated
Daucus carota L.	Peu41	Austria, V, Reichsbrücke
Eryngium campestre L.	Peu26	cultivated
Foeniculum vulgare Mill.	Peu141, 145	trade samples

Table 1 (*continued*)

Sample (synonyms)	Reference no	Origin
Heracleum sp.	Peu27, 135	cultivated/Austria, C, Turracher Höhe
Laserpitium latifolium L.	Peu48, 50, 70	Austria, LA, Ybbsitz (2)/Gainfarn
Laserpitium siler L.	Peu28, 42	cultivated/LA, Mödling
Levisticum officinale W. D. J. Koch	Peu29, 144	Austria, cultivated
Ligusticum mutellinoides Vill.	Peu55	Austria, St, Rettlkirchspitze
s odorata (L.) Scop.	Peu30	cultivated
Petroselinum crispum (Mill.) Fuss	Peu139	cultivated
Pimpinella peregrina L.	Peu31	cultivated
Pimpinella saxifraga L.	Peu43	Austria, V, Alte Donau
Seseli annuum L.	Peu44	Austria, V, Stammersdorf
Seseli libanotis (L.) W. D. J. Koch	Peu45	Austria, LA, Bisamberg
Silaum silaus (L.) Schinz & Thell.	Peu32	cultivated
Smyrnium perfoliatum L.	Peu52	WU: cultivated
Aconitum napellus s.l. L.	Aco1	cultivated
	Aco2	WU: Austria, LA, Reichenau/Rax
	Aco3	WU: Austria, Va, Zalim Tal
Gentiana lutea L.	G31-2, G37	cultivated
	G75	Austria, Va, Spullersee
G. pannonica Scop.	G10, G71	Austria, LA, Schneeberg/Rax
G. punctata L.	G54	Austria, St, Idlereckscharte
	G60	Austria, V, Zamangspitze
G. purpurea L.	G61, G63	Austria, V, Kreuzjoch
Persicaria bistorta (L.) Samp	Peu204-5	WU: Austria, LA, Terz/Weitra
Veratrum album L.	G19	Austria, LA, Schneeberg
	G57, G78, G80	Austria, St, Idlereckscharte/Rosenkogel (2)
	G74	Austria, Va, Spullersee
V. nigrum L.	G72	cultivated
	G81	Austria, LA, Haidlhof

RBGE: Royal Botanic Garden Edinburgh, Scotland.
WU: Herbarium of the University of Vienna, Austria.
Austrian provinces: B – Burgenland, C – Carinthia, LA – Lower Austria, S – Salzburg, St – Styria, (N-)T – (Northern) Tyrol, V – Vienna, Va – Vorarlberg.

pannonica Scop., *Persicaria bistorta* (L.) Samp. (syn. *Polygonum bistorta* L.) and the very toxic plants *Aconitum napellus* L. agg. and *Veratrum album* L. are frequent.

P. ostruthium is native to the mountains of Central and South Europe and often escaped from cultivation. The natural habitats are in montane to subalpine (alpine) elevations, in tall herb communities, moist shrub vegetation, stream-banks and mountain meadows [6] and [7]. At least in the Alps, the above-mentioned species of *Gentiana*, *Aconitum* and *Veratrum* can co-occur with *P. ostruthium* and therefore the incidence of admixtures is easily possible in wild collected plant material.

High-resolution melting curve analysis (HRM) is a very simple post-PCR method based on the melting behaviour of double-stranded DNA, dependent on length, GC-content and base composition of DNA molecules. During a slow temperature increase, the melting of double-stranded DNA to its single strands can be observed using a fluorescent dye present in the reaction. For the detection of single nucleotide polymorphisms (SNPs) short amplicons of less than 100 bp are preferred, but HRM analysis of fragments with several hundred base pairs is also possible [14]. Multiplexing of several primer pairs allows the analysis of several DNA fragments in one reaction, as long as the melting temperatures of the different amplicons are clearly distinct. Multiplexing of specific primers enables the detection of minor proportions of several adulterations in one analysis, what is not possible with standard sequencing methods. Compared to sequencing, HRM analysis is faster, less labour intensive and therefore the cheaper method [15].

Here we present a DNA-based identification method by HRM to distinguish *P. ostruthium* from other (European) species of the genus as well as from several other genera of the Apiaceae, traded or growing wild in Central Europe. The analysis of more or less closely related out-group samples will prove the specificity of the assays and the reliability of the results. A multiplex PCR/HRM was designed to detect adulterations with *Gentiana*, *Veratrum* and *Aconitum* also in mixtures.

2. Material and methods

In total 172 plant samples were analysed, including 62 samples of *P. ostruthium* (L.) Koch (Apiaceae), 46 samples of 19 other *Peucedanum* s.l. species, 42 samples of 22 other genera of the Apiaceae and 22 samples of *Aconitum* (Ranunculaceae), *Gentiana* (Gentianaceae), *Persicaria* (Polygonaceae) and *Veratrum* (Melanthiaceae) (Table 1). The specimens were sampled from the Herbarium of the University of Vienna (WU; Austria), the Herbarium of the Royal Botanic Garden Edinburgh (RBGE; Scotland), Kew Gardens (England) or were collected from the wild. Representative voucher specimens of the wild collected samples are stored at the herbarium of the University of Vienna, Austria (herbarium numbers WU 0078629-630, WU 0078633-637, WU 0078676-687, WU 0078702). Five trade samples of "Radix Imperatoriae" or "Rhizoma Imperatoriae" were obtained 2009–2010 from different trade companies, the species-identity was confirmed by sequencing of the ITS region as described below. Six *P. ostruthium* plants were obtained from an Austrian nursery, morphological features confirmed their identity. Two packages of seeds labelled as "*P. ostruthium*" were obtained from a German seed trade company. The seeds were raised in the greenhouse of the Institute of Animal Nutrition and Functional Plant Compounds and at the Department of Pharmacognosy, University of Vienna.

Genomic DNA was extracted from dried specimens using a modified CTAB-protocol [16] based on Doyle [17]. This extraction included a mixture of 1 mL CTAB extraction buffer per sample, containing 27.4 mM cetyltrimethylammoniumbromide, 0.7 M NaCl, 13.5 mM beta-mercaptoethanol, 14.4 mM sodium dodecyl sulphate, 4.1 μg Proteinase K, 10 mg polyvinylpyrrolidone K30 (all reagents from Carl Roth, Karlsruhe, Germany), 1 mM EDTA pH 8 and 10 mM

TRIS-HCl pH8 (both Sigma–Aldrich, Vienna, Austria).

Three chloroplast markers (trnK 5' intron, psbA-trnH intergenic spacer and trnT-trnL intergenic spacer) were sequenced from six samples of *P. ostruthium* and one sample of *Peucedanum austriacum*, *Peucedanum cervaria*, *P. officinale* and *Foeniculum vulgare*, respectively. The nuclear internal transcribed spacers (ITS1 and ITS2) were sequenced from the same species and additionally one sample of *Peucedanum terebinthaceum* and one sample of *Angelica sylvestris* (Genbank accession numbers: KP682393–KP682434). For sequencing 15 μl PCR reactions were prepared using 1× PCR buffer B, 2.5 mM $MgCl_2$, 133 μM dNTPs, 0.6 U Taq HOT FIREPol® polymerase (all reagents from Solis BioDyne, Tartu, Estonia) and 600 nM of each primer (Life Technologies, Vienna, Austria; Table 2). The PCR profile included a denaturation step at 95 °C for 15 min, followed by 40 cycles (95 °C for 45 s, 55 °C for 45 s and 72 °C for 90 s) and a final elongation step at 72 °C for 9 min. PCR products were checked on 1.4% agarose gels, cleaned up with Exonuclease I and Shrimp Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) following the manufacturer's instructions and were sequenced by Microsynth (Vienna, Austria).

For primer design, published *Peucedanum* and *Angelica* sequences of nrDNA ITS1 and ITS2 were aligned using MEGA4 [27]. Primers with an optimal melting temperature of 55 °C suitable for a group of genera closely related to *P. ostruthium* were designed using Primer Express® 2.0 (Applied Biosystems, Foster City, CA, U.S.A.) (Table 2).

The HRM with pre-amplification was performed with a Rotor-Gene Q (Qiagen, Hilden, Germany). For a 10 μl PCR reaction 1 μl of genomic DNA (1:20–1:100 dilutions of the original DNA extract) was added to a master mix containing 1× HOT FIREPol® EvaGreen® HRM Mix (no ROX) (Solis BioDyne, Tartu, Estonia) and 150 nM forward and reverse primers (Life Technologies, Vienna, Austria). The PCR profile included a denaturation step at 95 °C for 15 min, followed by 40 cycles (95 °C for 10 s, 55 °C for 20 s and 72 °C for 20 s) with a final denaturation step at 95 °C for 30 s. The HRM analysis was subsequently performed by increasing the temperature from 70 °C to 90 °C by 0.1 °C/s. All reactions were analysed in duplicates.

The multiplex PCR/HRM assay included general primers amplifying ITS1, and specific primers for *Aconitum* (amplifying part of ITS2), *Gentiana* sectio *Gentiana* (amplifying part of the trnL-trnF intergenic spacer) and *Veratrum* (amplifying part of the trnL intron) (Table 2). The specific primers were accordingly designed as described above but for 60 °C.

The HRM conditions were similar as described above with the following modifications: The primer concentration was 100 nM for each general ITS and *Gentiana* primer, 150 nM for the *Veratrum* primers and 200 nM for the *Aconitum* primers. The PCR included 45 cycles with an annealing temperature of 60 °C. HRM analysis was performed from 70 °C to 95 °C with a temperature increase of 0.2 °C/s. Artificial admixture series of 0.001%, 0.01%, 0.1%, 1%, 10%, 50% and 90% of *Aconitum*, *Veratrum* or *Gentiana* DNA in *P. ostruthium* DNA were prepared and standardised to a final concentration of 5 ng/μl. To compare amplicon melting temperatures of different runs, one sample of *P. ostruthium* was included in each run to calibrate the results.

A univariate ANOVA was calculated with SPSS for Windows version 17.0 (SPSS Inc., Chicago, USA) for Apiaceae specimens with two melting maxima.

3. Results

In the sequenced chloroplast DNA (trnK 5' intron, psbA-trnH and trnT-trnL) some variability within *P. ostruthium* but no species-specific mutations were found. Species-specific mutations appeared in the nrDNA sequences of ITS 1 and ITS 2. Two primer pairs amplifying these mutations (Post_1 in ITS1 and Post_2 in ITS2) were developed and tested with an extensive sample set (Table 1).

3.1. HRM analysis with specific primers

Primer pair Post_1 divided the *Peucedanum* samples into four groups (Fig. 1A). Group I consisted of *P. cervaria* and *P. verticillare*, group II of *P. aegopodioides*, *P. alsaticum, P. terebinthaceum*, *P.* cf. *carvifolia* and the target species *P. ostruthium*, group III of *P. austriacum*, *P. formosanum*, *P. longifolium*, *P. officinale, P. obtusifolium*, *P. oligophyllum*, *P. oreoselinum*, *P. palustre, P. praeruptorum* and the *Angelica* specimens and group IV of *P. carvifolia, P. hispanicum* and *P. japonicum*.

The primer pair Post_2 divided the same sample set into five groups (Fig. 1B). Group I included *P. alsaticum*, group II *P. austriacum* and *P. verticillare*, group III *P. ostruthium* (most wild collected samples), *P.* cf. *carvifolia*, *P. cervaria* and *P. formosanum*, one sample of *P. praeruptorum* (Peu163), group IV *P. ostruthium* (few wild collected samples) and *A. sylvestris* and group V *P. ostruthium* (all trade samples) and the remaining *Peucedanum* and *Angelica* specimens.

Intraspecific variation in the *P. ostruthium* samples appeared as A/G transition within the amplicon of Post_1, whereas the sequenced samples showed either both bases with varying proportions or only an adenine in the according position of the sequence chromatogram. Within the amplicon of Post_2 a T/G transversion occurred, whereas the sequences of the wild collected

Table 2
Base composition of PCR, sequencing and HRM primers used in this study.

Locus	Forward primer	Sequence (5′–3′)	Reverse primer	Sequence (5′–3′)	References	
PCR and Sequencing						
ITS	ITS5	GGAAGGAGAAGTCGTAACAAGG	ITS4	TCCTTCCGCTTATTGATATGC	[18] and [19]	
trnK 5′ intron	matKf1	ATACTCCTGAAAGATAAGTGG	ccmp1r	CCGAAGTCAAAAGAGCGATT	[20] and [21]	
psbA-trnH	psbA3'f	GTTATGCATGAACGTAATGCTC	trnHf	CGCGCATGGTGGATTCACAATCC	[22] and [23]	
trnT-trnL	trnT a	CATTACAAATGCGATGCTCT	trnL b	TCTACCGATTTCGCCATATC	[24]	
Specific HRM Analysis					Amplicon size	References
ITS1	Post_1-F	CACGTCAACAATTTGGGCAAG	Post_1-R	CCACCTACCAGGGATTCGC	~66 bp	This study
ITS2	Post_2-F	ACTGGCCTCCCGTACCTTGT	Post_2-R	CGTCGCCGGAGACTCGT	~55 bp	This study
Multiplex PCR/HRM						
ITS1	17SE (=AB101)	ACGAATTCATGGTCCGGTGAAGTGTTCG	ITS2	GCTACGTTCTTCATCGATGC	~420–460 bp	[25]/[26] and [18]
ITS2	Aco_ITS2_F	CGTCGCGGTCAGTGGTG	Aco_ITS2_R	CAACGAGGACGACGCGTC	65 bp	This study
trnL IGS	Gent_trnLIGS23F	TCCTCGACCCTTTTGCCTATC	Gent_trnLIGS72R	AGATTACAAAGTTTTATCTAGGTCCTATTCG	50 bp	This study
trnL intron	Verat_trnLI112F	AATAAAAAAAGATAGGTGCAGAGACTCAAT	Verat_trnLI154R	TGTGGGCCATCCTTTCTTTAA	43 bp	This study

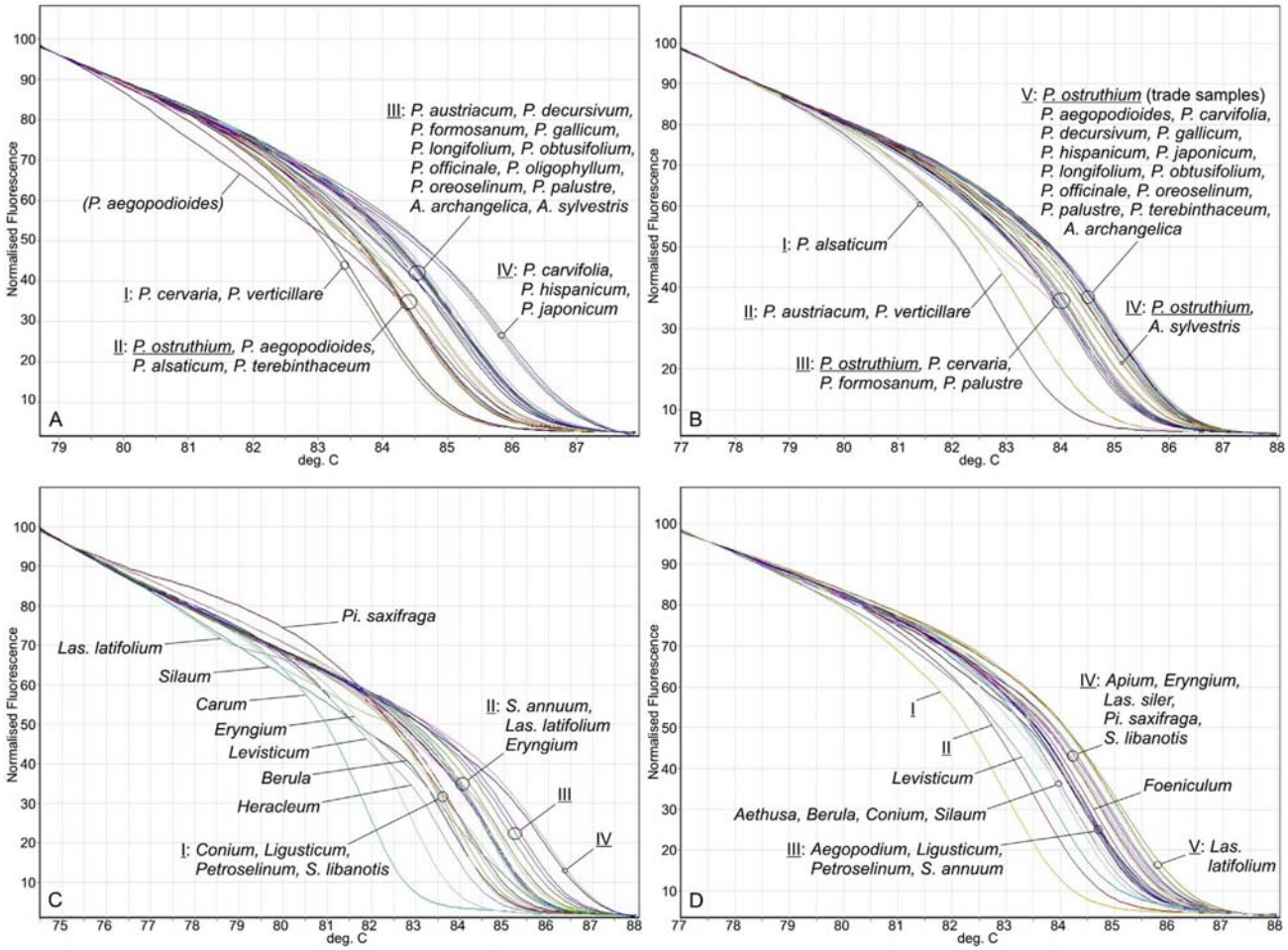

Fig. 1. High-resolution melting curve analysis of *Peucedanum*, *Angelica* and other Apiaceae samples. Roman numbers indicate the HRM curve groups of *Peucedanum* and *Angelica* samples for Post_1 and Post_2, respectively. A: HRM for *Peucedanum* and *Angelica* species based on the primer pair Post_1. B: HRM for *Peucedanum* and *Angelica* species based on the primer pair Post_2. C: HRM for other Apiaceae samples based on the primer pair Post_1. D: HRM for other Apiaceae samples based on the primer pair Post_2. Abbreviations: A.: *Angelica*, Las.: *Laserpitium*, P.: *Peucedanum*, Pi.: *Pimpinella*, S.: *Seseli*.

samples showed either both bases in varying proportions or mainly thymine, but the trade samples showed mainly a guanine. The influences of these variations on the HRM curves were relatively low for Post_1. The HRM curves of the heterozygote samples deviated slightly from the homozygote samples but showed no significant shift of the melting temperature, so this curves were treated as members of the same group. The polymorphism in the amplicon of Post_2 resulted in a significant melting temperature shift, making a classification into different groups necessary.

With both primer pairs, all amplifiable out-group samples (except *Seseli annuum* and *Eryngium campestre*) showed melting curves clearly distinct from those of *P. ostruthium* in at least one locus (Fig. 1C and D, data for not amplifiable samples not shown).

3.2. Multiplex PCR/HRM

The multiplex PCR/HRM included specific primers for short amplicons of *Aconitum*, *Gentiana* and *Veratrum* with relatively low melting temperatures (43–65 bp, 78.1–82.7 °C) and general ITS primers for relatively long amplicons with higher melting temperatures (approx. 420–460 bp, 85.4–94.2 °C) (Table 2 and 3). A preliminary test of the general ITS primers showed no amplification of fungal DNA (data not shown).

The melting curves of the Apiaceae specimens showed one (e.g. *Conium*, *Daucus*, *Foeniculum*, *Ligusticum*, *Petroselinum*), two (e.g. *Peucedanum*, *Angelica, Pimpinella, Seseli*) or three (*Eryngium*, *Heracleum* sp. and *Levisticum*) melting maxima (Table 3). Pipetting and technical inexactness caused a temperature shift of ±0.25 °C between different runs. Therefore the results were calibrated with a reference sample, which was included in each run. All 26 *P. ostruthium* samples showed two melting maxima of 88.59 ± 0.14 °C and 89.92 ± 0.14 °C, respectively. For an easier comparison, the results presented in Table 3 were calibrated to the mean value of the first maximum of the *P. ostruthium* samples. *Peucedanum* species showed either significantly different melting temperatures e.g. *P. aegopodioides* (−0.94 °C for the first melting maximum) or similar temperatures e.g. *P. terebinthaceum* (+0.23 °C).

