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Authentication of holy basil using markers relating to a toxicology-relevant compound

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Abstract

Holy Basil—*Ocimum tenuiflorum*—is one of the popular new "superfoods" thought to act as an antioxidant and to reduce stress and anxiety. However, it is often surrogated with other *Ocimum* species differing in their chemical profiles that may even pose health risks to the consumers. Moreover, even specific chemotypes of Holy Basil itself can be toxicologically relevant, because they sometimes contain the carcinogen compound methyleugenol. Using DNA barcoding based on plastidic markers, *O. tenuiflorum* can be differentiated from other species of *Ocimum*. However, this approach is still suboptimal in handling larger sample numbers and in tracing chemotypes that accumulate methyleugenol. We have, therefore, designed a trait-related DNA barcode based on the enzyme eugenol *O*-methyltransferase (EOMT), responsible for the synthesis of methyleugenol. We show that a multiplex PCR combining trait-related and trait-independent markers can differentiate *O. tenuiflorum* from other *Ocimum* species and identify methyleugenol chemotypes of *O. tenuiflorum*, even in dried material sold as mixtures.

Keywords Holy Basil · DNA barcode · Trait-related markers · Food security · Optimization

Introduction

From the turn of the millennium, the market for so-called "superfoods", i.e. foods with a supposed capacity to affect human health positively, has been growing impressively. However, the term "superfoods" raises more questions than it gives answers. Whether a plant-based product qualifies as "super" seems to depend, at least partially, on socio-economic, and not only on nutritional factors [1]. Whether these foods actually provide the promised value, for instance, if they can really boost the immune system, or if this is just propaganda, has been questioned [2]. It has also been questioned, whether some of these "superfoods" should be even avoided, given their potential harmful effects [3, 4].

Some of these "superfoods" are deeply rooted in traditional systems of healing and nutrition, but, due to globalisation, progressively leave their original cultural context, leading to confusion and even consumer deception. A typical example is the Holy Basil, also known as *Tulsi* or *Tulasi*,

corresponding to *Ocimum tenuiflorum* (often, the outdated synonym *Ocimum sanctum* is used), a member of the Lamiaceae family, with a long history in Ayurveda as a medicinal plant to treat a number of diseases [5]. Holy Basil is also widely used as a herbal product and has become a popular "superfood", sold mainly for its antioxidant capacity and to fight stress and anxiety [6]. As part of self-optimisation, it has become popular as so-called "adaptogen" [7]. However, one of the drawbacks of "superfoods" is that they are prone to food fraud, and Holy Basil is not an exception [8–10]. Food fraud, in the strict sense, is the deliberate and intentionally illicit replacement of a declared food product by a surrogate for the sake of economic profit, for example, by adulteration and mislabelling [11]. A softer version is surrogates that are partially accepted as cost-efficient or more easily available replacements for a desired product. For instance, *Vitex negundo* is accepted as surrogate for *O. tenuiflorum* in medicinal applications of Ayurveda, but not as food product [12], while Holy Basil in food products is often surrogated by other species of the genus *Ocimum*, such as *O. basilicum* [13]. This accepted surrogation is accentuated by discrepancies between vernacular and scientific nomenclature [14]. Although these surrogates are widely accepted, they have to be seen critically: some nutraceutical products based on *O. basilicum* have been found to contain

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estragole, methyleugenol and safrole, compounds that even though might not have a significant negative effect in health when consumed from herbal matrixes, are all genotoxic [15]. Based on these considerations, it is necessary to identify the authenticity of food products to maintain consumer safety. However, to authenticate commercial herbal products can be quite a challenging task.

There are a number of techniques that have been used for food authentication, involving microscopy, metabolomics and genomics [9, 13, 16–18]. Particularly for herbal products, light microscopy is useful as quite an inexpensive technique, by the identification of elements that can be used as a characteristic morphological marker [13, 19]. However, microscopy requires a lot of expertise and can be time consuming when working with a large amount of samples. Nevertheless, it is employed widely and successfully to detect adulterants in herbal products. A recent literature survey revealed that, globally, an estimated 40% of commercial herbal products tested by microscopical diagnostics turned out to be adulterated [19]. However, microscopy also faces certain limitations in processed products, because the preservation of anatomical features is often not sufficient for microscopic differentiation. Therefore, DNA-based authentication is progressively adopted as strategy to detect food fraud and a number of studies confirm that molecular methods have been successfully used for plant-based food authentication and traceability [4, 8, 14, 20–22]. Also, such molecular approaches have detected numerous cases of adulteration, as revealed by a recent global meta-study [23]. Technically, DNA technology is always based upon amplification of DNA by the Polymerase Chain Reaction (PCR), which is either followed by sequencing of differentiating molecular markers (so called genetic barcodes), or by a fingerprinting assay [24]. The most common fingerprinting strategy is Randomly Amplified Polymorphic DNA (RAPD), where differentiating pairs of arbitrary primers are used to differentiate species and adulterant [24]. Also, the use of restriction enzymes on the results of a PCR, restriction fragment length polymorphism (RFLP) has been helpful as an identification method [24, 25]. Likewise, multiplex PCR involving designed primers that yield a diagnostic side band depending on the species has been used successfully [25]. The drawback of these fingerprinting assays is that they require a null hypothesis—it is possible to discern unequivocally a declared species and a presumed adulterant. However, for unknown types of adulteration, the information content of fingerprinting is not sufficient. Here, the use of sequencing for authentication is mandatory. One of the keys to success for sequencing-based strategies is choosing the proper marker.