E. campestre and *S. annuum*, which had similar HRM curves with the *Peucedanum* primers, were clearly distinct with this assessment. *E. campestre* amplicons showed three melting maxima, the third more than 5 °C higher than *P. ostruthium*. The amplicon of *S. annuum* showed two melting maxima like *P. ostruthium*, but the first was slightly but significantly higher and the second lower than the respective maxima of *P. ostruthium*. All *P. ostruthium* samples showed a difference between their maxima of 1.3 ± 0.15 °C,

Table 3
Results of HRM and multiplex analysis. (n) number of samples. Columns Post_1 and Post_2 indicate amplification success with the two primer pairs, respectively. (+) Means good amplification; (~) means poor amplification (plateau phase in the PCR not reached) but HRM curve adequate; (−) means poor or no amplification and no species-specific HRM curve. Columns of peak 1 to 3 give the melting maxima of each sample. The values were calibrated to the mean value of all analysed *Peucedanum ostruthium* samples (88.59 °C). (*) means significant different values for samples with two melting maxima (P = 0.05), samples with (≠) indicate differences to *P. ostruthium* due to the different number of melting maxima. MV ± STD means mean value ± standard deviation [°C].

species	HRM assays			Multiplex assay			
	n	Post_1	Post_2	n	Peak 1	Peak 2	Peak 3
					MV ± STD	MV ± STD	MV ± STD
P. ostruthium	45	+	+	26	0 ± 0.14	1.32 ± 0.13	
Trade samples (roots and rhizomes)	5	+	+	5	−0.03 ± 0.06	1.44 ± 0.11	
Trade samples (plants)	6	+	+	2	0.02 ± 0.04	1.43 ± 0.04	
Trade samples (seeds)	6	+	+	2	−0.01 ± 0.13	1.18 ± 0.14	
P. aegopodioides	2	+	+	2*	−0.94 ± 0.08	1.23 ± 0.06	
P. alsaticum	1	+	+	1*	−0.44 ± 0.32	0.90 ± 0.24	
P. austriacum	3	+	+	3	−0.30 ± 0.21	1.64 ± 0.56	
P. carvifolia	3	+	+	3*	−0.26 ± 0.09	2.16 ± 0.06	
P. cervaria	7	+	+	7*	−0.23 ± 0.07	0.62 ± 0.11	
P. formosanum	1	+	+	1*	0.44 ± 0.09	1.16 ± 0.07	
P. gallicum	1	+	+	1*	−0.30 ± 0.03	1.83 ± 0.03	
P. hispanicum	1	+	+	1*	−0.04 ± 0.06	2.16 ± 0.12	
P. japonicum	1	+	+	1	0.28 ± 0.1	1.81 ± 0.08	
P. longifolium	2	+	+	2	0.05 ± 0.03	1.91 ± 0	
P. obtusifolium	1	+	+	1*	0.31 ± 0.06	2.01 ± 0.08	
P. officinale	5	+	+	5	−0.04 ± 0.16	1.66 ± 0.19	
P. oligophyllum	1	+	+	1*	−0.34 ± 0	2.08 ± 0.04	
P. oreoselinum	4	+	+	4	−0.02 ± 0.1	1.24 ± 0.12	
P. palustre	1	+	+	1	0.08 ± 0.26	1.80 ± 0.24	
P. praeruptorum	2	+	+	2*	0.43 ± 0.06	1.12 ± 0.07	
P. terebinthaceum	1	+	+	1	0.23 ± 0	1.46 ± 0.04	
P. verticillare	2	+	+	2*	−0.47 ± 0.09	1.14 ± 0.07	
Aegopodium podagraria	1	−	~	1≠	−0.83 ± 0.21		
Aethusa cynapium	1	−	+	1≠	0.20 ± 0.09		
Angelica archangelica	6	+	+	5*	−0.15 ± 0.16	2.05 ± 0.31	
A. decursiva	1	+	+	1*	−0.48 ± 0.12	1.38 ± 0.02	
A. sylvestris	3	2+/1−	2+/1~	3	−0.24 ± 0.19	1.27 ± 0.09	
Anthriscus cerefolium	1	−	−	1≠	−0.79 ± 0.04		
Apium graveolens	1	−	+	1	−0.04 ± 0.07	1.27 ± 0.07	
Berula erecta	1	+	~	1*	−1.06 ± 0	1.45 ± 0.16	
Carum carvi	2	-/~	~/-	2*	−1.79 ± 0.06	−0.23 ± 0.02	
Chaerophyllum bulbosum	1	−	−	1≠	0.35 ± 0.02		
C. tremulum	1	−	−	1≠	−0.28 ± 0.07		
Conium maculatum	1	~	+	1≠	0.20 ± 0.14		
Daucus carota	1	−	~	1≠	−0.83 ± 0.19		
Eryngium campestre	1	+	+	1≠	0.33 ± 0.15	1.03 ± 0.07	5.61 ± 0.2
Foeniculum vulgare	2	−	+	2≠	0.14 ± 0.15		
Heracleum sp.	2	−	−	2≠	−0.19 ± 0.24		
Laserpitium latifolium	3	+	+	3≠	−0.50 ± 0.18		
L. siler	2	−	+	2≠	−0.05 ± 0.14		
Levisticum officinale	2	~	+	2≠	−3.16 ± 0.13	−2.23 ± 0.14	0.47 ± 0.07
Ligusticum mutellinoides	1	+	+	1≠	0.21 ± 0.10		
Myrrhis odorata	1	−	−	1≠	0.44 ± 0.07		
Petroselinum crispum	1	+	~	1≠	0.02 ± 0.07		
Pimpinella peregrina	1	−	−	1*	0.40 ± 0.04	1.08 ± 0.07	
P. saxifraga	1	~	~	1	0.14 ± 0.14	0.86 ± 0.26	
Seseli annuum	1	+	+	1*	0.39 ± 0.08	1.01 ± 0.08	
S. libanotis	1	+	+	1	−0.24 ± 0.08	1.19 ± 0.10	
Silaum silaus	1	+	+	1≠	0.06 ± 0.04		
Smyrnium perfoliatum	1	−	−	1≠	−0.60 ± 0.02		
Persicaria bistorta				2*	0.40 ± 0.12	4.37 ± 0.14	
Aconitum napellus				3≠	−5.86 ± 0.18		
Gentiana lutea				4≠	−10.37 ± 0.10	0.73 ± 0.13	3.78 ± 0.18
G. pannonica				2≠	−10.48 ± 0.13	0.72 ± 0.1	3.82 ± 0.11
G. punctata				2≠	−10.05 ± 0.06	0.48 ± 0.03	3.97 ± 0.16
G. purpurea				2≠	−10.26 ± 0.18	0.43 ± 0.13	3.82 ± 0.25
V. album				5≠	−6.69 ± 0.16	−1.96 ± 0.14	
V. nigrum				2≠	−7.52 ± 0.05	−1.21 ± 0.06	

whereas *S. annuum* had only a difference of 0.6 ± 0.1 °C. *Apium graveolens*, which showed no adequate amplification with Post_1 and a melting curve of group IV with Post_2, was not significantly different in the multiplex assay. Therefore, *Apium* samples are conspicuous by the poor amplification of Post_1 only. The *Persicaria bistorta* samples showed two melting maxima, 0.4 and 4.37 °C higher than *P. ostruthium*. With the general primers, all samples were adequately amplified. With the specific primers, several outgroup samples showed a delayed or insufficient amplification in one or both loci (Table 1) indicating mutations in the primer binding sites of the specific primers [28].

A. napellus samples showed one PCR product amplified with the

specific primers with a melting temperature of 82.7 °C (−5.9 °C compared to *P. ostruthium*) but no amplification with the general primers. Additional analysis revealed that the general ITS primer 17SE (in combination with the primer ITS2) does not bind properly to *Aconitum* DNA and causes a delayed amplification (a delay of more than 7 cycles in a singleplex qPCR). Samples of *Gentiana* species showed two PCR products, one amplified with the specific primers with a melting temperature between 78.1 and 78.5 °C (−10.1 to −10.5 °C), and a second one amplified with the general primers with two melting maxima between 89.0 and 89.3 °C (+0.4–0.7 °C) and between 92.4 and 92.6 °C (+3.8–4 °C), respectively. *Veratrum* samples showed specific amplicons with melting temperatures of 81.1 °C (−7.5 °C, *Veratrum nigrum*) or 81.9 °C (−6.7 °C, *V. album*), the PCR products amplified with the general primers had melting temperatures of 87.4 °C (−1.2 °C, *V. nigrum*) or 86.6 °C (−2.0 °C, *V. album*). Artificial admixture series of 0.001%–90% of *A. napellus*, *G. lutea* or *V. album* DNA admixture to *P. ostruthium* DNA revealed different detection limits of 0.001% for *A. napellus* and *V. album*, and 10% for *G. lutea* (Fig. 2). In PCR reactions of all tested *Aconitum*–*Peucedanum* mixtures, products of the specific *Aconitum* primers and *Peucedanum* products of the general ITS primers appeared. The *Veratrum* mixtures showed *Veratrum* products with the specific primers, *Peucedanum* products with the general ITS primers and additionally *Veratrum* products with the general ITS primers, if at least 10% *Veratrum* was admixed. The 10% *Gentiana* admixtures showed all three amplification products. Admixtures of 1% *Gentiana* or less were not detectable and in mixtures of more than 50% *Gentiana*, no *Peucedanum* products appeared. A cross amplification of the specific primers was not observed.

3.3. Doubtful samples

The obtained seeds from a German seed trade company labelled as "*P. ostruthium*" showed curve type 3 in Post_1 and curve type 5 in Post_2, so different to *P. ostruthium* in the first locus. The comparison of the ITS sequence with published data using BLAST of NCBI resulted in only 98% identity with *P. officinale* and *P. ostruthium*, and morphological characters of the raised seeds (leaves 2–3 times pinnate similar to *P. cervaria*) showed, that the plants did not belong to one of this species.

The two samples obtained as *P. praeruptorum* showed different curve types to each other in ITS2 (sample 1: group III, sample 2: group V) indicating either misidentification or intraspecific variation. One sample identified as "*P.* cf. *carvifolia*" showed HRM curves like the wild collected *P. ostruthium* samples but melting curves like the other *P. carvifolia* samples in the multiplex assay.

4. Discussion

High-resolution melting curve analysis was used to detect species-specific single nucleotide polymorphisms in parts of the nuclear ribosomal DNA. With the application of two primer pairs (Post_1 and Post_2) the target species *P. ostruthium* can be unambiguously identified except from *P. terebinthaceum* (native to Eastern Asia [5]), *P. aegopodioides* (native to the Balkan Peninsula [6]) and two out-group samples (*E. campestre* and *S. annuum*). The application of the multiplex PCR/HRM can further discriminate *P. terebinthaceum*, the Apiaceae out-group samples (e.g. *Eryngium*, *Seseli* and references which were not amplified with the *Peucedanum* primers like *Aegopodium*, *Anthriscus*, *Carum*, *Chaerophyllum* and *Pimpinella*) and possible adulterations of *Persicaria*, *Gentiana, Aconitum* and *Veratrum*. Admixtures of only 0.001% *Aconitum* or *Veratrum* were detectable with this assay. The specimen of *P. aegopodioides* remained unresolved and *A. graveolens* was only conspicuous because of the failing amplification of Post_1.

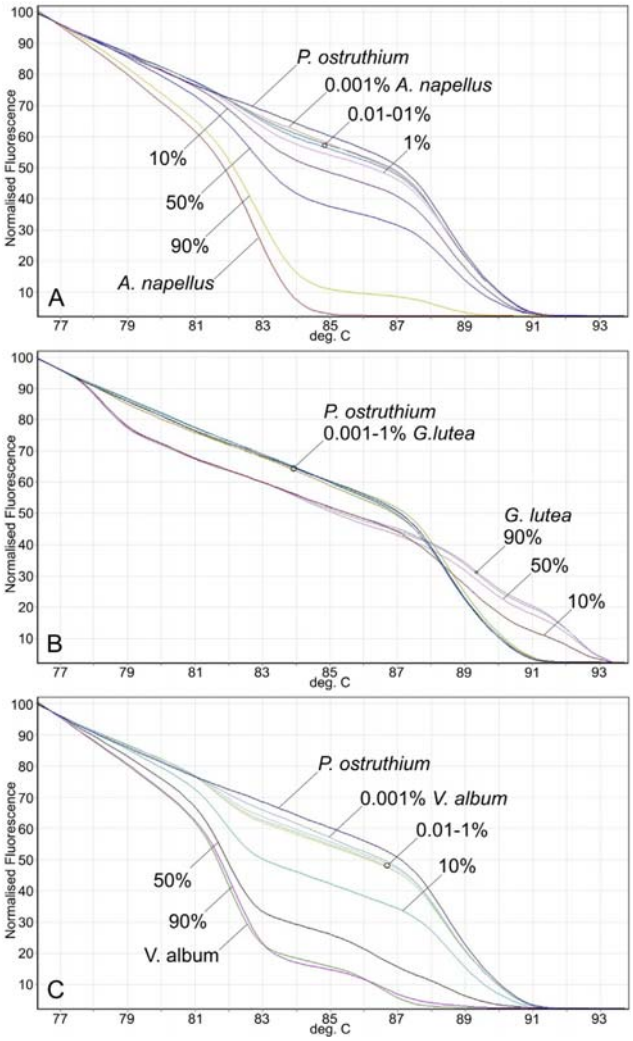

Fig. 2. Multiplex PCR/HRM analysis of pure samples and admixture series of 0.001–90% *Aconitum* (A), *Gentiana* (B) or *Veratrum* (C) DNA in *Peucedanum ostruthium* DNA.

Recently Liu et al. [29] investigated nearly 2000 species of 385 Apiaceae genera and found out that ITS (73.3% identification efficiency) or ITS2 (66.7%) in combination with psbA-trnH (82.2% and 80%, respectively) were most powerful DNA barcodes for the identification of Apiaceae specimens. Zhou et al. [30] demonstrated the suitability of ITS for the identification of *P. praeruptorum* specimens and the differentiation of its common substitutes and adulterants (all Apiaceae). Apart from the relatively high variability of ITS, the high copy number present in each cell increases the probability of PCR success.

However, standard DNA barcoding assays often do not address the problem of mixed sample material. It can be assumed that admixtures, occurring as double peaks or an overlay of two or more sequences, are visible in sequence chromatograms to a certain degree. The commonly used ITS primers published by White et al. [19] or Downie and Katz-Downie [18] co-amplify fungal DNA, what can result in a strong background noise in the sequence chromatograms, making the interpretation of admixtures more difficult. The primer 17SE, which was used in this study, showed no significant amplification of fungal DNA in preliminary tests, but we found

out, that e.g. *Aconitum* DNA was not properly amplified, and even proportions of more than 90% *Aconitum* would be overseen in a standard DNA barcoding assay.

Primers used for HRM analysis of SNPs are generally designed to amplify relatively short DNA fragments [14] what facilitates the amplification of degraded DNA, present in inadequately dried or old sample material [31] and [32]. Genus- or family-specific primers have the advantage not to co-amplify fungal DNA. But they can also lead to incorrect results, because DNA not amplifiable with those primers, and so the corresponding sample material (e.g. *Aegopodium*, *Anthriscus* or *Pimpinella*), is not detected. Therefore, a combination of specific and general assays delivers more reliable results than only one of them alone. It has to be kept in mind that unexpected adulterants could occur in trade samples. In doubtful cases, the amplification products of the multiplex PCR could be cleaned up after HRM analysis and sequenced with general ITS primers without the need of repeating the PCR. In this way, the advantages of HRM analysis, delivering fast results, could be combined with additional information from sequencing.

5. Conclusions

HRM analysis is a useful tool for the identification of *P. ostruthium* samples and the detection of (poisonous) admixtures. In our case, we found one trade sample (seeds) mislabelled as *P. ostruthium*. Hence molecular based methods are very helpful for the identification of trade material, especially where important morphological characters are rare or missing, e.g. for seeds, plantlets, roots and fine cut or ground plant material.

Acknowledgements

The authors gratefully acknowledge the Herbarium of the University of Vienna (WU), the Herbarium of the Royal Botanic Garden Edinburgh (RBGE) and Remigius Chizzola (University of Veterinary Medicine) for making their collections available. R. Chizzola helped in identifying the samples. Many thanks to Avni Hajdari, Brigitte Lukas, Johannes Saukel, Silvia Vogl and Christoph Wawrosch for collecting plant material. The authors thank Michaela Koch for her technical assistance. This work was financially supported by the Austrian Science Fund FWF (S 10708-B03).

References

[1] M. Grabowski, “Meisterwurz und Aderlass”. Anwendung und Wandel des ethnoveterinärmedizinischen Wissens im Großen Walsertal/Vorarlberg unter Hervorhebung der pflanzlichen Hausmittel und des religiösen Brauchtums (Diploma thesis), University of Vienna, Austria, 2010.
[2] P. Sarkhail, Traditional uses, phytochemistry and pharmacological properties of the genus *Peucedanum*: a review, J. Ethnopharm. 156 (2014) 235–270.
[3] S. Vogl, M. Zehl, P. Picker, E. Urban, C. Wawrosch, G. Reznicek, J. Saukel, B. Kopp, Identification and quantification of coumarins in *Peucedanum ostruthium* (L.) Koch by HPLC-DAD and HPLC-DAD-MS, J. Agric. Food Chem. 59 (2011) 4371–4377.
[4] M.G. Pimenov, M.V. Leonov, The Genera of Umbelliferae, Royal Botanical Gardens, Kew, 1993.
[5] e-flora of China, in: Z.Y. Wu, P.H. Raven, D.Y. Hong (Eds.), Peucedanum in Flora of China, vol. 14, 2005, p. 182, http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=124750 (accessed 25.02.15).
[6] T. Tutin, V. Heywood, N. Burges, D. Moore, D. Valentine, S. Walters, D. Webb, in: Flora Europaea, Rosaceae to Umbelliferae, vol. 2, Cambridge University Press, Cambridge, 1968, pp. 360–364.
[7] W. Adler, K. Oswald, R. Fischer, Exkursionsflora von Österreich, Eugen Ulmer, Stuttgart, Wien, 1994, p. 559.
[8] D.S. Katz-Downie, C.M. Valiejo-Roman, E.I. Terentieva, A.V. Troitsky, M.G. Pimenov, B. Lee, S.R. Downie, Towards a molecular phylogeny of Apiaceae subfamily Apioideae: additional information from nuclear ribosomal DNA ITS sequences, Plant Syst. Evol. 216 (1999) 167–195.
[9] K. Spalik, J.P. Reduron, S.R. Downie, The phylogenetic position of *Peucedanum* sensu lato and allied genera and their placement in tribe Selineae (Apiaceae, subfamily Apioideae), Plant Syst. Evol. 243 (2004) 189–210.
[10] P.J.D. Winter, A.R. Magee, N. Phephu, P.M. Tilney, S.R. Downie, B.E. van Wyk, A new generic classification for African peucedanoid species (Apiaceae), Taxon 57 (2008) 347–364.
[11] The Plant List, 2013. Version1.1. Published on the Internet, http://www.theplantlist.org/ (accessed 06.07.15).
[12] F. Berger, Handbuch der Drogenkunde. Erkennung, Wertbestimmung und Anwendung. Band 5: Radices, Wilhelm Maudrich, Wien, Bonn, Bern, 1960, p. 245.
[13] Hagers Handbuch der pharmazeutischen Praxis, in: fourth ed., in: P.H. List, L. Hörhammer (Eds.), Chemikalien und Drogen Teil A: N-Q, vol. 6, Springer, Berlin, Heidelberg, New York, 1977, p. 547.
[14] R. Vossen, E. Aten, A. Roos, J.T. den Dunnen, High-resolution melting analysis (HRMA) – More than just sequence variant screening, Hum. Mutat. 30 (2009) 860–866.
[15] M. Liew, R. Pryor, R. Palais, C. Meadows, M. Erali, E. Lyon, C. Wittwer, Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons, Clin. Chem. 50 (2004) 1156–1164.
[16] C. Schmiderer, B. Lukas, J. Novak, Effect of different DNA extraction methods and DNA dilutions on amplification success in the PCR of different medicinal and aromatic plants, Z. Arznei Gewürzpflanzen 18 (2013) 65–72.
[17] J. Doyle, DNA protocols for plants, in: G. Hewitt, A. Johnston, J. Young (Eds.), Molecular Techniques in Taxonomy, Springer Verlag, Berlin, 1991, pp. 283–285.
[18] S.R. Downie, D.S. Katz-Downie, A molecular phylogeny of Apiaceae Subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences, Am. J. Bot. 83 (1996) 234–251.
[19] T.J. White, T. Bruns, S. Lee, J. Taylor, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (Eds.), PCR Protocols: a Guide to Methods and Applications, Academic Press, San Diego, 1990, pp. 315–322.
[20] B. Heinze, A database of PCR primers for the chloroplast genomes of higher plants, Plant Methods 3 (2007) 4.
[21] K. Weising, R. Gardner, A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms, Genome 42 (1999) 9–19.
[22] T. Sang, D.J. Crawford, T.F. Stuessy, Chloroplast DNA phylogeny, reticulate evolution and biogeography of *Paeonia* (Paeoniaceae), Am. J. Bot. 84 (1997) 1120–1136.
[23] J.A. Tate, B.B. Simpson, Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species, Syst. Bot. 28 (2003) 723–737.
[24] P. Taberlet, L. Gielly, G. Pautou, J. Bouvet, Universal primers for amplification of three non-coding regions of chloroplast DNA, Plant Mol. Biol. 17 (1991) 1105–1109.
[25] Y. Sun, D.Z. Skinner, G.H. Liang, S.H. Hulbert, Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA, Theor. Appl. Genet. 89 (1994) 26–32.
[26] E.J.P. Douzery, A.M. Pridgeon, P. Kores, H.P. Linder, H. Kurzweil, M.W. Chase, Molecular phylogenetics of disease (Orchidaceae): a contribution from nuclear ribosomal ITS sequences, Am. J. Bot. 86 (1999) 887–899.
[27] K. Tamura, J. Dudley, M. Nei, S. Kumar, Molecular evolutionary genetics analysis (MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.
[28] J. Piñol, G. Mir, P. Gomez-Polo, N. Agustí, Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods, Mol. Ecol. Res. (2015), http://dx.doi.org/10.1111/1755-0998.12355 (online).
[29] J. Liu, L. Shi, J. Han, G. Li, H. Lu, J. Hou, X. Zhou, F. Meng, S.R. Downie, Identification of species in the angiosperm family Apiaceae using DNA barcodes, Mol. Ecol. Res. 14 (2014) 1231–1238, http://dx.doi.org/10.1111/1755-0998.12262.
[30] J. Zhou, W. Wang, M. Liu, Z. Liu, Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum* and its substitutes and adulterants by DNA-barcoding technique, Pharmacogn. Mag. 10 (2014) 385–390.
[31] E.L. Johnson, S.-H. Kim, S.D. Emche, Storage effects on genomic DNA in rolled and mature coca leaves, Bio Tech. 35 (2003) 310–316.
[32] J.A. Sikorsky, D.A. Primerano, T.W. Fenger, J. Denvir, Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method, Biochem. Biophys. Res. Commun. 323 (2004) 823–830.

Paper 5

Ruzicka J, Schmiderer C, Novak J (2016): DNA-based identification of *Valeriana officinalis* s.l.: A Multiplexed Amplification-Refractory Mutation System (MARMS) and High Resolution Melting Curve Analysis (HRMA). Plant Mol Bio Rep, Online First: DOI 10.1007/s11105-015-0967-6.

Author's contributions:

CS designed and tested the *Valeriana* primers for HRM analysis and revised the manuscript.

JR designed the MARMS primers, the *Veratrum* primers and developed the multiplex-HRM, performed most of the lab work and prepared the manuscript.

JN revised the manuscript.

Plant Mol Biol Rep
DOI 10.1007/s11105-015-0967-6

ORIGINAL PAPER

DNA-Based Identification of *Valeriana officinalis* s.l.: a Multiplexed Amplification Refractory Mutation System (MARMS) and High Resolution Melting Curve Analysis (HRMA)

Joana Ruzicka[1] · **Corinna Schmiderer**[1] · **Johannes Novak**[1]

Abstract The wide distribution of *Valeriana officinalis* as a herbal remedy as well as the considerably higher concentration of putative mutagenic valepotriate metabolites in other drug-delivering valerian species like *Valeriana procera* Kunth and *Valeriana jatamansi* Jones ex Roxb. illustrate the necessity of secure authentication of roots of *Valeriana officinalis* s.l., especially as the morphologically similar roots of the acutely toxic *Veratrum album* can be mistaken for those of *Valeriana officinalis*. We developed two DNA-based systems, a multiplex amplification refractory mutation system (MARMS), and a high-resolution melting curve analysis (HRMA) assay, both based on a sequence mutation within the atpB-rbcL region. With both methods, identification of *Valeriana officinalis* s.l. was possible. With the HRMA, the characteristic melting curve of 33 samples of *Valeriana officinalis* s.l. and of two commercial samples of Valerianae radix was distinct from the melting curves of all other *Valeriana* species (60 accessions), and from the closely related genera *Centranthus* and *Valerianella*. Since adulteration of *Valeriana* with toxic *Veratrum* species was reported previously, *Veratrum* primers were included in a multiplex PCR-HRM analysis. This system allowed the detection of a *Veratrum* admixture down to the level of 0.01 %. Although the advantages, in terms of sensitivity, specificity and practicality of the HRM for analysis of degraded plant material were superior to the MARMS assay, both methods are suitable for routine analysis. The results demonstrated the general ability of HRMA to detect specific (toxic) adulterations in drugs in a semiquantitative way.

Keywords High-resolution melting curve analysis · Identification · Multiplex amplification refractory mutation system · *Valeriana officinalis* · Adulteration · *Veratrum album*

Introduction

Valerianae radix originates from the roots of *Valeriana officinalis* sensu lato (European Pharmacopoeia 7.8 2013). According to Fischer et al. (2008), the *Valeriana officinalis* L. sensu lato complex comprises six taxa (*V. officinalis* ssp. *officinalis*, *V. officinalis* ssp. *vorarlbergensis*, *V. officinalis* ssp. *excelsea*, *V. officinalis* ssp. *sambucifolia*, *V. officinalis* ssp. *versifolia*, *V. officinalis* ssp. *tenuifolia*) with different ploidy levels ($2n = 14$, $2n = 28$, or $2n = 56$), which are spread over the European, Asian and North American continent (Hänsel et al. 1994; Heuberger et al. 2012). Since intermediate forms are common (Hänsel et al. 1994), the European Pharmacopoeia 7.8 (2013) allows *Valeriana officinalis* L. sensu lato as plant origin.

V. officinalis contains valerenic acid and valepotriates at approximately equal concentrations, whereas in two other drug-delivering species, *V. procera* Kunth and *V. jatamansi* Jones ex Roxb., the valepotriates dominate (Bos et al. 1996). This is of relevance as metabolites of the valepotriates (e.g., baldrinals) were found to possibly have cancerogenic and mutagenic effects. The purchase of roots, especially in a higher processed form, offers the possibility of mistakes or adulteration of the drug material, e.g., with the drug-delivering species *V. procera* Kunth, *V. jatamansi* Jones ex Roxb. or with *V. dioica* L.. Due to a similar habitus, the roots of *Veratrum album* can also be mistaken for *Valeriana officinalis* (Berger

✉ Joana Ruzicka
Joana.Ruzicka@vetmeduni.ac.at

[1] Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

Published online: 19 January 2016

1960; Schier and Schulze 1989; Hänsel et al. 1994; Frohne and Pfänder 2004). Although this is not so common, it could cause severe problems as *Veratrum album* is acutely toxic.

Therefore, suitable techniques for the correct authentication of the drug Valerianae radix have to be used. To date, Valerianae radix is identified by macroscopic and microscopic examination as well as by characterization of valerenic acid with thin-layer chromatography (European Pharmacopoeia 7.8 2013; Hänsel et al. 1994). These techniques are not reliable enough for a secure identification. The morphological examination of the source material is not applicable to herbal drugs that are further processed, and the correct chemical markers can even be present in substitutions (Palhares et al. 2015).

DNA-based techniques are promising as reliable and sensitive alternatives for an unequivocal verification even of ground or highly processed drug material (Heubl 2010; Sucher and Carles 2008; Yang et al. 2011; Yip et al. 2007). However, a DNA-based marker for the simultaneous identification of the source material *Valeriana officinalis* s.l. and possible substitutes or contaminants does not exist. Palhares et al. (2015) used DNA-barcoding by sequencing for quality control of Valerianae radix. Nevertheless, sequencing results were not satisfactory because of the poor DNA quality, which hampered the barcoding processes. The PCR-RFLP technique published by Slanc et al. (2006) for fingerprinting of a herbal tea mixture is based on ITS sequences, which distinguish *V. officinalis* sensu stricto from other *Valeriana* species but also from other taxa of the *Valeriana officinalis* s.l. complex. Therefore it is not suitable for the identification of Valerianae radix.

In the approach presented here, a multiplex amplification refractory mutation system (MARMS) and high-resolution melting curve analysis (HRMA) were developed on the basis of atpB-rbcL sequence differences found between *Valeriana* species. MARMS is a multiplex PCR approach with allele-specific primers. HRMA can be seen as an offshoot of qPCR technology (Mader et al. 2008), and is widely known as a sensitive and rather quick method to detect mutations by measuring a decrease in fluorescence while continuously melting the DNA, which leads to characteristic melting curves of each amplicon (Hofinger et al. 2009; Vossen et al. 2009; Wu et al. 2009). Both methods presented here, but especially the HRMA, do not, in contrast to other DNA based techniques, demand high quality DNA and are suitable for the degraded DNA present in highly processed plant material. An additional advantage of both methods is that they not only identify *V. officinalis* but also detect admixtures with adulterants. Molecular markers targeted on the species of interest only can be a tool for the exclusion of substitutions but will never detect any adulteration. DNA barcoding is an alternative but, in cases with more than two different sequences in a sample, results can be unsatisfactory because of low sequence quality.

Materials and Methods

Plant Material

Voucher specimens from different herbarium collections [Herbarium of the University of Hamburg, Germany (HBG); Herbarium of the University of Vienna, Austria (WU)] and seed material [Botanical Garden and Botanical Museum Berlin Dahlem, University of Berlin, Germany (BGBM); Botanical Garden of the University of Hohenheim, Germany (BG HOH); Botanical Garden of the University of Graz, Austria (BG UniGraz)] were used for the analyses. Additionally, wild plants of *Valeriana* spp. from different localities in Austria were sampled, and vouchers were deposited in the herbarium of the institute (Table 1). In total, 93 accessions of *Valeriana* species, three outgroup species, and two commercial samples of Valerianae radix were analysed with both methods presented here.

DNA Extraction

Approximately 0.5 cm^2 of dried leaf material was ground to fine powder with a swing mill (Retsch MM301, Haan, Germany), and the DNA was extracted using a modified CTAB protocol (Doyle 1991).

Sequencing

For amplification of the atpB-rbcL-IGS, the primers atpB-rbcL 2 and atpB-rbcL 10 (Table 2) were used in a first approach. For subsequent sequencing, specific primers VA_atpb29F and VA_atpb860R were designed based on our first sequencing results (Table 2). For a 20-µl PCR reaction, 1 µl genomic DNA (~5 ng) was added to a master mix containing 1× PCR buffer B [80 mM Tris–HCl pH 9.4 – 9.5 at 25 °C; 20 mM $(NH_4)_2SO_4$; 0.02 % w/v Tween-20], 2.5 mM $MgCl_2$, 0.1 mM dNTP, 400 nM F- and R-primer and 0.8 U *Taq* polymerase (HOT FIREPol®DNA Polymerase I, Solis Biodyne, Tartu, Estonia). A thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA) was programmed with an initial heating step of 15 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C, with a final extension for 7 min at 72 °C. For purification, the PCR reactions were treated with 0.3 µl EXO1 and 1.2 µl SAP (Fermentas, Burlington, ON, Canada) after PCR. PCR mixes were incubated at 37 °C for 1 h and the reaction was stopped by heat-inactivation of 15 min at 85 °C. PCR quality and purity were checked via gel electrophoresis. The sequence reaction was performed by an external company (IBL, Gerasdorf, Austria). PCR products (in a total volume of 20 µl, undiluted) and sequencing primers (10 pmol/µl and 10 µl per reaction) were sent to the company separately following the company's recommendations. All sequences were

Table 1 Samples used in this study, collection details, voucher information and GenBank accession numbers

Species	No. of individuals	Origin/voucher	Voucher number	Accession number
Valeriana alliariaefolia Vahl.	1	Georgia, Reg. Khevi, georgische Heerstraße, from Gveleti to Derdoraki. 1800–2100 m. Gröger & Lobin 104 – 3, 2000	WU0025936	
Valeriana alliariifolia Adams	2	TR-0-B-2518780: Turkey, Prov. Rize, Dilek Dağlari, Ayder Ilica, Camlihemşin, 1300 m, leg. Ern 6587 (BGBM)		KP205403
		Georgia, Prov. Kartli, western part of the Trialetic Mts., northern slope of the Kokhta gora mountain ca. 2 km SE Bakuriani. 43°32′ E, 41°45′ N. 1800–1900 mm. Schönswetter & Tribsch, 15.7.1997	WU0078609	
Valeriana alpestris Steven	2	Georgia, Khevi, Dvaletskij hrebet, Kazbek: northern slope E of the Devdoraki glacier. 2250-2400 m, Schneeweiß 5526, 23.8.2000 (WU)	WU0078608	KP205404
		Georgia, Prov. Kartli, western part of the Trialetic Mts: Ts'khratskaro pass ~8 km SSW Bakuriani (Bakuriani). 43°30′ E, 41°40′30″ N. 1800–1900 m. Schönswetter & Tribsch, 17.7.1997 (WU)	WU0078607	
Valeriana bertiscea Pančić	1	Montenegro, Komovi Mts.: Kom Kučki: western and southern slopes; 1900–2487 m. 19°38′30″ E, 42°40′50″ N. Frajman & Schönswetter 11565, 23.8.2006 (WU)	WU0078606	
Valeriana capitata Pall. ex Ledeb.	2	Russian Federation, Taymyrskiy (Dolgano-Nenetskiy) Avtonomnyy Okrug. Taimyrsky Autonomous Okrug, Severo-Sibirskaya Nizmennost (North-Siberian Lowland): "Ary-Mas" nature reserve, ©. 50–60 km NNW Khatanga), right riverside of Novaya, along the river and up to ca. 3 km S of river; 10–50 m. E 101°51′49″, N 72°27′52″. (Itinerary number: Taimyr-04-08). Schönswetter & Tribsch T342, 27.7.2004 (WU)	WU0078605	
		Russian Federation. Taymyrskiy (Dolgano-Nenetskiy) Avtonomnyy Okrug. Taimyrsky Autonomous Okrug, Severo-Sibirskaya Nizmennost (North-Siberian Lowland): Khatanga, forest tundra c. 4 km E of village; 20–30 m. E 102°34′27″, N 71°58′11″. (Itinerary number: Taimyr-04-01). Schönswetter & Tribsch T22, 15.7.2004 (WU)	WU0078604	
Valeriana cardamines M. Bieb.	1	Georgia, Prov. Khevi, Caucasus: E of Kazbegi. 44°40′ E, 42°39′N. 1850–1950 m. Schönswetter & Tribsch, 20.7.1997 (WU)	WU0078603	
Valeriana celtica L. ssp.norica Vierh.	3	Austria, Carinthia, Gurktaler Alps, 13°43′ E, 46°52′35″ N. 1758–2440 m. Schönswetter & Tribsch 3752, 20.8.1999 (WU)	WU0029080	KP205405
		Austria, Styria, Rosenkogel, 2 Individuals, C. Schmiderer, 21.06.2009	WU0078543	KP205406
		Austria, Carinthia, Plöckenpaßstraße, path from Gh. Plöckenhaus to Kleinen Pal (N 46°37′05″; E 12°56′53″); 945 msm., C. Schmiderer & B. Lukas, 18.07.2009	WU0078544	KP205407
Valeriana chaerophylloides Sm.	1	Peru, Lima, Loma de Pachacamac 25 km s.e. of Lima (center). Fosberg 56237, 4.7.1976 (WU)	WU0078602	
Valeriana dioica L.	6	unknown (BGUniGraz)[a]		KP205408 KP205409
		Germany, 1954 (HBG)		
		Germany, 1891 (HBG)		
		Germany, 1894 (HBG)		
		Austria, Lower Austria, Eastern Alps, valley of the green Feichtenbach S of Schallhof (S Pernitz). 460 m. Till W. & Till S., s.n. 16.5.1993 (WU)	WU0078600	
		Rumania, Cluj/Alba, Munţii Apuseni, Muntele Mare: from Staţiunea Muntele Băişorii in direction of Scariţa (SW Băişoara). 1100–1300 m. c. 23°20′ E 46°30′ N. Schönswetter & Tribsch 10736, 18.6.2005 (WU)	WU0078601	
Valeriana dioscoridis Sm.	2	Turkey, ca. 53 km SSE Izmir, in Efes (archaeological excavation Ephesos), 20 m. Walter 02/0121, 26.3.2002 (WU)	WU0078580	KP205410
		Turkey a. 53 km SSE Izmir, in Efes (archaeological excavation Ephesos), NW-slopes of the Panayir Dag. 20–200 m. Walter 02/0131, 26.3.2002 (WU)	WU0078579	

Table 1 (continued)

Species	No. of individuals	Origin/voucher	Voucher number	Accession number
Valeriana effusa Griseb.	1	Argentinia, Prov. Tucumán, Dept. Lules, along the road from San Pablo to Villa Nogues, 2 km ESE of Villa Nogues, 1050 m. Till, 10015, 4.2.1993 (WU)	WU0078598	
Valeriana globulariaefolia Ramond ex DC.	1	France, Hautes-Pyrenées/Huesca, Col du Pourtalet - Pic d'Aneou - Le Campana d'Aneou (Itinerarnummer 180). Schneeweiß, Schönswetter & Tribsch, 6447, 16.7.2001 (WU0020729)	WU0020729	KP205411
Valeriana mexicana	1	Mexico, Tlaxcala, Botanical garden, C. Schmiderer, 20.09.2009		KP205412
Valeriana montana L.	6	unknown (BGUniGraz)		KP205413
		Switzerland, 1893 (HBG)		
		Germany, Allgäuer Alps, 1893 (HBG)		
		Austria, Tyrol, 1872 (HBG)		
		Austria, Carinthia, Karawanken, Bärental, Greimler 19970621/ 8, 21.6.1997 (WU)	WU0078591	KP205414
		Spain, Prov. Huesca, Camino de Santiago, Canfranc Estación, on the path towards Castillo Fuerte Col de Ladrones. Chizzola s.n., 13.6.2001 (WU)	WU0078592	
Valeriana officinalis L.	25	DE-0-B-1950606: Germany, Baden-Württemberg, district Villingen-Schwenningen, Niederschach, lime, leg. Dürbye 3325 (BGBM)		KP205415
		DE-0-B-2033703: Germany, North Rhine-Westfalia, district Soest, Lippetal-Hultrop, mud, leg. Raus s.n. (BGBM)		
		unknown (BGHOH)		KP205416
		Clone 1, Nell et al. 2010		KP205417
		Clone 5, Nell et al. 2010		
		Clone 9, Nell et al. 2010		
		Austria, Upper Austria, Bad Goisern, 1997 (HBG)		
		Finland, 1982 (HBG)		
		Austria, Lower Austria, Semmering, Semmering city, Individuum 1, C. Schmiderer,10.05.2009		
		Austria, Lower Austria, Semmering, near Greis am Semmering, Individuum 4, C. Schmiderer, 10.05.2009		
		Austria, Lower Austria, Kl. Erlauftal, Ewixenbach-Tal ca. 3 km SE Wang, ca. 360 m. Tod s.n. (2/2), 15.9.2001 (WU)	WU0022102	
		Austria, Lower Austria, Rax, ca. 3 km NW of Reichenau a.d. Rax, 1550–1700 m. Schumacher s.n., 9.7.1999 (WU)	WU0078593	
		Austria, Lower Austria, Wiener Becken, Feuchtgebiet SE of Oberwaltersdorf, WSW of Grillenbühel, 210 m s. m. Till s.n., 12.8.1998 (WU)	WU0078594	
		Austria, Upper Austria, Böhmerwald, Klaffer, bank of the pond (E 13°52′3″ N 48°41′55″), 600 m s.m. Kleesadl 843, 13.7.1996 (WU)	WU0078595	
		Austria, Burgenland, southern part of the Leithagebirge, path between Müllendorf and Zechmeisterkreuz, 250–370 m s. m. Till s.n., 12.5.1994 (WU)	WU0078596	
		Austria, Lower Austria, Semmering, Semmering city, Ind.2, C. Schmiderer, 10.5.2009		
		Austria, Lower Austria, Semmering, Semmering city, Ind.3, C. Schmiderer, 10.5.2009		
		Austria, Lower Austria, Semmering, near Greis am Semmering, Ind.5, C Schmiderer, 10.5.2009		
		Austria, Lower Austria, Semmering, near Greis am Semmering, Ind.6, C. Schmiderer, 10.5.2009		

Table 1 (continued)

Species	No. of individuals	Origin/voucher	Voucher number	Accession number
		Austria, Lower Austria, Ötscher, C. Schmiderer, 26.07.2009	WU0078574	
		Austria, Lower Austria, Ötscher, C. Schmiderer, 26.07.2009		
		Austria, Lower Austria, Ötscher, Ind 1, C. Schmiderer, 02.08.2009	WU0078575	
		Austria, Lower Austria, Ötscher, Ind 2, C. Schmiderer, 02.08.2009	WU0078576	
		Austria, Möglingbach near Mödling, river side, (1/3), T. Pacher, 28.06.2009	WU0078568	KP205418
		Austria, Vorarlberg, alongside the path between Zug im Lechtal and Spullersee (½), C. Schmiderer & B. Lukas, 16.07.2009	WU0078566	
V. officinalis subsp. *sambucifolia* (J. C. Mikan ex Pohl) Čelak.	1	Austria, Lower Austria, Ötscher, C. Schmiderer, 26.07.2009	WU0078573	
V. officinalis ssp. *tenuifolia*	5	France, Dep. Puy de Dôme, 1967 (HBG)		
		France, Dep. Puy de Dôme, 1967 (HBG)		
		France, Dep. Ariège, 1967 (HBG)		
		Germany, Rhineland, Schweizer Tal, 1967 (HBG)		
		Austria, South Tyrol, St. Valentin, riverside, 1956 (HBG)		
Intermediate form of V. officinalis ssp. *sambucifolia and V. officinalis* ssp. *excelsea*	1	Germany, Bavaria, Berchtesgadener Land, Bischofswiesen, 1966 (HBG)		
Valeriana pratensis Dierb. ex Walter	1	FR-0-B-2592202: France, Dép. Hautes Alpes, Massif des Ecrins, La Meje, S La Grave, 1450 m, leg. Dürbye al. 1997 (BGBM)		KP205419
Valeriana procera Kunth	2	Mexico, Valley of Toluca, 1892 (HBG)		
		Mexico, Mexico City, Botanical Garden, University of Mexico City, C. Schmiderer, 09.09.2009		
Valeriana pyrenaica L.	2	FR-0-B-2152082: France, Dép. Pyrénées-Atlantiques, Gabas, silicate, 1200 m, leg. Ern 7220 (BGBM)		KP205420
		France, Dep. Pyrenees-Orientales, 1961 (HBG)		
Valeriana saliunca All.	1	Italy, Rocca d'Orel, between M. Pianard and M. Bussaia SE Entracque (Prov. Cúneo): southern ridge. Gutermann et al. Iter alpinum 1998 32426, 5.7.1998 (WU)	WU0078590	
Valeriana saxatilis L.	7	Italy, Trento/Lombardia, 2000 (HBG)		
		Austria, Lower Austria, valley of the Weißenbach ca. 4 km SW of Gutenstein, 520–540 m. Till, s.n., 6.6.1996 (WU)	WU0078586	KP205421
		Italy, Trentino-Alto Adige, Brescia, Brescian Alps: Cimba Tombea: northern slope of the west ridge; 2850–1950 m. Schneeweiss et al. 3927, 15.6.2000 (WU)	WU-HBV 0078588	
		Italy, Lombardia, Brescia, Brescian Alps: Dosso Alto W Bagolino: SW ridge and summit; 2000–2064 m. Schneeweiss et al. 3936, 16.6.2000 (WU)	WU-HBV 0078587	
		Austria, Lower Austria, valley of the Weißenbach, 4 km SW of Gutenstein, 580-600 m. Till, s.n., 18.5.1997 (WU)	WU0078585	
		Austria, Carinthia, Karawanken, Vellacher Kotschna, Offner Hütte. E & G Gölles Gö716, 1.7.1989 (WU)	WU0078589	KP205422
		Austria, Lower Austria, Schneeberg, Fadensteig, C. Schmiderer, 03.08.2009(WU)		
Valeriana saxicola C.A.Mey.	1	Georgia, Khevi, Mt'iulet'is k'edi: valley of the Arsykomydon SE Ketrisi; 2200–2900 m. Schneeweiß, 5525, 24.8.2000 (WU)	WU0078581	KP205423

Table 1 (continued)

Species	No. of individuals	Origin/voucher	Voucher number	Accession number
Valeriana scandens L.	2	Bolivia, 1909 (HBG)		
		San Salvador, 1891 (HBG)		KP205424
Valeriana simplicifolia (Rchb.) Kabath	1	Germany, Brandenburg, 1864 (HBG)		
Valeriana sitchensis ssp. *scouleri* (Rydb.) F.G.Meyer	1	USA, Rocky Mountains, British Columbia, 1904 (HBG)		
Valeriana tomentosa Kunth	1	Ecuador, Loja, Paso de la Carretera Loja-Saraguro, ~10 km al sur de Saraguro. 3100–3150 m. Larsen & Dall 242, 6.9.1985 (WU)	WU0078584	
Valeriana tripteris L.	12	Austria, Lower Austria, Ybbsitz, C. Schmiderer, 09.05.2009	WU0078550	KP205425
		Austria, Lower Austria, Ybbsitz, C. Schmiderer, 09.05.2009	WU0078551	
		Austria, Lower Austria, Scheibbs, ca. 6 km O of Gaming, Vordere Tormäuer, Stampf & Tod 13, 8.6.1993 (WU)	WU-HBV 0078582	
		Austria, Lower Austria, Ybbstaler Alps, Dürrenstein, Lechnergraben, E & G Gölles Gö849, 16.5.1990 (WU)	WU0078578	
		Austria, Lower Austria, Haraseck, SE Ramsau, (SE Hainfeld), between Mariental and Harasecker, 520–560 m. Till, s.n. 23.5.1994 (WU)	WU0078583	
		Austria, Lower Austria, Semmering, Semmering city, C. Schmiderer, 10.5.2009	WU0078552	
		Austria, Lower Austria, Semmering, Semmering city, C. Schmiderer, 10.5.2009	WU0078553	
		Austria, Lower Austria, Semmering, Semmering city, C. Schmiderer, 10.5.2009	WU0078554	
		Austria, Vorarlberg, alongside the path between Zug im Lechtal and Spullersee (N 47°10′36,3″; E 10°05′58,4″); 1972 msm., C. Schmiderer & B. Lukas, 16.07.2009	WU0078559	
		Austria, Carinthia, Plöckenpaßstraße, (2/2), C. Schmiderer & B. Lukas, 18.07.2009	WU0078560	
		Austria, Tyrol, Weissensee NE of Fernpass, chalk, (N 47°21′53,8″; E 10°52′27,4″); 1083 msm., C. Schmiderer & B. Lukas, 14.07.2009	WU0078546	
		Austria, Carinthia, Plöckenpaßstraße S Gh. Eder, roadside (N 46°38′02,6″; E 12°57′10,8″); 992 msm., C. Schmiderer & B. Lukas, 18.07.2009	WU0078547	
Valerianella coronata (L.) DC.	1	Unknown (BGBM)		KP205426
Valerianella locusta (L.) Laterr.	1	DE-0-B-0432407: Germany, Brandenburg, district Märkisch-Oderland, Strausberg, 66 m, leg. Dürbye 3516 (BGBM)		
Centranthus ruber (L.) DC.	1	Unknown (BGHOH)		KP205427
Valerianae radix	1	Austria, 1210 Vienna, Donau-Apotheke		
Valerianae radix	1	Italy, 52037 Sansepolcro, Aboca		

[a] Two sequence variants, sequence identity proofed by Blast search

Table 2 Primers used in this study. Destabilised nucleotides included in specific primers are underlined

Primer name	Sequence (5′->3′)	Tm (°C)	Reference
atpB-rbcL 2	GAAGTAGTAGGATTGATTCTC	58	Savolainen et al. 1993
atpB-rbcL 10	CATCATTATTGTATACTCTTTC	56	Savolainen et al. 1993
atpb29F	TCYGYCTAAAATTTTTKGCGAA	62	This paper
atpb860R	GGAATGCTGCCAAGATATCAGTAT	64	This paper
VA_atpB69F	TGTCCGATAGCGGGTKGAT	61	This paper
VA_atpB771R	GCACTTGCTTTAGTCTCTGTTTGT	64	This paper
VALOFF_F	ATGAAAGAGTATACAATAAGATGTATTTGCT	60	This paper
NONVALOFF_R	GCTTGATTATTAGACCATAATATATGATTTG	59	This paper
Valeriana_HRMF1	CATATATATGAAAGAGTATACAATAATGATGT	53	This paper
Valeriana_HRMR1	CTTGATTATTAGACCATAATATTTGATTC	54	This paper
Verat_trnLintron112F	AATAAAAAAAGATAGGTGCAGAGACTCAAT	59	Mader et al. 2011
Verat_trnLintron154R	TGTGGGCCATCCTTTCTTTAA	58	Mader et al. 2011

edited with Chromas (Technelysium, Tewantin, Australia) and aligned with the Clustal W algorithm of the Mega 4 software (Tamura et al. 2007). Blast homology searches were performed to assure the correctness of the sequences. Allele-specific primers and HRM primers were designed based on the differences between these sequences using PrimerExpress® (Applied Biosystems) (Table 2).

Multiplex Amplification Refractory Mutation System

A 15-µl reaction was prepared using 1 µl genomic DNA, 1× PCR buffer B [80 mM Tris–HCl pH 9.4 – 9.5 at 25 °C; 20 mM $(NH_4)_2SO_4$; 0.02 % w/v Tween-20], 2.5 mM $MgCl_2$, 0.1 mM dNTP, 400 nM primer VA_atpB69F, 400 nM of primer VA_atpB771R, 130 nM specific primer VALOFF, 600 nM specific primer NONVALOFF and 0.6 U *Taq* Polymerase (HOT FIREPol®DNA Polymerase I, Solis Biodyne). Deliberately destabilised nucleotides were included into the specific primers to ensure absolute specificity (Table 2). The PCR program was performed on the MastercyclerGradient (Eppendorf, Wesseling-Berzdorf, Germany) and consisted of an initial heat activation of 95 °C for 15 min, followed by 30 cycles of 95 °C for 45 s, 56 °C for 45 s, with a ramp of 3 °C/s, 72 °C for 90 s and one cycle with 72 °C for 9 min. An aliquot (3 µl) of the amplification product was separated on a 2 % agarose gel and detected under UV-light after staining in ethidium bromide. The size of the amplicons was compared to a standard 100 bp DNA ladder.

High Resolution Melting Curve Analysis

HRMA with pre-amplification was performed in a real-time PCR machine (Rotor-Gene 6000, Corbett Life Science, Sydney, Australia) using the following protocol: 5 min at 95 °C, 45 cycles of 10 s at 95 °C for denaturation, 20 s at 55 °C for annealing, and 20 s at 72 °C for extension. For the HRM analysis a temperature between 65 °C and 75 °C was screened with increments of 0.1 °C and a hold of 1 s at each step. The 10-µl reaction mixture contained 2 µl HRM Mastermix (Solis Biodyne), 150 nM Valeriana_HRMF1 and Valeriana_HRMR1- primer each, and 1 µl genomic DNA. Each sample, as well as non-template controls, was analysed in duplicate. For the Multiplex HRM, the primers Verat_trnLintron112F and Verat_trnLintron154R (Mader et al. 2011) were used additionally. Apart from that, the same protocol and program were applied as for the HRMA; all primers were used in an equal concentration. In contrast to the singleplex HRMA, the temperature range for the multiplexed HRMA lay between 65 °C and 85 °C. To simulate a contamination, mixtures of equimolar concentrations of *Veratrum album* and *Valeriana officinalis* DNA were made in a 90 %, 50 %, 5 %, 0.1 % and 0.01 % admixture of *Veratrum album* DNA to a *Valeriana officinalis* DNA sample, and in an admixture of 70 %, 50 % and 30 % *V. tripteris* DNA to a *V. officinalis* sample.

Primer Validation

Primer specificity was checked by a PrimerBLAST search to verify the uniqueness of the primer binding sites, and by gel electrophoresis of the PCR products on a 2 % agarose gel. The reproducibility of the assays was assessed by testing as many samples as possible in several trials. The MARMS assay was additionally performed on different cyclers [MastercyclerGradient and MastercyclerPersonal (both Eppendorf, Wesseling-Berzdorf, Germany), GeneAmp 9700 (Applied Biosystems)]. During this validation, we detected that differences in the ramp time influenced the banding pattern, which necessitated optimisation of the transition times when thermocyclers of different providers were used.

Results

Sequence Characterization

In order to identify species–specific mutations within *Valeriana*, the cp atpB-rbcL IGS of one sample per species was sequenced directly as far as possible. In case of difficult taxa, or of species of special interest, more than one sample per species was sequenced. In a few cases, old herbarium material led to poor DNA quality due to DNA degradation. This caused negative PCR results (cp region) and precluded direct sequencing.

Both new and previously published (GenBank) sequences were used together to develop the MARMS and HRMA primers.

Multiplex Amplification Refractory Mutation System

In the atpB-rbcL-IGS region, one single nucleotide polymorphism (SNP) was detected that discriminated *V. officinalis* s.l. from all other *Valeriana* species (Fig. 1). This transition (C/T) was used for the generation of two specific primers in opposite direction for both sequence variants. MARMS was performed in combination with two primers common to all *Valeriana* species, revealing a typical banding pattern of 229 bp and ~744 bp for *V. officinalis* and of 577 bp and ~744 bp for all other *Valerian* species with the exception of *V. celtica* ssp. *norica* and *V. saxatilis* (Fig. 2). The shorter PCR products of 229 bp and 577 bp specifically discriminate *V. officinalis* from other species of the genus *Valeriana*, whilst the 744 bp band is the product of the common primers serving as an internal control for PCR and DNA quality. In the case of *V. celtica* ssp. *norica* and *V. saxatilis*, the specific product failed to amplify due to additional sequence variations at the primer binding site of the NONVALOFF primer. In both species, only primers common to the genus *Valeriana* produced an amplification product. Due to intraspecific polymorphisms within *V. saxatilis*, this common product can vary in length in samples of this species. Length polymorphisms of the atpB-rbcL region are common within the genus *Valeriana*. Therefore, slight length variations (~10–12 bp) of the amplicons of the common primers, as well as of the NONVALOFF primer, are possible (i.e. lane 3, Fig. 2) but do not hamper the identification. In contrast, the PCR product of *Valerianella* is, at ~500 bp, significantly shorter than that of *Valeriana* and can therefore be distinguished easily from that of the genus *Valeriana*. Our samples of *V. chaerophylloides*, *V. effusa*, *V. saliunca*, *V. simplicifolia*, *V. sitchensis* ssp. *scouleri* and V. *tomentosa* failed to give positive PCR results with the

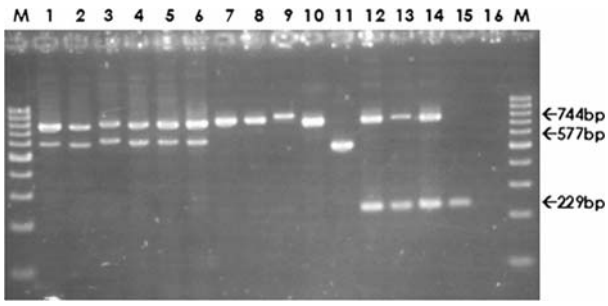

Fig. 2 MARMS of selected samples of *Valeriana* spp. and *Valerianella* with primers VA_atpb69F, VA_atpb771R, VALOFF_F and NONVALOFF_R on a 2 % agarose gel. Lanes: *1 - 6*: *V. alliariifolia*, *V. alpestris*, *V. bertiscea*, *V. capitata*, *V. cardamines* and *V. procera* as representative samples for all tested *Valeriana* species apart from *V. officinalis*, *V. pyrenaica*, *V. saxatilis* and *V. celtica*; *7 V. celtica* ssp. *norica*; *8*, *9 V. saxatilis*; *10 V. pyrenaica*; *11 Valerianella coronata*; *12–15 V. officinalis* s.l.;*16* non-template control, *M* 100 bp DNA ladder

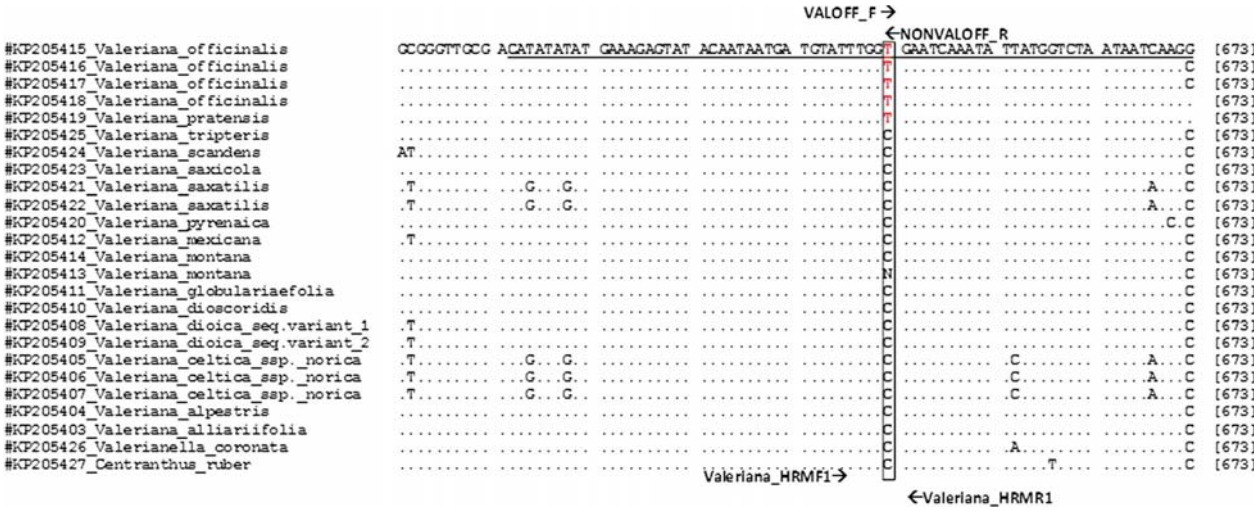

Fig. 1 Partial atpB-rbcL alignment with representative sequences. The decisive point mutation is highlighted in *brackets* and high-resolution melting (HRM) as well as multiplex amplification refractory mutation system (MARMS) primers are given in *bold* with *arrows* indicating primer direction

multiplex approach. Furthermore, direct sequencing of these samples was also not possible. This was due to severe DNA degradation and clearly shows the necessity of a system working with DNA fragments shorter than 600 bp for processed or old plant material.

Furthermore, we detected that differences in ramp time influenced the banding pattern. This was noted when thermocyclers of different providers were used, necessitating adjustment of transition times.

High-Resolution Melting Curve Analysis

HRM primers were designed to amplify a short product of 68 bp in *Valeriana* species as well as in *Valerianella* and *Centranthus ruber* spanning the SNP of interest. The C > T point mutation in this small fragment leads to a ΔT_m of 0.7 °C between 'wild type' and mutation ('variant') and therefore to a significant shift in the melting curves. All analysed samples of *V. officinalis* (thymine variant) had a T_m value of ~69.3 °C, whereas all other samples [cytosine ('wild type')] had a higher T_m value of ~70 °C. This shift allowed an unambiguous identification of all samples (Fig. 3). To test for possible adulterants, artificial DNA mixtures of *V. officinalis* and V. *tripteris* were tested at concentrations of 30 %, 50 % and 70 %, respectively. As expected, the melting curves of all mixtures lay between the curves of the pure *V. officinalis* and *V. tripteris* samples. Increasing concentrations of contaminating DNA were characterised by a continuous increment of T_m (Fig. 4). The mixture with 30 % 'contaminating' *V. tripteris* DNA just separated from the pure *V. officinalis* samples (Fig. 4). Therefore, an admixture with other *Valeriana* species with a DNA proportion of less than 30 % cannot be detected. For the simultaneous detection of a possible contamination of Valerianae radix with *Veratrum album*, a second primer combination specific for *Veratrum* (Mader et al. 2011) was added to the HRM mastermix without further optimisation. The resulting multiplex protocol therefore contained the specific valerian primers, which led to two curve types within the genus *Valeriana* (T_m ~69.3 °C and 70 °C), and *Veratrum* primers, which resulted in curve types specific for *Veratrum album* (T_m ~81.7 °C) and *Veratrum nigrum* (T_m ~81.2 °C) (Fig. 5). Again, artificial mixtures of *V. officinalis* and *Veratrum album* samples were prepared. Admixtures of *Veratrum album* to a *V. officinalis* DNA in dilution series of 90, 50, 5, 1, 0.1 and 0.01 % were tested. Although all admixtures were clearly separated from both, the pure *V. officinalis*

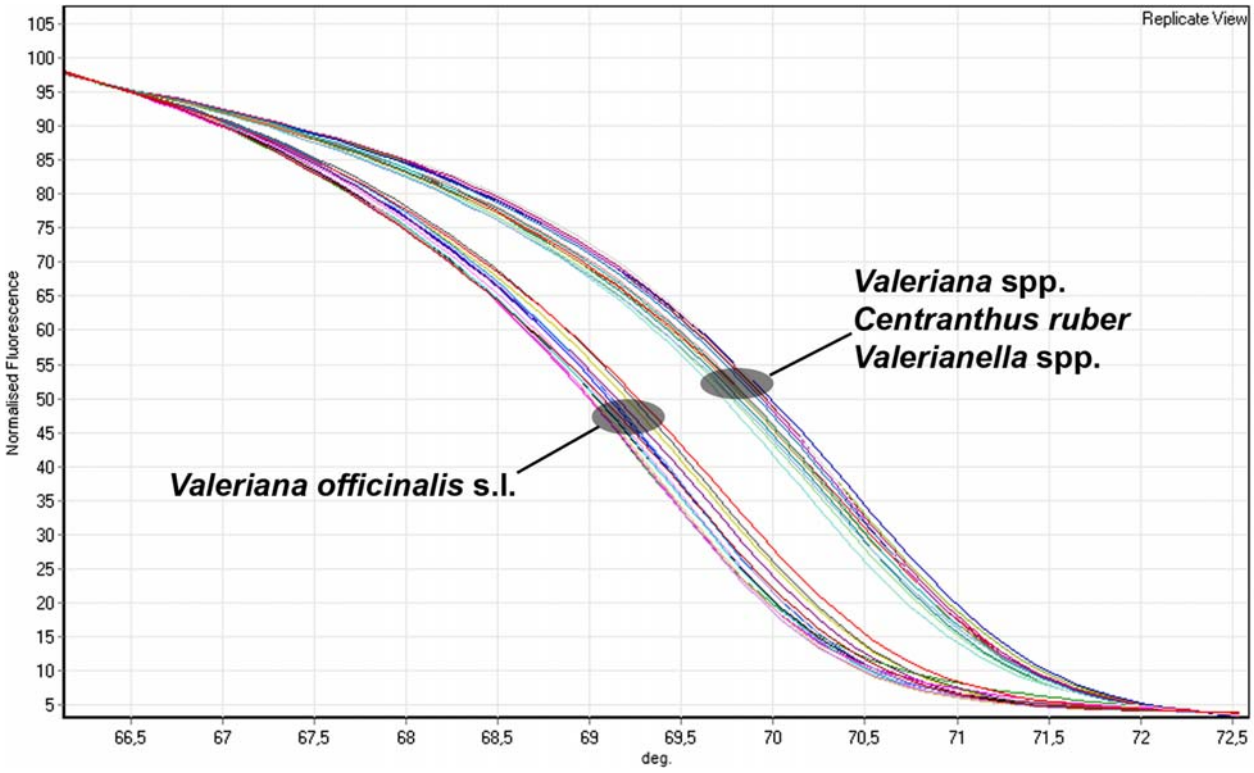

Fig. 3 Melting curves of selected samples of *Valeriana officinalis* s.l., other *Valeriana* species, *Centranthus ruber* and *Valerianella* species using the Valeriana_HRMF1 + R1 primers. The melting points of the curves of *V. officinalis* are approximately 0.7 °C lower than those of *Valeriana* spp., *Centranthus ruber* and *Valerianella*

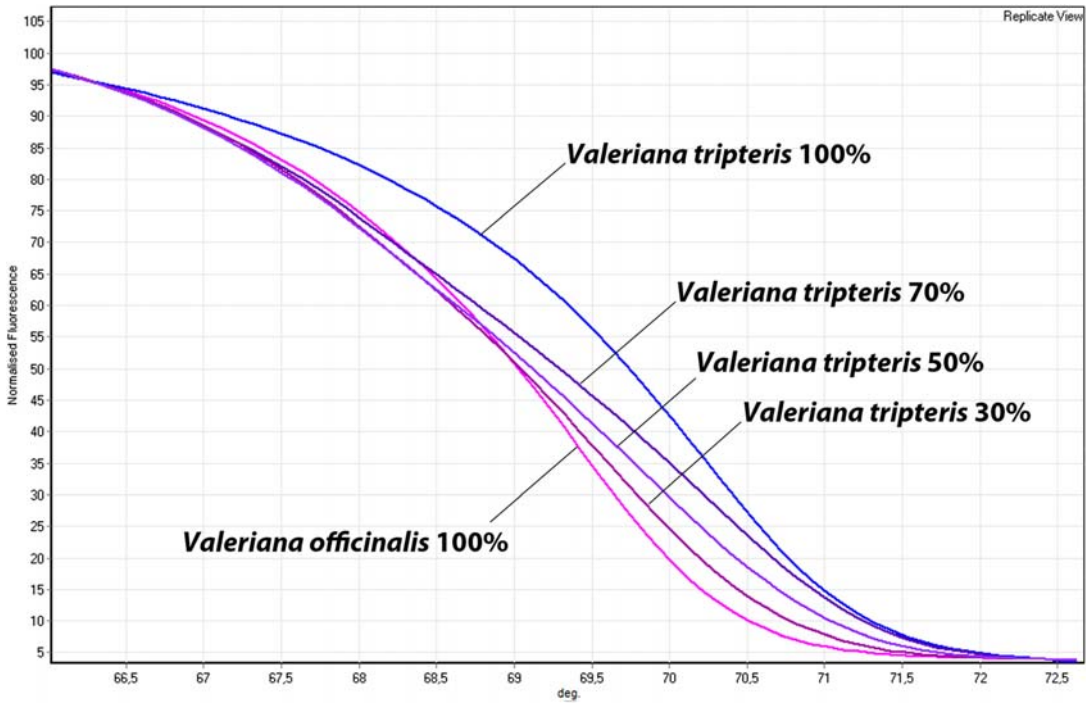

Fig. 4 Melting curves of a DNA admixture series of 70 %, 50 % and 30 % *Valeriana tripteris* in *Valeriana officinalis* DNA elevating between the HRM curves of the pure *Valeriana officinalis* and *Valeriana tripteris* samples

and the *Veratrum* sample, concentrations from 1 % to 50 % could not be separated from each other. Only the 0.1 % and 0.01 % *Veratrum* concentrations showed different melting temperatures than the other artificial mixtures. Anyhow, although a distinct quantification of the contamination's degree at the middle range was not successful, the detection limit of 0.01 % *Veratrum album* in a *Valeriana officinalis* sample was very low and reproducible. A contamination with *Veratrum album* can be distinguished easily from an admixture with another valerian species as both are designated by totally different curve types.

Discussion

Detection of Adulterations With Species from the Same Genus

One of the main aspects of quality control is to detect adulterants added either intentionally or unintentionally. Highly processed herbal raw materials are especially easy targets for adulteration since classical morphological techniques are often no longer applicable. Any adulteration with other drug-delivering *Valeriana* species like, e.g. *V. procera* Kunth, *V. jatamansi* Jones ex Roxb. or *V. dioica* L., can therefore remain undetected and can affect the harmlessness of the drug Valerianae radix.

Two DNA-based identification methods, a MARMS and a HRMA, are presented here based on one species specific-sequence mutation within the atpb-rbcL intergenic spacer differentiating *V. officinalis* s.l. and other *Valeriana* species. Both techniques have the possibility to detect both sequence variants quite quickly and cheaply and are therefore suitable methods for routine control.

In the case of MARMS, two specific primers were designed to bind selectively to both variants of the SNP of interest. In the case of HRM analysis, the primer pair flanks the region of interest and is bound to both variants, therefore allowing amplification of both DNA variants with only one primer combination.

Although both methods were designed for the same sequence variation, *V. celtica* and *V. saxatilis* could have been amplified with the HRM primers while the MARMS primers did not give positive results. Both species had additional mutations at the primer binding site, but, in contrast to the HRM analysis, destabilized nucleotides were included in both specific primers of the MARMS (Table 2) to guarantee absolute specificity. *V. celtica* (Bell 2004; Hidalgo et al. 2004) and *V. saxatilis* (this work), closely related to each other, are already quite distant to other *Valeriana* species. Therefore, it is not surprising that these two species possess additional mutations affecting this assay.

Anyhow, adulteration with *V. celtica* and *V. saxatilis* does not play a decisive role in quality control of the drug

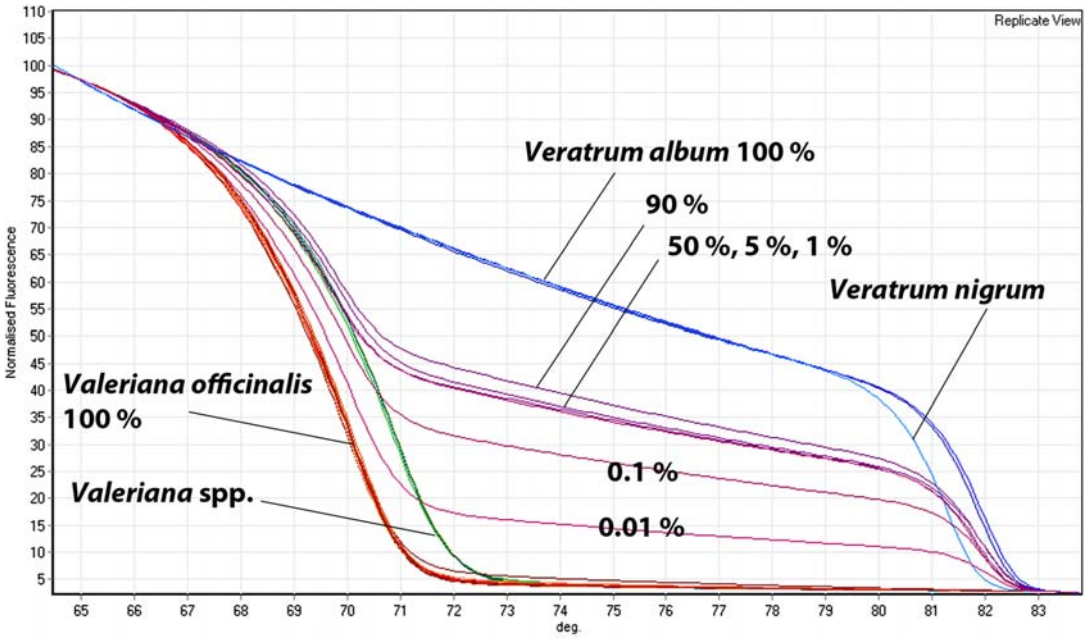

Fig. 5 HRM curves of the multiplex PCR containing Valeriana- and Veratrum- primers. The graph indicates *Valeriana officinalis*, *Valeriana* spp., *Veratrum album* and *Veratrum nigrum* samples, as well as an admixture series of 0.01 %, 0.1 %, 1 %, 5 %, 50 %, and 90 % *Veratrum album* in a *Valeriana officinalis* DNA sample. The melting curves of *Veratrum album* and *Veratrum nigrum* show a ΔT_m of 0.5 °C, whereas the melting points of *Valeriana* are at minimum 11.2 °C lower than those of *Veratrum*. All mixtures of *Veratrum album* and *Valeriana officinalis* lie between the pure samples. The 'contamination' with 0.01 % *Veratrum album* can be distinguished clearly from the pure *Valeriana officinalis* sample. Higher concentrations of *Veratrum album* (1–50 %) have identical HRM curves but can be separated easily from the pure samples

Valerianae radix. The essential oil of the alpine plant *V. celtica* is used to produce a soap speciality in Central Europe ('Speikseife') (Novak et al. 1998, 2000), but the rhizome and roots are so small (therefore expensive) that this plant will never be an intentional adulterant of *V. officinalis*.

Furthermore, *V. celtica* and *V. saxatilis* can be differentiated easily from *V. officinalis* via the alternative molecular method presented here: HRMA. The differentiation from *V. dioica*, closely related to *V. officinalis* (Bell 2004; Bell et al. 2012; Hidalgo et al. 2004), was unproblematic with both systems. *V. procera* and V. *mexicana* were also easily detected.

PCR Optimization

Changes in the reaction conditions or the PCR program may affect the banding pattern of a multiplex PCR approach (Henegariu et al. 1997; Saunders et al. 2001). Different salt concentrations of the buffer, the ramp time between annealing and extension step, and the length of the extension time, to name just a few, can differentially affect the elongation of longer or shorter PCR products. The transition rate between heating and cooling steps normally differs between thermocyclers. This transition time between annealing and extension had to be controlled and adjusted here for our multiplex PCR approach when cyclers of different providers were used. With a new generation of thermocyclers it is possible to imitate other PCR machines, thus simplifying the introduction of new methods.

DNA Degradation

Tissue disruption, heat and chemical processes have negative effects on DNA quality and DNA breaks into smaller fragments (DNA degradation). Given the difficulty of extracting high molecular weight DNA, protocols adapted to small DNA fragments are essential. Universal primers for direct sequencing are designed mainly to allow sequencing of long fragments, and thus to gain as much information as possible in one run. The amplification of strongly degraded DNA with these primers often fails, necessitating re-PCR or cloning. DNA barcoding of herbal products of *V. officinalis* in particular proved to be rather difficult because of poor DNA quality gained from these root products (Palhares et al. 2015). Out of 35 samples of *V. officinalis*, only 19 were successfully sequenced by the authors. For the MARMS developed in this study, the *V. officinalis* specific primer was designed to amplify a short segment of ~250 bp. This length proved short enough for a successful amplification even in very old herbarium material and in both commercial samples of Valerianae radix in a single PCR approach; the lack of the long 744 bp product of the common primers while the specific band can clearly be visualised strongly indicates degradation of the

DNA. The specific amplicon of the NONVALOFF primers is, at 577 bp, significantly longer than that of the VALOFF specific primers. This inhibited the amplification of degraded material in *V. chaerophylloides*, *V. effusa*, *V. saliunca*, *V. simplicifolia*, *V. sitchensis* ssp. *scouleri* and *V. tomentosa*. Therefore, a secure identification can be hampered by degradation of the DNA when herbal material is used. If solely the short amplicon of the VALOFF specific primer is amplified but none of the longer products occurs, DNA degradation of the source material has to be verified using universal primers that produce an amplicon of at least 500–600 bp in length. This additional work and expense is essential to gain unequivocal results. Unfortunately, there are only a few DNA-based techniques that are not influenced by DNA degradation (Yip et al. 2007).

We solved the problem of negative PCR results caused by DNA degradation by introducing a second approach, HRM analysis. The short ~68 bp product of the HRM allows amplification even of strongly degraded material so that, in our case, all samples could be analysed with this method. Even *V. chaerophylloides*, *V. effusa*, *V. saliunca*, *V. simplicifolia*, *V. sitchensis* ssp. *scouleri* and *V. tomentosa*, which failed to give positive results with the sequencing primers as well as with the MARMS primers, were amplified and classified correctly. Furthermore, HRMA is not as sensitive to DNA contamination with plant secondary compounds, making it ideal for a high throughput analysis of drug material.

Detection of Adulterations With Species from Other Genera

Similar to an adulteration with other *Valeriana* species, admixtures of species from other genera can be found. DNA-barcoding proved to be a good tool for the detection of substitutions (Palhares et al. 2015). Nevertheless, poor DNA quality derived from herbal medicines, and the lack of consistent primer binding sites in a wide range of plant genera, are limiting factors for the applicability of this technique (Palhares et al. 2015). In the case of documented or known contamination, the development of a specific identification system is recommended, because the detection of specific mutations is more sensitive than tests for unknown admixtures with universal primers. Intoxication with *Veratrum album* by consuming Valerianae radix has been reported previously (Berger 1960; Schier and Schulze 1989; Hänsel et al. 1994; Frohne and Pfänder 2004). For detection of this specific admixture, a multiplexed HRMA approach was performed, combining the ability to identify the *Valeriana* species of choice as well as an adulteration with *Veratrum* species, and may serve here as an example of detecting specific adulterations. Mader et al. (2011) already proved that an identification system based on a multiplex HRMA can be very effective. The authors established a HRMA to detect a possible *Veratrum nigrum* adulteration in *Helleborus* samples, and detected contaminations in ratios down to 1:200,000. We used the same *Veratrum* primers in our assay for the identification of *Veratrum album* in *Valeriana officinalis*. Concentrations down to 0.01 % could be identified easily, lower ratios were not tested. The lethal dosage of *Veratrum album* for humans is 20 mg of the alkaloids (1–2 g dried roots per person) (Frohne and Pfänder 2004). To assume an intake of 2–3 g of the drug Valerianae radix as recommended (Hänsel et al. 1994), a lethal intoxication with *Veratrum album* would be the consequence of an adulteration at a ratio of 1:1. An adulteration with *Veratrum* at concentration rates of 0.01 % or even lower would hardly cause severe intoxication. Therefore, the detection limit of *Veratrum* concentrations down to 0.01 % provided by the HRM analysis guarantees the safe use of the drug Valerianae radix.

Quantification of Adulterants

The quantification of possible adulterants in herbal remedies by molecular methods is still challenging and unresolved. The ratios between the amplicons of ‘drug’ and ‘adulterant’ only allow limited conclusions on the actual ratio of contamination in the drug for several reasons. First, extraction of equal amounts of DNA of the drug and the adulterant cannot be fully guaranteed, especially when different plant parts of both species are present in the mixed sample. Second, molecular markers usually used for the identification of herbal remedies (cp markers or ITS) do not exist in constant copy number, as the number of plastids per cell can vary among taxa and organs, and the nuclear ITS is a multicopy gene with variable copy numbers. Single copy genes or constant low copy genes could resolve this problem, but, as a matter of fact, there is very little information about genes holding a constant copy number over a wide range of plant genera (Small et al. 2004). Therefore, further research is needed to clarify whether pragmatic calibration curves using standard mixtures can provide enough security for an exact quantification or whether single copy genes are actually required.

Although both assays presented here are able to detect adulterations, the detection limit of 30 % for adulterations with other *Valeriana* species was not ideal. In contrast to that, the detection limit of a contamination with *Veratrum album* was, at 0.01 %, very low. The almost equal curves of samples adulterated with more than 1 % *Veratrum* may be explained by the primer concentrations used. The relatively low primer concentrations, limiting the PCR, in combination with the excess of dNTPs present in the reaction, allow amplification of both species independently of the introduced DNA proportion (1–50 % *Veratrum*) as long as there is enough DNA of both variants in the reaction. The curves of the 0.01 and 0.1 % *Veratrum* contamination are clearly distinct because in this case the number of cycles is not sufficient for the amplification

of PCR products in equal concentrations. An increase in the primer concentration or the use of primers in unequal concentrations would presumably improve the resolution of the assay, but also worsen the detection limit. Anyhow, due to the high toxicity of *Veratrum* species, an adulteration with 1 % of *Veratrum album* is not acceptable. Therefore the exact determination of concentrations higher than that is of secondary importance, whereas a low limit of detection (0.01 %) is essential.

Conclusion

The two DNA-based identification systems developed in this study provide a basis to guarantee secure identification of *V. officinalis*. Both methods proved to be suitable for routine controls. Although the advantages of HRM analysis outperform MARMS, the latter is, in our opinion, still justified as a screening method, mainly because no special equipment is needed as for HRMA.

Acknowledgements This work has been carried out with financial support from Aboca (Sansepolcro, Italy) and FP7 grant No. 245199 (Plantlibra), which are gratefully acknowledged.

Compliance with Ethical Standards

Funding This study has received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement No. 245199 (Plantlibra). This report does not necessarily reflect the Commission's views or its future policy in this area.

Conflict of Interest The corresponding author has received research grants from Aboca (Sansepolcro, Italy).

References

Bell CD (2004) Preliminary phylogeny of Valerianaceae (Dipsacales) inferred from nuclear and chloroplast DNA sequence data. Mol Phylogenet Evol 31:340–350

Bell CD, Kutschker A, Arroyo MTK (2012) Phylogeny and diversification of Valerianaceae (Dipsacales) in the southern Andes. Mol Phylogenet Evol 63:724–737

Berger F (1960) Handbuch der Drogenkunde. Issue 5, Maudrich, Wien, pp 490–507

Bos R, Woerdenbag HJ, Hendriks H, Zwaving JH, De Smet P, Tittel G et al (1996) Analytical aspects of phytotherapeutic valerian preparations. Phytochem Anal 7:143–151

Doyle J (1991) DNA protocols for plants. In: Hewitt G, Johnston A, Young J (eds) Molecular techniques in taxonomy. Springer, Berlin, pp 283–285

European Pharmacopoeia 7th edition (7. 8) (2013); http://online.pheur.org. Accessed 9 September 2013

Fischer MA, Oswald K, Adler W (2008) Exkursionsflora für Österreich, Liechtenstein und Südtirol, 3rd edn, Biologiezentrum der Oberösterr. Landesmuseen, Linz

Frohne D, Pfänder HJ (2004) Giftpflanzen, 5th edn. Wissenschaftliche Vertragsgesellschaft, Stuttgart, pp 265–268

Hänsel R, Keller K, Rimpler H, Schneider G (1994) Drogen P-Z. In: Hagers Handbuch der Drogenkunde, Springer, Berlin, pp 1079–1095

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997) Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23:504–511

Heuberger H, Heubl G, Müller M, Seefelder S, Seidenberger R (2012) Relationships and ploidy levels of selected accessions as initial genetic material for breeding of medicinal valerian (*Valeriana officinalis* L. s.l.). Z Arznei-Gewurzpfla 17:28–37

Heubl G (2010) New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. Planta Med 76: 1963–1974

Hidalgo O, Garnatje T, Susanna A, Methez J (2004) Phylogeny of Valerianaceae based on *matK* and ITS markers, with reference to *matK* individual polymorphism. Ann Bot 93:283–293

Hofinger BJ, Jing HC, Hammond-Kosack KE, Kanyuka K (2009) High-resolution melting analysis of cDNA-derived PCR amplicons for rapid and cost-effective identification of novel alleles in barley. Theor Appl Genet 119:851–865

Mader E, Lukas B, Novak J (2008) A strategy to setup codominant microsatellite analysis for high-resolution-melting-curve-analysis (HRM). BMC Genet 9:69–77

Mader E, Ruzicka J, Schmiderer C, Novak J (2011) Quantitative high-resolution melting analysis for detecting adulterations. Anal Biochem 409(1):153–155

Nell M, Wawrosch C, Steinkellner S, Vierheilig H, Kopp B, Lössl A et al (2010) Root colonization by symbiotic arbuscular mycorrhizal fungi increases sesquiterpenic acid concentrations in V*aleriana officinalis* L. Planta Med 76:393–398

Novak J, Franz CM, Novak S (1998) Essential oils of Rhizomes and Rootlets of *Valeriana celtica* L. ssp. *norica* Vierh. from Austria. J Essent Oil Res 10(6):637–640

Novak J, Novak S, Bitsch C, Franz CM (2000) Essential oil composition of underground parts of *Valeriana celtica* ssp. from Austria and Italy. Flavour Fragr J 15(1):40–42

Palhares RM, Gonçalves Drummond M, Dos Santos Alves Figueiredo Brasil B, Pereira Cosenza G, Das Graças Lins Brandão M, Oliveira G (2015) Medicinal plants recommended by the World Health Organization: DNA barcode identification associated with chemical analyses guarantees their quality. PLoS ONE 10(5), e0127866

Saunders GC, Dukes J, Parkes HC, Cornatt JH (2001) Interlaboratory study on thermal cycler performance in controlled PCR and random amplified polymorphic DNA analyses. Clin Chem 47(1):47–55

Savolainen V, Manien JF, Douzery E, Spichiger R (1993) Molecular phylogeny of families related to Celastrales based on *rbcL* 5'flanking sequences. Mol Phylogenet Evol 3(1):27–37

Schier W, Schulze W (1989) Aktuelle Verfälschungen von Arzneidrogen – Baldrianwurzel, Enzianwurzel und Nieswurzelstock (Adulterations of drugs. Valerian and gentian roots and sneezewood). Deut Apothekerzeitung 129:1540–1542

Slanc P, Ravnikar M, Štrukelj B (2006) Identification of individual herbal drugs in tea mixtures using restriction analysis of ITS DNA and real-time PCR. Pharmazie 61:912–915

Small RL, Cronn RC, Wendel JF (2004) Use of nuclear genes for phylogeny reconstruction in plants. Aust Syst Bot 17(2):145–170

Sucher NJ, Carles MC (2008) Genome-based approaches to the authentication of medicinal plants. Planta Med 74:603–623

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Vossen RHA, Aten E, Roos A, Den Dunnen JT (2009) High-Resolution Melting Analysis (HRMA)—more than just sequence variant screening. Hum Mutat 30(6):860–866

Wu SB, Tavassolian I, Rabiei G, Hunt P, Wirthenson M, Gibson JP et al (2009) Mapping SNP-anchored genes using high-resolution melting analysis in almond. Mol Genet Genomics 282:273–281

Yang Y, Zhai Y, Liu T, Zhang F, Ji Y (2011) Detection of *Valeriana jatamansi* as an adulterant of medicinal *Paris* by length variation of chloroplast psbA-trnH region. Planta Med 77:87–91

Yip PY, Chau CF, Mak CY, Kwan HS (2007) DNA methods for identification of Chinese medicinal materials. Chin Med 2:9. doi:10.1186/1749-8546-2-9

Paper 6

Mader E, Ruzicka J, Schmiderer C, Novak J (2011): Quantitative high-resolution melting analysis for detecting adulterations. *Analytical Biochemistry* 409 (1): 153-155.

Contrary to the published version, the formatting of the supplemental material was partially adapted to this work.

Author's contributions:

JR designed and tested the *Veratrum* primers.

CS designed and tested the *Helleborus* primers.

EM designed and tested the multiplex-PCRs and prepared the manuscript.

JN revised the manuscript.

Analytical Biochemistry 409 (2011) 153–155

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

Quantitative high-resolution melting analysis for detecting adulterations

Eduard Mader *, Joana Ruzicka, Corinna Schmiderer, Johannes Novak

Institute for Applied Botany, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Wien, Austria

ARTICLE INFO

Article history:
Received 3 July 2010
Received in revised form 1 October 2010
Accepted 1 October 2010
Available online 12 October 2010

ABSTRACT

Admixtures of different plant species are a common problem in raw materials for medicinal use. Two exemplary assays were developed to admixtures in *Helleborus niger* with high-resolution melting analysis. HRM proved to be a very sensitive tool in detecting admixtures, able to detect a ratio of 1:1000 with unknown species, and of 1:200,000 with *Veratrum nigrum*. The example proves the ability of HRM for quantification in multiplex PCR.

The method is not limited to detecting adulterations. It can also be used to quantify a specific target by integrating a second amplicon in the assay as internal standard.

Recent technological advances in recording diminishing fluorescence during DNA denaturation ("melting") opened a wide range of applications of "high-resolution melting analysis" (HRM or HRMA)[1] [1]. Increased resolution and precision of the instruments and the development of saturating DNA dyes facilitated the use of HRM for genotyping (SNP, SSR markers) [2–4], for methylation analysis, as an alternative to gel electrophoresis, and for quantification (copy number variants and mosaicism) [1]. Some ideas for using HRM for (semi-)quantitative analysis were presented in [5–7], most of them concerning DNA methylation studies.

Detection and quantification of DNA from nontarget species or cultivars in a sample of plant-derived material are currently exercised in the analysis of GMO admixtures. The approach in the present study is similar, since a pair of genetic markers is used: one targeting the intended reference material and the other targeting material representing the usually unwanted adventitious presence.

When developing such an assay two cases must be distinguished:

(1) testing for unknown admixture(s);
(2) testing for specific admixture(s) (e.g., toxic plants or admixtures commonly present in trade samples).

For both cases *Helleborus niger*, a medicinal plant in homeopathy [8], was used as the reference drug species in the present study. In pharmacopoeias, the tests for identity and purity are based on macro- and microscopical as well as phytochemical methods. DNA-based methods of identification (commonly referred to as DNA barcoding) are rather new approaches not yet part of regulations for medicinal plants.

In a previous study Schmiderer et al. [9] developed a HRM method for the positive identification of *H. niger*. To expand the possibilities of this method for a purity assessment, an (additional) assay which also detects species from other genera and taxa is needed. *Veratrum nigrum* is a common admixture to *H. niger*, mainly due to the same English common name "Black Hellebore." Therefore one assay was designed to detect an adulteration by *V. nigrum*. A second assay should target a broad range of plant taxa to detect admixtures with unknown adulterants.

Genomic DNA was extracted from leaf tissue of *H. niger* and all other species separately using a modified CTAB protocol [10]. The DNA quantification was done with a Bio-Rad Versa Fluor™ fluorometer and the fluorochrome Hoechst 33258 (Bio-Rad; Vienna, Austria). Contamination of samples was simulated by mixing a dilution series of DNA extracts of *V. nigrum* and a premixed sample (KS2) of eight different species (Supplementary material, Table 2) to a DNA extract of *H. niger*.

For the detection of *V. nigrum* admixtures a duplex assay was used, containing specific primers for *Veratrum* (Verat_trnLintron112F/154R) and specific primers for *H. niger* (Hel_matK3F/R) in a ratio of 1:1 (see Supplementary material for further data).

For the detection of a contamination with an unknown species a second duplex PCR assay was developed, containing a universal primer for the matK gene [11] and the same specific primer for *H. niger* noted above. Here a ratio of universal to specific primer of 1.5:1 was used after optimization (data not shown). In both cases the T_m value of the *H. niger* amplicon was intentionally lower than that of the amplicon of the adulteration(s). The reason is to have first a complete dissociation of the *H. niger* amplicon, followed by a phase where amplicons of the adulteration(s) are still

* Corresponding author. Fax: +43 1 25077 3190.
E-mail address: eduard.mader@vetmeduni.ac.at (E. Mader).

[1] *Abbreviations used*: HRM or HRMA, high-resolution melting analysis; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

0003-2697/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2010.10.009

mainly present as dsDNA, until finally all DNA is melted and fluorescence reaches its basic level.

The PCRs were prepared at a volume of 10 μl and contained 0.4 U Taq HOT FIREPol polymerase and 1 μl 10× Buffer B2 (Solis BioDyne, Tartu, Estonia), 3.5 mM $MgCl_2$, 0.1 mM dNTP mix (25 μM each dNTP), 0.1 mM of each specific primer, and 0.15 mM of the universal primers in the assay for unknown contaminations, 0.6× fluorescent dye EvaGreen (Biotium Inc., Hayward, CA, USA), and distilled water. A 0.5 μl DNA solution (1:50 from original extract) of *Helleborus* samples and 0.5 μl of contamination dilutions were added to each reaction. All reactions were done in duplicate. All runs were done in two or more repetitions to evaluate interrun variation.

The PCR cycling was performed on a RotorGene 6500 (Corbett Research Pty Ltd, Sydney, Australia) and started with an initial phase of 15 min (for the Taq HOT FIREPol polymerase) at 95 °C, then 45 cycles of 10 s at 95 °C, 20 s at 56 °C, and a 20 s elongation step at 72 °C. High-resolution melting was carried out immediately following PCR from 70 to 90 °C at steps of 0.1 °C, each step with a 1 s hold.

HRM curves were analyzed with the Rotor-Gene Q Software Version 1.7 (Qiagen, Hilden, Germany); further calculations were performed with the Software R, Version 2.8.0 (The R Foundation, Vienna, Austria).

In the developed assay the PCR products of pure *H. niger* samples showed melting curves with a single inflection point at a T_m value of 77.5 °C (Fig. 1). The curves of pure *V. nigrum* samples (pure contamination sample) also showed one inflection point at 81.5 °C. All mixed samples showed both melting domains in their curves, resulting in two inflection points. Dilution ratios from 1:1 to 1:50 possessed roughly the same curve shape.

The samples mixed in ratios down to 1:200,000 clearly showed the presence of *V. nigrum* through elevated fluorescence at temperatures between 77.5 and 81.5 °C compared to pure *H. niger* (Supplementary material, Fig. 1a).

Other *Helleborus* species (*H. foetidus*, *H. odorus*, *H. lividus*) were clearly separated from *H. niger* by the T_m value of their amplicons, and in mixtures with *H. niger* new curve forms due to heteroduplex formation emerged (Supplementary material, Fig. 1a). All of these heteroduplexes showed T_m values lower than that of *H. niger*, whereas *V. nigrum* amplicons show a higher T_m, which prevents confusion with these potential adulterations.

The second duplex PCR assay with a universal primer for the matK gene produced complex melting curves with two visible inflection points at 79.9 °C and at 81.2 °C for the so-called contamination mixture KS2 consisting of eight different species. The pure *Helleborus* samples again showed melting curves with one inflection point at 77.7 °C. The melting curves of the mixed samples had a complex shape, showing the melting domain of *Helleborus* and one or two inflection points in the region between 79.9 and 81.2 °C. Mixtures with dilution ratios from 1:1 down to 1:1000 (vol/vol) signaled the presence of non-*Helleborus* DNA by an elevated fluorescence in the intermediate temperature zone around 79 °C. The mixing ratios of 1:2000 and 1:5000 resulted in curves congruent to that of *Helleborus*.

The fluorescence level at a predefined temperature between the melting domains of the *Helleborus* amplicon and the amplicons of the universal primer was used as a measure for the level of contamination (for calibration (R^2 = 0.98) refer to Fig. 2).

Both assays demonstrated that low levels of adulterations can be efficiently detected by HRM. The high sensitivity of HRM for sequence variation opens many options for fine tuning of the quantitative design of multiplex assays by selecting optimal T_m differences for the amplicons, adjusting primer ratios, considering differential efficiencies of primer pairs, etc. By limiting the primers for *H. niger* the reaction for the adulteration(s) can be promoted and sensitivity increases. The efficiency for the highly sensitive *V. nigrum* adulteration, e.g., was by 0.1 higher than that for *H. niger*.

The level of fluorescence after dissociation of the *H. niger* amplicon can be regarded as a quantitative measure of contamination with adulteration(s). The highly significant correlation between the level of fluorescence at a predefined temperature and the logarithm of the contamination percentage allowed the development of a standard curve (Fig. 2).

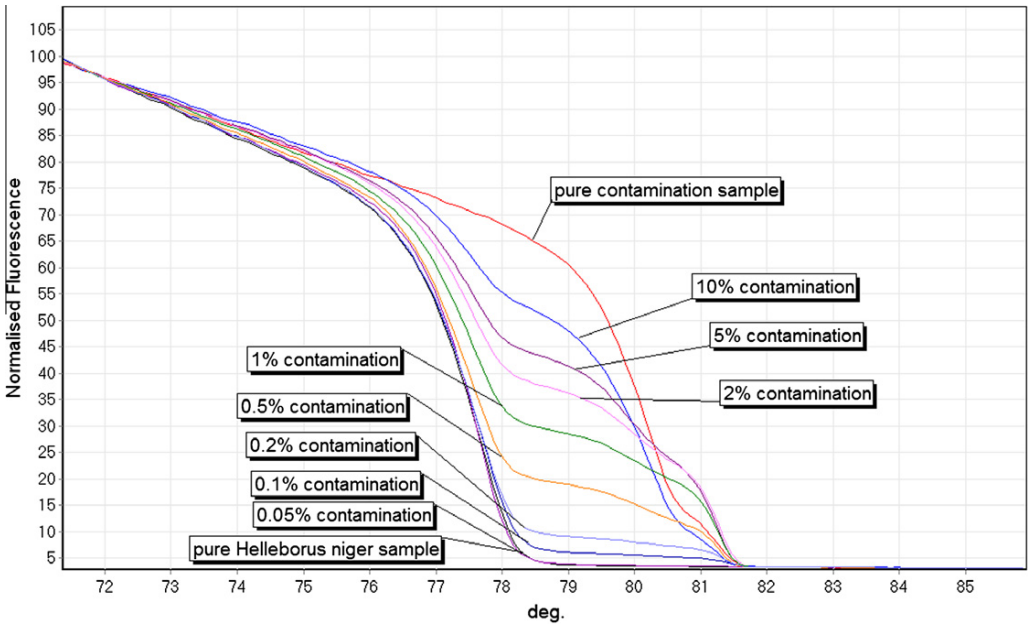

Fig.1. High-resolution melting curves of the universal primer assay (KS2). The *Helleborus niger* amplicon is dissociating between 77 and 78 °C; the amplicons of the universal primer dissociate between 80 and 82 °C. A contamination can be detected down to 0.1%.

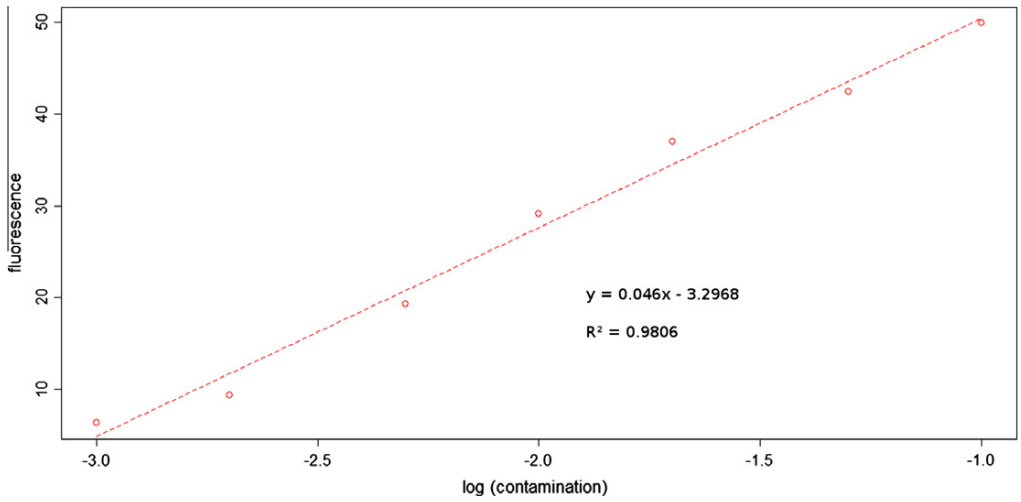

Fig.2. Linear regression on the basis of fluorescence levels in the intermediate temperature zone between two melting domains. *y*-axis, fluorescence level at 78.8 °C of a dilution series of premixed contamination DNA mixed with a *Helleborus niger* sample; *x*-axis, logarithm of the ratio of contamination.

A major drawback for the detection of an unknown adulteration is the availability and binding efficiency of universal primers that may not react with all species. Therefore we suggest assays designed for a specific contamination, which may be a species such as in the presented method (like *V. nigrum*) or specific on higher level taxa (e.g., Poaceae, Solanaceae).

Furthermore there is a wider field of application for multiplex HRM, for example, host/pathogen quantification [12] or GMO detection.

Since HRM is an inexpensive and fast method, combination with other technologies as a prescreening tool could be a good idea. Unknown adulterations detected this way by HRM, for example, could be subsequently identified by next generation sequencing.

Considering this method for future routine analysis, another point is essential: The ratios between the amplicons of two species only allow limited inferences on the actual ratio of admixture in plant material or drugs. Variations of the number of cells per weight and the number of plastids per cell may be substantial among taxa, organs, and stages of development. Further research in this area is needed to clarify if and how calibration measurements can eliminate this problem.

The presented method is not limited to detecting adulterations. It can also be used to quantify a specific target by integrating a second "artificial" target as internal standard. In this sense, delta-delta-C_t calculation of real-time PCR could be replaced by HRM without use of labeled primers or probes.

Acknowledgment

This work has been carried out with financial support from the FAH/AIF – Forschungsvereinigung der Arzneimittel-Hersteller e.V. (Project No. 15182-N), which is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.10.009.

References

[1] R. Vossen, E. Aten, A. Roos, J.D. Dunnen, High-resolution melting analysis (HRMA): more than just sequence variant screening, Hum. Mutat. 30 (2009) 860–866.
[2] J. Lin, C. Tseng, Y. Chen, C. Lin, S. Chang, H. Wu, J. Cheng, Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis, J. Clin. Microbiol. 46 (2008) 1090–1097.
[3] L. Zhou, L. Wang, R. Palais, R. Pryor, C.T. Wittwer, High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution, Clin. Chem. 51 (2005) 1770–1777.
[4] A.E. Croxford, T. Rogers, P.D.S. Caligari, M.J. Wilkinson, High-resolution melt analysis to identify and map sequence-tagged site anchor points onto linkage maps: a white lupin (*Lupinus albus*) map as an exemplar, New Phytol. 180 (2008) 594–607.
[5] F. Malentacchi, G. Forni, S. Vinci, C. Orlando, Quantitative evaluation of DNA methylation by optimization of a differential-high-resolution melt analysis protocol, Nucleic Acids Res. 37 (2009) 44.
[6] A. Lorente, W. Mueller, E. Urdangarin, P. Lazcoz, A. von Deimling, J. Castresana, Detection of methylation in promoter sequences by melting curve analysis-based semiquantitative real time PCR, BMC Cancer 8 (2008) 61.
[7] R. Palais, M. Liew, C. Wittwer, Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping, Anal. Biochem. 346 (2005) 167–175.
[8] Homöopathisches Arzneibuch 2004 (HAB 2004), Amtliche Ausgabe, Stuttgart, Deutscher Apotheker Verlag, 2004.
[9] C. Schmiderer, E. Mader, J. Novak, DNA-based identification of *Helleborus niger* by high-resolution melting analysis. Planta Med. (2010) (EFirst).
[10] J.J. Doyle, J.L. Doyle, Isolation of plant DNA from fresh tissue, Focus 12 (1990) 13–15.
[11] K. Weising, R.C. Gardner, A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms, Genome 42 (1999) 9–19.
[12] G. Valsesia, D. Gobbin, A. Patocchi, A. Vecchione, I. Pertot, C. Gessler, Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction, Phytopathology 95 (2005) 672–678.

Quantitative High Resolution Melting Analysis for Detecting Adulterations

Eduard Mader, Joana Ruzicka, Corinna Schmiderer and Johannes Novak

Supplement

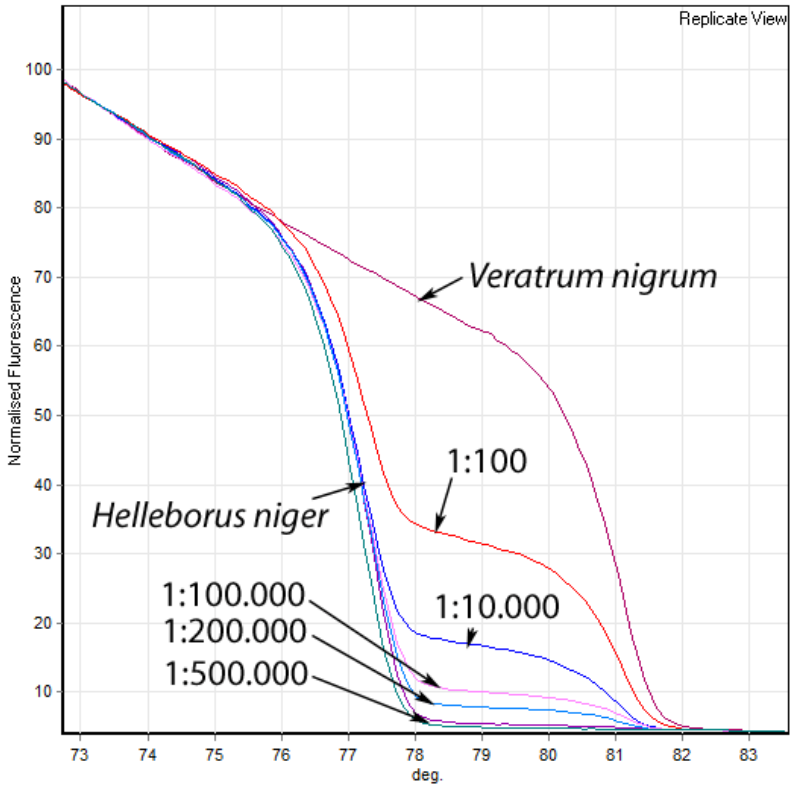

Figure 1a: High resolution melting curves of dilution series of *Veratrum nigrum* mixed with *Helleborus niger*. Adulteration with *Veratrum nigrum* can be detected down to a ratio of 1 : 200,000.

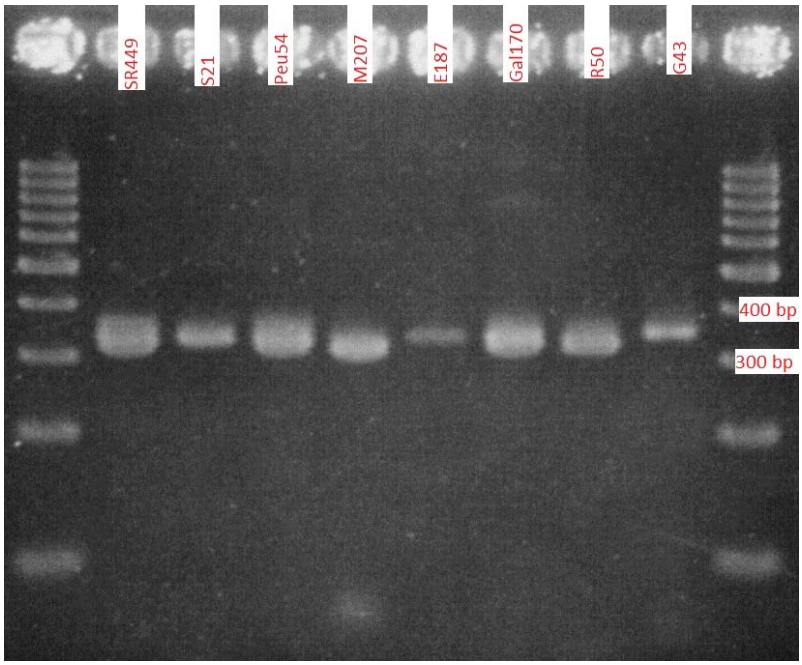

Figure 3: Amplification bands of the contained species in the contamination mixture with primer pair syst_matK1f / syst_ccmp1r. Left to right: size marker, *Origanum onites, Salvia pomifera, Peucedanum ostruthium, Melampyrum sylvaticum, Epilobium angustifolium, Galphimia glauca, Rumex alpinus, Veratrum nigrum,* size marker.

Table 1. Primers / loci used in the PCR duplex assays.

Primer name	Reference sequences	Locus	Sequence (5' – 3')	Primer T_m	Amplicon length (bp) and G/C content
Hel_matk3F	AJ414328.1	matK	F: ATCCCTTCATGCATTATTTCCG	56 °C	129
Hel_matk3R	gi:23095820		R: TGAGACCAAAAGTAAAAATGATATTCCC	56 °C	32,9% GC
Verat_trnL intron112F	DQ517461.1 gi:100271287	trnL intron	F: AATAAAAAAAAGATAGGTGCAGAGACTCAAT	59 °C	116 38,3% GC
Verat_trnL intron154R			R: TGTGGGCCATCCTTTCTTTAA	58 °C	
Syst_matK1f	NC_000932	matK/	F: ATACTCCTGAAAGATAAGTGG	58 °C	333 (*Arabidopsis*)
Syst_ccmp1r	gi:7525012	intron	R: CCGAAGTCAAAAGAGCGATT	58 °C	31,8% GC

Table 2. Species / samples used for the contamination mixture.

Species	Internal sample code
Origanum onites	SR449
Salvia pomifera	S21
Peucedanum ostruthium	Peu54
Melampyrum sylvaticum	M207
Epilobium angustifolium	E187
Galphimia glauca	Gal170
Veratrum nigrum	G43
Rumex alpinus	R50

Paper 7

Lukas B, Schmiderer C, Novak J: DNA-Metabarcoding as a tool for quality control of botanicals. *Food Control*, submitted April 14th, 2015.

Contrary to the submitted manuscript, the formatting was partially adapted to this work and the figures and tables were positioned within the main text.

Author's contributions:

BL and CS performed the lab work and revised the manuscript.

JN analysed the data and prepared the manuscript.

DNA-Metabarcoding as a Tool for Quality Control of Botanicals

Brigitte Lukas, Corinna Schmiderer, Johannes Novak

Abstract

Quality control of botanicals consists of identifying the raw material to avoid unwanted admixtures or exchange of material as well as proofing abiotic and biotic contaminations that have to fulfill certain limits. So far, the processes for identity of plant materials and microbial contaminations are separated by the methods used. Species identification by their DNA ('DNA-barcoding') has the potential to supplement existing macro-, microscopic and phytochemical methods of identification. The advent of next-generation sequencing (NGS) offers completely new possibilities like the identification of whole communities, termed 'DNA-metabarcoding'. Here we present an assessment to identify plants and fungi of two commercial sage samples (*Salvia officinalis* L., Lamiaceae) by a standard DNA barcoding region, the internal transcribed spacer (ITS). DNA was extracted and ITS1 and ITS2 pre-amplified. The two samples were barcoded with a short sample recognition oligonucleotide and submitted to next-generation sequencing (Illumina MiSeq). The sequences were 'collapsed' (identical sequences combined into one) and sequences with a coverage of more than 9 submitted to identification. The main species in both was identified as *Salvia officinalis*. The spectrum of accompanying plant and fungal species, however, was completely different between the samples. The species composition was also depending on the primer set used what clearly shows the need for harmonization. This NGS approach for quality control is suitable for routine analysis and gives deeper insight into the real species composition of biological contaminations. Therefore, it would allow a better knowledge-based risk-assessment than any other method. However, the method is economically feasible in routine analysis only if a high sample throughput can be guaranteed.

Highlights

- Next-generation sequencing as a tool for quality control of botanical raw materials.
- Combination of identification of the target species as well as of biotic contaminations ('DNA-metabarcoding').
- Two sage (*Salvia officinalis* L., Lamiaceae) samples were analyzed by pre-amplifying ITS1 and ITS2 before sequencing by Illumina MiSeq.
- Extensive information about the species composition allows a knowledge-based risk-assessment.
- The resulting list of species is also dependent from the primer combinations used.

Keywords

DNA-metabarcoding, quality control, identity, botanicals, medicinal and aromatic plants

Introduction

Adulteration of botanicals or the substitution of the correct species by undesired or even toxic species occurs either accidentally or deliberately (Mader, Ruzicka, Schmiderer, & Novak, 2011) (Sanzini, Badea, Santos, Restani, & Sievers, 2011) (Ouarghidi, Powell, Martin, de Boer, & Abbad, 2012) (Newmaster, Grguric, Shanmughanandhan, Ramalingam, & Ragupathy, 2013). The raw materials are originating either from wild collection or field cultivation. They are growing under natural conditions influenced by abiotic and biotic factors and cultivated or wild collected with standard agronomical practices. Therefore they cannot be free of biotic contaminations. Hence, the implementation of methods for species identification of biotic contaminations by plants or microorganisms is a principal necessity in quality control. The term quality control summarizes a number of methods like macroscopic and microscopic examinations, physical and chemical analysis (e.g. thin-layer chromatography), the content of foreign matter, ash, extractable matter, water and volatile matter, pesticide residues, heavy metals, microorganisms, etc.). These methods are often regulated in pharmacopoeias (e.g. the European Pharmacopoeia (Council of Europe, 2013)).

DNA-based identification is becoming increasingly popular because of its ability to identify species in a state difficult to identify by other means (e.g. identification of root drugs or processed materials) where morphological or chemical methods do not provide sufficient resolution. These methods have therefore the potential to complement existing methods for the identification of plant species used and partially even biological contaminants in botanicals (Mader et al., 2011; Newmaster et al., 2013; Techen, Parveen, Pan, & Khan, 2014).

Amongst different other methods to identify a sample by its DNA, DNA sequencing can still be regarded as the method delivering the utmost possible information. This approach is known under the term ‘DNA barcoding’. One of the many DNA barcodes proposed is the internal transcribed spacer (ITS) situated between genes coding for ribosomal RNA located in the nucleus, a marker that is applicable in both, plants (Techen et al., 2014) and fungi (Schoch et al., 2012; Seifert, 2009).

High-throughput DNA sequencing (or next-generation sequencing, NGS) is a major step in delivering DNA sequence information by highly parallelizing the sequencing process thus producing up to millions of different sequences concurrently. These technologies are lowering the costs per sequenced base pair dramatically thus enabling new applications so far unthinkable. Exemplarily,

NGS became very popular in studying complex microbial community structures, termed as 'DNA-metabarcoding' (Schmidt et al., 2013; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012).

Here we use DNA-metabarcoding in order to analyze the plant and fungal community of two trade samples of sage (*Salvia officinalis* L., Lamiaceae), an important medicinal and aromatic plant. The aim of the study is to combine two assessments in quality control of plant raw materials in one analysis: (1) identification of the target species (sage) and (2) detection of biological (plant and fungal) contaminations.

Material and Methods

Plant material

Two fine cut samples of sage leaves (*Salvia officinalis* L.) originating from wild collection in Albania where kindly provided by Kräutermix (Abtswind, Germany). The samples were drawn as provided directly from the Albanian exporters before any further processing.

DNA extraction

Genomic DNA was extracted from dried samples using a modified CTAB-protocol based on Doyle (Doyle, 1991), with modifications as described by Schmiderer et al. (Schmiderer, Lukas, & Novak, 2013) ('CTAB-protocol I').

Primers and PCR

The nuclear ribosomal internal transcribed spacers (ITS) were pre-amplified for NGS with two primer pairs to obtain ITS1 and ITS2 separately because of the short reading lengths in subsequent NGS sequencing with the Illumina technology. ITS1 was amplified with the primers ITS5 (5'-GGAAGGAGAAGTCGTAACAAGG) and ITS2 (5'-GCTACGTTCTTCATCGATGC). ITS2 was amplified with the primers ITS3 (5'-GCATCGATGAAGAACGTAGC) and ITS4 (5'-TCCTTCCGCTTATTGATATGC) (Downie & Katz-Downie, 1996; White, Bruns, Lee, & Taylor, 1990). All primers were synthesised by Invitrogen, Lofer, Austria.

PCR amplification of ITS1 and ITS2 was carried out with 1:100 dilutions of extracted DNA. For a 50 µL PCR reaction 3.3 µL of DNA was added to a master mix containing 2 U of HOT FIREPol® DNA Polymerase, 1x PCR buffer B, 0.133 mM dNTPs (all reagents from Solis BioDyne, Tartu, Estonia), 0.6 mM forward- and reverse-primers. The PCR products were purified with enzymes (Exonuclease 1 and Shrimp Alkaline Phosphatase, Fermentas, St. Leon-Rot, Germany) in accordance to the

manufacturer's protocol. The DNA contents of the amplification products were determined fluorometrically and adjusted to 200 ng/µl.

NGS using the Illumina technology was outsourced (LGC Genomics, Berlin, Germany), where the two sage samples were barcoded before sequencing. The two samples were combined with other samples into one run in a way that the capacity used for the two samples was around 2% of the capacity of one Illumina-MiSeq run.

First bioinformatic steps (demultiplexing, clipping of Illumina adapters, combination of forward and reverse reads and primer sorting) were performed at LGC Genomics. Demultiplexing of all samples was done with CASAVA 1.8 (Illumina, San Diego, USA), reads with a final length < 20 bases were discarded and forward and reverse reads were combined using FLASH (Magoc and Salzberg 2011) with a minimum overlap of 10 bases. Equal sequences were combined (clustered or 'collapsed') and the frequencies of the collapsed sequences were recorded using Galaxy (Goecks, Eberhard, Too, Nekrutenko, & Taylor, 2013). Only collapsed sequences with more than 10 equal sequences were combined and compared with the NCBI-NR database using BLAST® of NCBI (National Center for Biotechnology Information, Bethesda, USA). BLAST results were further processed with MEGAN version 5.7.1 (Huson, Mitra, Ruscheweyh, Weber, & Schuster, 2011) with the LCA-algorithm ('lowest common ancestor') using the programs' standard parameters (LCA: min. score: 50, max. expected: 0.01, top percent: 10, min support percent: 0.01, min support: 1, LCA percent: 100, min complexity: 0.0).

The results were cross-checked with Primer-BLAST (Ye et al., 2012) to explore reasons for differential species composition between ITS1 and ITS2.

Results

ITS1 and ITS2 of two trade samples of sage (*Salvia officinalis* L., Lamiaceae) were amplified, barcoded and sequenced by NGS (Illumina) as part of a sample set covering about 2% of the capacity of one Illumina-MiSeq run. In total 163,902 (ITS1) and 232,577 (ITS2) sequences were obtained for sample 1 and 97,159 (ITS1) and 102,513 (ITS2) sequences for sample 2 (Table 1). Identical sequences were 'collapsed', i.e. replaced by one sequence with their frequencies recorded in the collapsed sequence name. After removing singletons, sample 1 resulted in 132,869 and 166,370 sequences for ITS1 and ITS2, respectively. Although adjusted to the same DNA content before sequencing, sample 2 resulted in significantly lower numbers of sequences with 75,499 and 56,896 reads for ITS1 and ITS2, respectively.

Table 1: Basic statistics of the NGS sequencing of two sage samples in two ITS regions (ITS1, ITS2).

Sample	total no. of reads	no. of reads without singletons	collapsed sequences blasted*)	% sequences represented by blasting (w/o singletons)	ratio sequences blasted to total reads sequenced (w/o singletons)
Sample 1					
ITS1	163,902	132,869	1,219	88%	0.92%
ITS2	232,577	166,370	1,748	87%	1.05%
Sample 2					
ITS1	97,159	75,499	655	84%	0.87%
ITS2	102,513	56,896	475	81%	0.83%

*) criteria for blasting: collapsed sequences with at least 10 identical reads

In an attempt to exclude erroneous sequences as far as possible, only those collapsed sequences with a frequency of more than 9 underlying sequences were blasted. This stringent criterion increased also the speed of identification significantly. Although stringent, the blasted sequences represented between 81% and 88% of the original sequences (without singletons) (Table 1). By the combined approach of collapsing sequences and blasting only sequences above a certain coverage the gain in efficiency was around 100-fold as indicated by the ratio of blasted sequences to reads (without singletons).

The main part of both samples was identified unambiguously as *Salvia officinalis*. The percentages of *S. officinalis* specific DNA-sequences were different between ITS1 and ITS2 and between the samples. Sample 1 consisted of 96.6% (ITS1) and 86.4% (ITS2) sage specific sequences, sample 2 of 97.7% (ITS1) and 95.2% (ITS2) sage specific sequences, respectively.

The species composition of the contaminations with plants or fungi as revealed by ITS1 showed *Nigella sativa* and *Bupleurum baldense* as major accompanying species in sample 1 (Figure 1), followed to a much lower sequence representation by *Cuscuta* sp., one species from the sub-tribe Centaureinae, *Helichrysum* sp. and *Trifolium* sp.. Only one fungus (*Alternaria alternata*) was prominent here. The same species composition was seen by ITS2 with the difference that the fungi were emphasised showing *Alternaria* sp. at a higher proportion and with the addition of *Cladosporium* sp., and *Aureobasidium pullulans* to the major contaminations (Figure 2).

The contamination of Sample 2 consisted of *Artemisia* sp., *Bromus* sp., *Daucus* sp., *Brachypodium distachyon*, one not closer determinable species of the Convolvulaceae, *Lactuca* sp., *Rubus* sp., *Melissa* sp. and *Malva* sp.. Very prominent fungi were *Alternaria alternata* and *Cladosporium* sp., fungi with minor sequence representation were *Chaetomium* sp., *Aspergillus versicolor*, *Aspergillus fumigatus* and *Golovinomyces biocellatus* (Figure 3). Sequences of ITS2 did not detect the plants *Artemisia* and *Bromus*, but determined *Melissa officinalis* and *Convolvulus arvensis* to the species

level. Also in the fungi composition, a similar shift could be observed. *Cladosporium* sp., *Chaetomium* sp., the two *Aspergillus* species and *Golovinomyces biocellatus* were not identified, while other fungi like *Septoria tanaceti* and *Mycosphaerella delegatensis*, just to name the two major new species, could be detected (Figure 4).

Figure 1: Word cloud of sample 1 identified by ITS1 (without *S. officinalis*). **Figure 2**: Word cloud of sample 1 identified by ITS2 (without *S. officinalis*). **Figure 3**: Word cloud of sample 2 identified by ITS1 (without *S. officinalis*). **Figure 4**: Word cloud of sample 2 identified by ITS2 (without *S. officinalis*).

Discussion

The approach of DNA-metabarcoding demonstrated that it is possible to identify many different plants and fungi in one assessment. That gives the possibility to look comprehensively for toxic plants or fungi accompanying the sample by pre-amplifying ITS. Generally, both internal transcribed spacers, ITS1 and ITS2, are equally suitable for fungal identification (Blaalid et al., 2013), the same can be expected from plants. However, the selectivity of the identification process is primer dependent, i.e. the choice of primers will lead to changes in the identification profile and quantity of the sequences for each species. The primers used to amplify ITS were originally designed for the amplification of fungal ribosomal RNA genes (White et al., 1990) and were later modified through a small amount of base exchanges (one base in primer ITS2 and primer ITS3, 2 bases in primer ITS5) to facilitate amplification of ITS of higher plants (Downie & Katz-Downie, 1996). The three bases difference between plants and fungi in ITS1 (amplified with primers ITS5 and ITS2) led to a severe underestimation of the fungal community compared to ITS2 with only one base difference (primers ITS3 and ITS4). The so-called ‘universal’ primers do not bind to all plant species and primer-template

mismatches reduce the amplification efficiency depending on the location and number of mismatches within the primer binding site. This can lead to an underestimation of species up to several orders of magnitude or even no amplification (Gobbers et al., 1997; Piñol, San Andrés, Clare, Mir, & Symondson, 2014). Due to the strong dependency of this method to primer specificity, the primer sets used for quality control needs to be carefully selected and possibly harmonized if results should be comparable between laboratories.

The Illumina sequencing method itself is principally quantitative, where the numbers of sequences of a species represents the abundance of this species' DNA in the sample matrix. However, due to the pre-amplification, our data is only of qualitative nature. Therefore we preferred word clouds to show results instead of a more exact quantitative data presentation like tables or bar charts. Sequence quantities can give some directions and allow a first risk estimation, not more. The data is not quantitative due to several reasons, (i) varying primer specificity as discussed above. (ii) varying amplification efficiency of the PCR due to varying DNA sequences, (iii) a variable copy number of the multi-copy ITS between species, (iv) heterogeneous DNA extraction efficiency from different plant or fungal matrices and (v) possibly unequal DNA degradation between taxa.

Quantitative PCR, however, could be used following a two-step strategy. First, an NGS approach is used to identify possible risks and in a second step, a detected specific, risk-related contamination may be quantified with a specific qPCR assay. Results from quality control will decide about unblocking batches within a production process. This influences the economics of the production process. Therefore, these results should be available as soon as possible. But even such a two-step procedure as described above could be performed within a reasonable time (within two to three weeks) when well organized.

Due to the enormous numbers of sequences arising from NGS most of them are present in multiple replicates. However, there are huge numbers of unique sequences (singletons) where their uniqueness may arise from sequencing errors. It is now common practice to remove singletons and clusters with a low copy number (usually below 5 copies) before downstream analysis, although they may represent taxa with minor proportions (Lindahl et al., 2013). The cut-off value depends on the research question. If the 'true' diversity has to be examined, only singletons or no sequences at all will be excluded. In this study, our cut-off value was set to 9 copies, because from the practical point of a possible routine analysis this sequencing depth delivered sufficient information with a fast bioinformatic assessment.

In NGS one run is very costly. The costs, however, can be divided to several samples. For this, the samples can be 'barcoded' (a short sample specific DNA sequence attached before sequencing in order to recognize the sample affiliation of each sequence after sequencing). Therefore, the method presented here has the potential to become a routine method only if the number of samples is high enough to be able to run many of them in parallel. We used here about 2% of the capacity of one run for each sample with a sufficient amount of sequences obtained. So when running 100 samples in parallel, the costs can be estimated to be about 200 € for sequencing plus an additional 100 € if using commercial computer clusters for bioinformatics (Ripp et al., 2014). A more pragmatic high cut-off could further lower these costs.

NGS has the potential to bring together different methods of quality control into one assessment, namely identification of the target species as well as identification of biotic contaminations including bacteria, fungi and plants. Nowadays microbial contamination is tested by sum tests, counting colony-forming units. Just for very critical species like *Salmonella*, species specific tests are foreseen. In this assay besides plants only fungi were determined, but it would be also possible to include DNA barcoding primers for bacteria (Janda & Abbott, 2007), insects (Jinbo, Kato, & Ito, 2011) and additionally specific primers for very toxic species like *Salmonella* sp. or *Senecio* sp. in order to increase selectivity for toxic species. A problem in detecting toxic species, however, is often their spotwise distribution in raw materials. Therefore, good sampling strategies like sufficient sub-sampling need to be employed. A 'spotwise' sub-sampling strategy can easily be integrated into this NGS-approach by either mixing the sub-samples again to a 'master-sample' or extracting and barcoding DNA from the sub-samples separately.

Next-generation sequencing has the potential to improve quality control of medicinal and aromatic plants by identifying the target species as well as biotic contaminations in samples. Due to primer specificity of 'universal' primers a careful selection (harmonization) process should be started to determine the best suitable primer pairs for this approach.

References

Blaalid R, Kumar S, Nilsson RH, Abarenkov K, Kirk PM, Kauserud H (2013): ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources* 13 (2): 218-24.

Council of Europe (2013): European pharmacopoeia. (8th Edition). Strasburg: EDQM.

Downie SR, Katz-Downie DS (1996): A molecular phylogeny of Apiaceae subfamily Apioideae: Evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* 83 (2): 234-51.

Doyle J (1991): DNA protocols for plants. In: Hewitt G, Johnston A, Young J (Eds.): *Molecular techniques in taxonomy*. Berlin: Springer. pp. 283-5.

Gobbers E, Fransen K, Oosterlaken T, Janssens W, Heyndrickx L, Ivens T, Vereecken K, et al. (1997): Reactivity and amplification efficiency of the NASBA HIV-1 RNA amplification system with regard to different HIV-1 subtypes. *Journal of Virological Methods* 66 (2): 293-301.

Goecks J, Eberhard C, Too T, Nekrutenko A, Taylor J (2013): Web-based visual analysis for high-throughput genomics. *BMC Genomics* 14 (1): 397.

Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC (2011): Integrative analysis of environmental sequences using MEGAN4. *Genome Research* 21 (9): 1552-60.

Janda JM, Abbott SL (2007): 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology* 45 (9): 2761-4.

Jinbo U, Kato T, Ito M (2011): Current progress in DNA barcoding and future implications for entomology. *Entomological Science* 14 (2): 107-24.

Lindahl BD, Nilsson RH, Tedersoo L, Abarenkov K, Carlsen T, Kjøller R., Kõljalg U, et al. (2013): Fungal community analysis by high-throughput sequencing of amplified markers – a user's guide. *New Phytologist* 199 (1): 288-99.

Mader E, Ruzicka J, Schmiderer C, Novak J (2011): Quantitative high-resolution melting analysis for detecting adulterations. *Analytical Biochemistry* 409 (1): 153-155.

Newmaster SG, Grguric M, Shanmughanandhan D, Ramalingam S, Ragupathy S (2013): DNA barcoding detects contamination and substitution in north american herbal products. *BMC Medicine* 11: 222.

Ouarghidi A, Powell B, Martin GJ. de Boer H, Abbad A (2012): Species substitution in medicinal roots and possible implications for toxicity of herbal remedies in morocco. *Economic Botany* 66 (4): 370-82.

Piñol J, San Andrés V, Clare EL, Mir G, Symondson WOC (2014): A pragmatic approach to the analysis of diets of generalist predators: The use of next-generation sequencing with no blocking probes. *Molecular Ecology Resources* 14 (1): 18-26.

Ripp F, Krombholz CF, Liu Y, Weber M, Schäfer A, Schmidt B, Köppel R, Hankeln T (2014). All-food-seq (AFS): A quantifiable screen for species in biological samples by deep DNA sequencing. *BMC Genomics* 15 (1): 639.

Sanzini E, Badea M, Santos AD, Restani P, Sievers H (2011): Quality control of plant food supplements. *Food and Function* 2 (12): 740-6.

Schmiderer C, Lukas B, Novak J (2013): Effect of different DNA extraction methods and DNA dilutions on the amplification success in the PCR of different medicinal and aromatic plants. *Zeitschrift Für Arznei- und Gewürzpflanzen* 18 (2): 65-72.

Schmidt PA, Bálint M, Greshake B, Bandow C, Römbke J, Schmitt I (2013): Illumina metabarcoding of a soil fungal community. *Soil Biology and Biochemistry* 65: 128-32.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, et al. (2012): Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proceedings of the National Academy of Sciences of the United States of America* 109 (16): 6241-6.

Seifert KA (2009): Progress towards DNA barcoding of fungi. *Molecular Ecology Resources* 9 (Supplement 1): 83-9.

Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E (2012): Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology* 21 (8): 2045-50.

Techen N, Parveen I, Pan Z, Khan IA (2014): DNA barcoding of medicinal plant material for identification. *Current Opinion in Biotechnology* 25: 103-10.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds.): PCR protocols: A guide to methods and applications. New York: Academic Press. pp. 315-22.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T (2012): Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13: 134.

Conclusions

The DNA-based identification of plant material is a valuable tool for quality control, especially in samples, where traditional morphological or phytochemical methods reach their limits. It can be assumed that trade samples composed of e.g. roots, seeds, fine cut or ground plant materials often cannot be identified to species level by their morphology because species specific features are missing or destroyed by processing (e.g. by cutting or grinding). It can be also assumed, that phytochemical methods often cannot distinguish phytochemically similar (closely related) species or do not detect the presence of 'inconspicuous' species, which contain no remarkable secondary metabolites. Minor admixtures or traces of undesired plant species, like allergenic or poisonous plants (e.g. *Aconitum* spp. or *Veratrum* spp. in *Helleborus niger*, *Valeriana officinalis* or *Peucedanum ostruthium* rhizomes), may be easily overseen with morphological or phytochemical analysis, but are easily detectable with appropriate DNA-based methods (Mader et al., 2011; Schmiderer et al., 2015b).

For the application as routine testing methods, it would simplify the whole analysis process to standardise as many working steps as possible. But the comparison of different DNA extraction methods showed that different sample matrices need to be treated differently (Schmiderer et al., 2013). Although in our assessment both tested CTAB extraction methods and the DNeasy kit lead to a higher PCR amplification success than the other two tested kits, some materials were in general problematic (valerian roots, cinnamon bark and capsules) and other extraction methods may deliver better results. The amount of extractable DNA per dry matter strongly varied between the used extraction methods, plant species, plant parts, and the storage time. According to the heterogeneous results a unique DNA extraction method for all plant materials or a standardised DNA concentration for PCR cannot be recommended.

High-resolution melting curve analysis is a valuable tool for the identification of plant specimens. Short amplicons including species-specific mutations like one SNP (transversion of G or C to A or T and *vice versa*) or few SNPs allowed the unambiguous identification of the species of interest. E.g. the amplicons of a part of the *Calendula* trnK 5' intron showed a melting temperature difference of approx. 0.8 °C due to a C/A exchange, making an unambiguous designation of each sample easily possible. G/C or A/T exchanges are known to cause only minor melting temperature shifts (Liew et al., 2004) and were therefore not used for HRM analysis. As expected, species-specific mutations for *Helleborus niger* (Ranunculaceae), *Calendula officinalis* (Asteraceae) and *Valeriana officinalis* (Valerianaceae) were only found in introns (e.g. trnK 5' intron) or intergenic spacers (e.g. trnL-trnF, psbA-trnH) but not in coding sequences (rbcL gene, matK gene) (Schmiderer et al., 2010; Schmiderer

et al., 2015a; Ruzicka et al., 2016). *Peucedanum ostruthium* (Apiaceae) showed no species-specific mutations in the sequenced chloroplast markers (trnK 5' intron, trnT-trnL and psbA-trnH) but in the internal transcribed spacers. Additional intraspecific variability and the presence of heterogeneous ITS copies complicated the analysis (Schmiderer et al., 2015b).

As demonstrated for *Valeriana officinalis* (Ruzicka et al., 2016), adulterants can be detected with two basically different methods: 1. closely related species with similar DNA sequences, like other *Valeriana* species, can be amplified with the same primer pair as the target species. The resulting amplicons differ in only one or few base pairs, what causes a shift of the melting temperature. If one sample is composed of two species with slightly different sequences a heteroduplex formation occurs. The resulting melting curves have an additional inflection point (caused by the lower melting temperature of the heteroduplices) what causes a change of the melting curve shape compared to both pure samples. In the assessment for *Valeriana* the detection limit of the adulteration was 30%. It is likely that the analysis of admixtures with lower percentages of the adulterant would have revealed a lower detection limit. Similar unpublished assays revealed a detection limit of approx. 10% admixtures. Melting curves of samples with minor admixtures are so similar to that of the pure sample, that the curves cannot be unambiguously distinguished. 2. The second method enables the detection of distantly related or unrelated species and comprised a duplex-PCR with two specific primer pairs, one amplifying *Valeriana* DNA, the other amplifying the adulterant, in this case *Veratrum* spp. The primer pairs were designed to bind to different loci, so a heteroduplex formation did not occur, and that the amplicons have clearly distinct melting temperatures. If the *Valeriana* primers are present in a limiting amount, even traces of *Veratrum* DNA (0.01%) can be amplified and unambiguously detected.

For the detection of adulterations similar experiments with duplex-PCRs were performed before (Mader et al., 2011). One experiment was designed to detect *Veratrum nigrum* as adulterant of *Helleborus niger* – both named 'black hellebore' in English – and included specific primers for each genus. A second experiment was tested for unknown admixtures of *Helleborus niger*. This experiment included the same *Helleborus* specific primers and general primers (matKf1 and ccmp1r) amplifying the trnK 5' intron. A primer-BLAST analysis showed that all published Ranunculaceae sequences have several primer-template mismatches, what very likely hinders the amplification of *Helleborus* DNA with these primers (personal observation; so far no corresponding *Helleborus* sequence has been published). Hence the presence of trnK amplicons indicates the presence of admixtures, and the proportions of the trnK-amplicons correlated well with the proportions of admixtures. With both experiments it was possible to detect traces of the adulterants.

A satisfying multiplex approach with even four primer pairs was developed for *Peucedanum* and its adulterants *Aconitum napellus*, *Veratrum album* and *Gentiana* spp. (Schmiderer et al., 2015b). Theoretically multiplexing of more primer pairs would be possible, but the formation of primer dimers and the ability to distinguish the different amplicons is limiting.

For the last paper the composition of two trade samples of Salviae officinalis folium was analysed by DNA metabarcoding (Lukas et al., submitted). Previous morphological and phytochemical analysis (De Mori, 2015) showed that the samples differed in their essential oil content (approx. 1.5% and 2.1%, respectively), mainly due to the proportions of stems, grasses and unidentifiable material. The metabarcoding approach, in this case the PCR amplification of the internal transcribed spacers with general primers followed by Illumina sequencing, allowed the detection and identification of several adulterations to species or genus level, belonging either to plants (e.g. *Artemisia*, *Lactuca*, *Bupleurum*, *Daucus*, *Nigella*, *Cuscuta*, *Bromus*, *Brachypodium*, *Rubus*, *Malva*, *Trifolium*) or to fungi (e.g. *Alternaria*, *Aureobasidium*, *Aspergillus*, *Cladosporium*, *Mycosphaerella*). Of course the presence of sage stems, building the major part of the 'foreign matter', as revealed by morphological analysis, could not be detected with this analysis.

A perfect assay for DNA-based identification of plant samples has to fulfil several requirements. 1. Primarily the used plant species need(s) to be identified (correct species and/or substitution), and 2. (other) adulterations should be detected and if possible also identified. 3. As far as e.g. the European Pharmacopoeia tolerates minor impurities (commonly 2%) a quantification of the adulterants would be necessary. 4. For routine analysis a test should be cheap, fast and easy to perform, what is of course in conflict with the other requirements.

In the last years many DNA-based assays were developed to identify plant specimens and to authenticate traded plant material. Many of them seem to work well, but the assay design often allows only limited conclusions, which need to be critically interpreted. For the setup of a new approach often only very restricted sample sets are used, which allow only limited or no conclusions about the intra- and interspecific variability, hence the reliability of the method. E.g. Choo et al. (2009) used three specimens of *Angelica decursiva* (syn. *Peucedanum decursivum*), three specimens of *Peucedanum praeruptorum* and two specimens of *Anthriscus sylvestris* (all Apiaceae) to develop SCAR markers for the discrimination of these species. In my opinion, on the one hand the low sample numbers were not sufficient to reflect the intraspecific variability, and on the other hand the testing of only this three species does not allow the identification of them, especially because *Angelica* and *Peucedanum* are species-rich and complex genera. It is likely that the developed primers bind to DNA of other related species and hence deliver unreliable results. Marieschi et al. (2012) published a

similar approach to detect plant adulterants of saffron, including i.a. *Calendula officinalis*. Obviously the species-specificity of the developed SCAR marker was not verified because probably only one specimen of *C. officinalis* but no specimens of closely related species or genera were tested. So also for this assessment it could be easily possible that the primers developed for the SCAR marker either do not bind to all *C. officinalis* specimens (accordingly they would not be detected) or they could also bind to DNA of several other species. Although in this case it may be secondary for a consumer which species exactly was used as adulterant, in the strict sense an identification of the adulterant is not feasible. The same dilemma appears for the assessment of Jiang et al. (2014), who developed a barcode-HRM method to authenticate saffron and its adulterants. The authors did not cross-check, that the used psbA-trnH sequences of *C. officinalis* are identical with several other published sequences of *C. arvensis* and *C. suffruticosa* (NCBI; personal observation). As far as the access to plant specimens and their screening can be time consuming and laborious, a previous *in silico* examination by means of databases could be helpful to review the primer specificity and enables a cheap and easy overview of the (species) specificity of the approach (like Primer-BLAST of GenBank; Ye et al., 2012) – as far as according sequences are already published. Apparently it is often not considered, that a trade sample could consist of a species mixture and at least little impurities are very likely (e.g. the presence of 'weeds' or co-harvested species). So for the setup of a new method it should be checked, to which degree substitutes or admixtures could be detected.

Several publications dealing with DNA barcoding for the authentication of trade samples present their results in a way that analysed sample materials belonged either to the correct species or to a substitute (Palhares et al., 2015, Stoeckle et al., 2011). 'Wrong' positive results (in this case meaning the detection of other species than indicated on the label) are commonly accepted without additional revision although the composition of PCR products does not necessarily reflect the composition of the raw material. Piñol et al. (2015) tested the reliability of DNA metabarcoding and demonstrated very well that the results reflected the qualitative composition of the original sample (all species mixed in a mock sample were detected) but not the quantitative composition. Primer-template mismatches, especially in the 3'-end of the primers, affected the previously generated PCR products up to five orders of magnitude, so a quantitative inference is not adequate with this method. It can be concluded, that a DNA barcoding analysis for a sample mixture composed of 99% species A (showing several primer-template mismatches) and 1% of species B (without mismatches) would result in an unambiguous sequence chromatogram showing species B only. Due to an unequal amplification success for different primer pairs and different amplification efficiencies DNA barcoding is of limited suitability for the analysis of mixed samples. Especially unexpected DNA barcoding results need to be cross checked and verified; otherwise the enthusiasm in finding mislabelled or

even fraudulent products may lead to questionable reports (e.g. Newmaster et al., 2013; criticised by Grafner et al., 2013).

The analysis of plant products like capsules or pills containing phytochemical extracts (liquid or dry extracts) is in my opinion very problematic and only little expedient. On the one hand I assume that phytochemical extracts contain relatively few DNA and that standard DNA extraction methods are not suitable to gain DNA of sufficient quality, quantity and purity. Additionally, the present DNA could be degraded due to extensive processing of the plant material. On the other hand, for the production of dry extracts and finished products the use of carriers or fillers (e.g. soy, rice or potato) is common and their DNA could be detected instead of the medicinal plant's DNA (Grafner et al., 2013). Hence the failure of a subsequent PCR or the amplification of other DNA than the pharmaceutically active species is possible but that does not necessarily reflect the composition or the quality of the product.

For a correct quantitative evaluation a link from amplified DNA to the proportion of the dry matter of each present species is necessary. For such calculations many factors would need to be considered. The amount of DNA per dry mass is dependent from plant species and plant organs. It can be assumed that young leaves consisting of cells with small vacuoles and thin cell walls contain more DNA per dry mass than cells of a trunk with thick, lignin-containing cell walls. It is likely that also the proportion of extractable DNA varies because the rupture of woody cell walls is more difficult. The genome sizes greatly vary between different taxa (in land plants approx. 63 Mbp to 149 Gbp; Greilhuber and Leitch, 2012), the number of plastid DNA copies per plastid (approx. from 10 to several hundreds), plastids/chloroplasts per cell (from 1 in Algae to ≈100) and the number of nuclear gene copies per cell vary (e.g. nrDNA ≈400-44,000) (Milo and Philips, 2015; Rogers and Bendich, 1987). The quality or degradation of DNA depends on several factors, i.a. the age of the plant material (e.g. young *vs.* senescent leaves; Sakamoto and Takami, 2014), drying conditions, storage time and storage conditions (e.g. temperature, humidity) of the plant material. The DNA extraction method including the prior sample preparation (grinding) and the storage conditions of extracted DNA also influences the degradation (Rossmanith et al., 2011). So far it is not known if the progression of DNA degradation is similar for all plants or if significant differences exist. As already mentioned, the PCR efficiency is influenced by the applied primers, primarily due to primer-template mismatches and the formation of amplicon secondary structures. GC rich templates and the formation of strong secondary structures can even cause a complete failure of the PCR amplification (Frackman et al., 1998). Several of these factors may vary greatly between samples and could be

influenced by unequal harvesting, processing and storage making a correct calculation very complex or even impossible.

An alternative to estimate a sample composition or the detection limit of adulterants would be the analysis of admixture series (Schmiderer et al., 2015a and 2015b). The admixing of raw plant materials would obviate several uncertainty factors (e.g. DNA extraction efficiency, PCR efficiency) and allow at least a semi-quantitative estimation. For the setup of a new method admixing of extracted DNA can be used as an intermediary step. On the one hand admixing of known DNA amounts better allows conclusions about competition effects during PCR; on the other hand the preparation of the DNA admixtures is easier to perform, like measuring of especially small proportions, homogenisation and the need of fewer raw materials. A comparison of both admixing methods could demonstrate uncertainty factors like different DNA extraction efficiencies. Although the method of admixing is a practicable approach, certain incertitude remains (e.g. variable composition of plant parts, influence of unequal degradation) which can distort the results.

For the development of a new DNA-based approach, it is very important to consider all demands which should be fulfilled by the test. The more precise the scope of the work is the easier is it to choose an appropriate method and to design an approach. But for following analyses of (trade) samples it has to be considered, for which purposes the approach was developed, and hence which conclusions can be drawn from the results.

References

Choo BK, Moon BC, Ji Y, Kim BB, Choi G, Yoon T, Kim HK (2009): Development of SCAR markers for the discrimination of three species of medicinal plants, *Angelica decursiva* (*Peucedanum decursivum*), *Peucedanum praeruptorum* and *Anthricus sylvestris*, based on the internal transcribed spacer (ITS) sequence and Random Amplified Polymorphic DNA (RAPD). *Biol Pharm Bull* 32 (1): 24-30.

De Mori A (2015): Analisi morfologiche, fitochimiche e genetiche di due campioni commerciali di *Salvia officinalis* [Morphological, phytochemical and genetic analyses of two *Salvia officinalis* trade samples]. Diploma thesis, Universitá degli studi di Trieste.

Frackman S, Kobs G, Simpson D, Storts D (1998): Betaine and DMSO: Enhancing agents for PCR. *Promega Notes* 65: 27. http://at.promega.com/~/media/files/resources/promega%20notes/65/betaine%20and%20dmso-%20enhancing%20agents%20for%20pcr.pdf?la=de-de. Accessed: November 30th, 2015.

Grafner S, Blumenthal M, Reynaud DH, Foster S, Techen N (2013): ABC Review and Critique of the research article "DNA barcoding detects contamination and substitution In North American herbal products" by Newmaster et al. *HerbalEGram* 10 (11).

Greilhuber J and Leitch IJ (2012): Genome size and the phenotype. In: Plant genome diversity. Vol. 2. Vienna: Springer; pp 323-44.

Jiang C, Cao L, Yuan Y, Chen M, Jin Y, Huang L (2014): Barcoding melting curve analysis for rapid, sensitive, and discriminating authentication of saffron (*Crocus sativus* L.) from its adulterants. *BioMed Res Int* 2014: 809037.

Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C (2004): Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem* 50: 1156-64.

Lukas B, Schmiderer C, Novak J (submitted): DNA-Metabarcoding as a tool for quality control of botanicals. *Food Control*, submitted April 14th, 2015.

Mader E, Ruzicka J, Schmiderer C, Novak J (2011): Quantitative high-resolution melting analysis for detecting adulterations. *Anal Biochem* 409: 153-55.

Marieschi M, Torelli A, Bruni R (2012): Quality control of saffron (*Crocus sativus* L.): Development of SCAR markers for the detection of plant adulterants used as bulking agents. *J Agr Food Chem* 60: 10998-11004.

Milo R and Philips R (2015): Cell biology by the numbers (draft). Garland Science. http://book.bio-numbers.org/how-large-are-chloroplasts/. Accessed: November 30th, 2015.

Newmaster SG, Grguric M, Shanmughanandhan D, Ramalingam S, Ragupathy S. DNA barcoding detects contamination and substitution in North American herbal products. *BMC Medicine*

11: 222. doi:10.1186/1741-7015-11-222.

Palhares RM, Gonçalves Drummond M, dos Santos Alves Figueiredo Brasil B, Pereira Cosenza G, das Graças Lins Brandão M, Oliveira G (2015): Medicinal plants recommended by the World Health Organization: DNA barcode identification associated with chemical analyses guarantees their quality. *PLoS ONE* 10 (5): e0127866. doi:10.1371/journal.pone.0127866.

Piñol J, Mir G, Gomez-Polo P, Agustí N (2015): Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Mol Ecol Resour* 15: 819-30.

Rogers SO, Bendich AJ (1987): Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. *Plant Mol Biol* 9: 509-20.

Rossmanith P, Röder B, Frühwirth K, Vogl C, Wagner M (2011): Mechanisms of degradation of DNA standards for calibration function during storage. *Appl Microbiol Biotechnol* 89: 407-17. doi: 10.1007/s00253-010-2943-2.

Ruzicka J, Schmiderer C, Novak J (2016): DNA-based identification of Valeriana officinalis s.l.: a Multiplexed Amplification-Refractory Mutation System (MARMS) and High Resolution Melting Curve Analysis (HRMA). Plant Mol Bio Rep, Online First: DOI 10.1007/s11105-015-0967-6.

Sakamoto W, Takami T (2014): Nucleases in higher plants and their possible involvement in DNA degradation during leaf senescence. *J Exp Bot* 65 (14): 3835-43.

Schmiderer C, Mader E, Novak, J (2010): DNA-based identification of *Helleborus niger* by high-resolution melting analysis. *Planta Med* 76 (16): 1934-7.

Schmiderer C, Lukas B, Novak, J (2013): Effect of different DNA extraction methods and DNA dilutions on amplification success in the PCR of different medicinal and aromatic plants. *Z Arznei-Gewürzpfla* 18(2): 65-72.

Schmiderer C, Lukas B, Ruzicka J, Novak J (2015a): DNA-based identification of *Calendula officinalis* (Asteraceae). *Appl Plant Sci* 3 (11): 1500069. http://dx.doi.org/10.3732/apps.1500069.

Schmiderer C, Ruzicka J, Novak J (2015b): DNA-based identification of *Peucedanum ostruthium* specimens and detection of common adulterants by high-resolution melting curve analysis. *Mol Cell Probes* 29 (6): 343-50.

Stoeckle MY, Gamble CC, Kirpekar R, Young G, Ahmed S, Little DP (2011): Commercial teas highlight plant DNA barcode identification successes and obstacles. *Sci Rep* 1: 42. doi: 10.1038/srep 00042.

Wallace LJ, Boilard SMAL, Eagle SHC, Spall JL, Shokralla S, Hajibabaei M (2012): DNA barcodes for everyday life: Routine authentication of Natural Health Products. *Food Res Int* 49: 446-52.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012): Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinf* 13: 134.

Abstract

The DNA-based identification of plant material is a valuable tool for quality control, especially in samples, where morphological or phytochemical methods reach their limits and are not able to assign the plant material to species level. For this thesis three areas of the DNA-based identification were investigated:

1) For the application as routine testing methods, it would be simplifying the whole analysis process to standardise as many working steps as possible. Hence five DNA extraction methods followed by subsequent PCR were compared using different plant materials (like roots, leaves, flowers and barks). But the analysis showed that different sample matrices need to be treated differently to obtain optimal results (Schmiderer et al., 2013).

2) The use of polymerase chain reaction (PCR) followed by high-resolution melting curve analysis (HRM) was proven to be a suitable method for the DNA-based identification of plant specimens. For selected medicinal plant species (*Helleborus niger*, Ranunculaceae [Schmiderer et al., 2010; Mader et al., 2011]; *Calendula officinalis*, Asteraceae [Schmiderer et al., 2015a]; *Peucedanum ostruthium*, Apiaceae [Schmiderer et al., 2015b]; *Valeriana officinalis*; Valerianaceae [Ruzicka et al., 2016]) and their reported adulterants HRM-suitable PCR primers were designed in such a way that 1) primers were flanking species specific mutations (for the target species), 2) those mutations resulted in well distinguishable HRM curves and 3) other species of the genus or closely related genera were amplified approximately equally well with the same primers. For the detection of unrelated species (e.g. *Aconitum napellus* or *Veratrum album* as possible adulterants of *Peucedanum ostruthium*), adulterant-specific primer pairs were designed and multiplexed with the target species-specific primers. The developed approaches allowed both, the authentication of the analysed target species and the detection of adulterants. Multiplexing of different primer pairs allowed the detection of adulterants down to the proportion of 0.001% (e.g. *Aconitum* or *Veratrum* in *Peucedanum*).

3) In order to detect unknown herbal or fungal adulterations two ‘Salviae officinalis folium’ trade samples were analysed by DNA metabarcoding (Lukas et al., submitted). The metabarcoding approach included the separate PCR amplification of both internal transcribed spacers with general primers followed by Illumina sequencing. The obtained sequences revealed the presence of several adulterants belonging to diverse plant families or fungi genera. The unequal results of ITS1 and ITS2 demonstrated that the qualitative and quantitative composition of the obtained sequences is influenced by the applied primers. Apart from species, which were not at all amplified with the used primers, the metabarcoding results were only of qualitative nature.

Zusammenfassung

Die DNS-basierte Identifizierung von Arzneipflanzen ist besonders bei Proben, bei denen morphologische oder phytochemische Methoden an ihre Grenzen stoßen und eine Artbestimmung nicht möglich ist, eine wertvolle Möglichkeit der Qualitätskontrolle. Für diese Dissertation wurden drei verschiedene Aspekte der DNS-basierten Artidentifikation untersucht:

1) Da es für Routineanalysen eine Arbeitsvereinfachung wäre, wenn möglichst viele Arbeitsschritte vereinheitlicht werden könnten, wurde die Auswirkung unterschiedlicher DNS-Extraktionsmethoden auf den PCR-Erfolg untersucht. Dafür wurde DNS mit fünf Methoden aus verschiedenen Drogenmatrices (wie Wurzeln, Blätter, Blüten und Rinden) extrahiert und mittels PCR analysiert. Der Vergleich der Ergebnisse zeigte jdoch, dass verschiedene Proben unterschiedlich behandelt werden müssen um optimale Erfolge zu erzielen (Schmiderer et al., 2013).

2) Die Verwendung der PCR gefolgt von einer Hochauflösenden Schmelzkurvenanalyse (high-resolution melting curve analysis, HRM) zeigte sich als nützliche Methode für die DNS-basierte Artidentifizierung von Pflanzenproben. Für ausgewählte Zielarten (*Helleborus niger*, Ranunculaceae [Schmiderer et al., 2010; Mader et al., 2011]; *Calendula officinalis*, Asteraceae [Schmiderer et al., 2015a]; *Peucedanum ostruthium*, Apiaceae [Schmiderer et al., 2015b]; *Valeriana officinalis*, Valerianaceae [Ruzicka et al., 2016]) wurden PCR-Primer mit folgenden Eigenschaften bzw. Zielen entwickelt: 1) Die Primer flankieren die für die Zielart spezifische Mutation. 2) Die in den Amplikons enthaltenen Mutationen verursachen gut unterscheidbare HRM-Kurven. 3) Proben von anderen Arten der Gattung oder nahe verwandten Gattungen können ungefähr gleich gut amplifiziert werden wie die Zielart. Für die Detektion nicht verwandter Pflanzenarten (z.B. *Aconitum napellus* oder *Veratrum album* als mögliche Verunreinigungen von *Peucedanum ostruthium*) wurden eigene Primer entwickelt und mit jenen der Zielarten in einer Multiplex-PCR/HRM verwendet. Die entwickelten Methoden erlaubten sowohl die Authentifizierung der untersuchten Zielarten als auch die Detektion von Verunreinigungen. Das Multiplexen von Primerpaaren ermöglichte die Detektion von Verunreinigungen bis zu einem Anteil von nur 0,001% (z.B. *Aconitum* oder *Veratrum* in *Peucedanum*).

3) Um unbekannte pilzliche oder pflanzliche Verunreinigungen in zwei ‚Salviae officinalis folium' Handelsproben nachweisen zu können, wurden diese mit einem ‚DNA Metabarcoding' Ansatz untersucht (Lukas et al., eingereicht). Dieser Ansatz beinhaltete die separate Amplifikation beider Internal Transcribed Spacers mithilfe von Standard-PCRs, gefolgt von einer Illumina Sequenzierung der PCR-Produkte. Die erhaltenen Sequenzen bewiesen Verunreinigungen der Salbeiproben mit etlichen Arten verschiedenster Pflanzen und Pilze. Die ungleichen Ergebnisse beider Proben von jeweils ITS1 und ITS2 zeigten, dass die qualitative und quantitative Zusammensetzung der erhaltenen

Sequenzen von den verwendeten Primern abhängig ist. Abgesehen von den Arten, die mit den verwendeten Primern nicht amplifiziert werden konnten, konnten von den Metabarcoding-Ergebnissen nur Aussagen über die Artenzusammensetzung der Proben gemacht werden, nicht aber über deren mengenmäßige Anteile.

Acknowledgements

Many persons contributed directly or indirectly to the success of this thesis, who I cordially would like to thank.

I'm deeply grateful to the colleagues of the Laboratory of Molecular Systematics (Museum of Natural History Vienna). With their professional, kind and patient way Werner Mayer†, Elisabeth Haring, Barbara Däubl, Mihaela Pavlicev and Luise Kruckenhauser were able to arise my interest in molecular biology. Without you, I possibly would have never started this work.

I cordially thank my supervisor Johannes Novak, my colleagues Brigitte Lukas, Joana Ruzicka, Eduard Mader and Paolo Grassi (Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Vienna) and my colleagues of the VetCore Facility, Ralf Steinborn, Martin Hofer and Georg Mair, for all their support and the helpful discussions at any time.

Several guest scientists, students and technical assistants helpfully contributed to parts of my work or my projects, namely Zehra Aytaç (Eskişehir Osmangazi University), Najada Kadiasi (Agricultural University of Tirana), Aurelia Gupte, Alexandra Huber, Michaela Koch, Katja Schilling, Olivia Tan and Juliana Wohlmuther (University of Veterinary Medicine).

I gratefully acknowledge Walter Till and Heimo Rainer (Herbarium of the University of Vienna), the Leibniz Institute of Plant Genetics and Crop Plant Research Gatersleben, the Royal Botanic Garden Edinburgh, the Royal Botanic Gardens Kew and Remigius Chizzola (University of Veterinary Medicine) for making their collections available for my research.

I also gratefully acknowledge all colleagues and friends who collected sample material for me, most of them gratuitous in their leisure time: Brigitte Lukas, Joana Ruzicka, Johannes Novak and Karin Zitterl-Eglseer (University of Veterinary Medicine), Avni Hajdari (University of Prishtina), Johannes Saukel and Sylvia Vogl (University of Vienna), Monika Nell, Thomas Pacher, Erich Unterweger and Jutta and Regina Sekara. I especially thank Brigitte Lukas for our memorable collection trips across the Mediterranean area and Austria.

I gratefully thank Birgit Grohs and Monika Saul for the professional and kind project management and project handling and the members of the Forschungsvereinigung der Arzneimittel Hersteller e. V. (FAH) for pleasant and constructive collaboration in context of the IGF project 15182 N.

Projects involved in this thesis were financed by the Austrian Science Fund FWF (S 10708-B03), the German Federal Ministry of Economics and Technology and the German Federation of Industrial

Research Associations (IGF project number 15182 N) and the European Community's Seventh Framework Program (FP7/2007–2013) under grant agreement n_245199. The completion of this thesis was financially supported by the Public Employment Service Austria.

I cordially thank my parents for supporting me in all essential decisions of my life, the enabling of my diploma studies and for one decade patience awaiting the completion of my thesis.

I gratefully and sincerely thank Rosabelle Samuel that she never gave up asking me about the progress of my thesis and even more for the final motivation to finish it. Without you, I probably would have never finished this work.