A global network of scientists has discussed extensively what characteristics DNA barcodes should have. For example, they should allow for high discrimination power between species, but be conserved within a species; the amplified region should give taxonomic information to identify samples; markers should deliver consistent results; and the aimed amplification product should be sufficiently short to allow for amplification even under conditions of DNA degradation [26, 27]. Therefore, mostly neutral markers that are not under selective pressure have been commonly used in DNA barcoding. However, there are cases, where it is important to discriminate categories within a species or across closely related species differing with respect to a trait of interest. In the case of functional food, these would be genetic markers coding for the value-giving compounds, or, in the negative case, markers monitoring the presence of hazardous adulterants. Such trait-related markers represent a fingerprint for a desirable or non-desirable trait, even below the level of a species. Such trait-related markers are already commonly used for improving traits of crops, for example, in marker-assisted breeding, by helping in the selection of plants that are resistant to stresses such as salinity, cold, and drought [28, 29].

In case of *Ocimum* species, a non-desirable trait is the presence of methyleugenol (ME), a compound of toxicological relevance. ME is a phenylpropene and a carcinogenic compound generated by the enzyme eugenol *O*-methyltransferase (EOMT) [15, 30]. A sequence comparison revealed that *bona fide* EOMT homologues show variation and cluster into distinctive clades [31]. Thus, this enzyme is a good candidate for developing DNA barcoding based on trait-related markers in *Ocimum* species. In our previous work [14], we had explored the limits of neutral markers for the discrimination of *Ocimum* species, particularly *O. tenuiflorum*. In the present research, we aim to optimise a current DNA barcoding method and to explore the possibility to expand resolution, using EOMT as trait-related marker to discriminate ME-accumulating chemotypes in commercial samples as contribution to consumer safety.

Materials and methods

Plant material

The study made use of the *Ocimum* species collection established at the Botanical Garden of the Karlsruhe Institute of Technology (Table 1). Each species had been previously authenticated using both, classic taxonomical identification, and plastidic DNA-barcoding markers [14].

Commercial samples

The commercial samples originated from local stores in Karlsruhe, Germany and were coded by an ID for the purpose of this study (Table 2). Commercial sample

Table 1 Reference plant material used in this research; scientific name; common name; ID of the voucher specimen cultivated in the Botanical Garden of the KIT; Genbank accession numbers of the sequences from the chloroplastidic *psbA-trnH* region, and the partial sequences of the enzyme eugenol *O*-methyltransferase (EOMT)

Identity	Common name	KIT ID	psbA-trnH	EOMT
O. tenuiflorum L.	Krishna tulsi	5751	MF784540	MW582310
O. tenuiflorum L.	Krishna tulsi	8257	MF784544	–
O. tenuiflorum L.	Krishna tulsi	8097	MF784541	–
O. tenuiflorum L.	Krishna tulsi	8099	MF784542	–
O. tenuiflorum L.	Rama tulsi	8256	MF784543	–
O. basilicum L.	Sweet basil	5192	MF784535	–
O. basilicum L.	Sweet basil	9056	MW582309	MW582311
O. x africanum Lour.	Lemon basil	7537	MF784538	–
O. x africanum Lour.	Lemon basil	5748	MF784537	–
O. americanum L.	Hoary basil	7811	MF784536	–
O. gratissimum L.	Clove basil	5749	MF784560	–
O. campechianum Mill.	Wild sweet basil	7564	MF784557	–
O. kilimandscharicum Gürke.	Camphor basil	7810	MF784539	–
Mentha x piperita L.	Pepper mint	5393	MH753571	–

Table 2 Commercial samples and declared content used to test DNA barcodes

Sample ID	Description of product label
TulComm 0001	Tea that contains different types of Holy Basil, not mixed with other types of herbs. No specifications on the *Ocimum* species
TulComm 0002	Tea that contains different types of Holy Basil, not mixed with other types of herbs. No specifications on the *Ocimum* species
TulComm 0003	Holy Basil tea not mixed with other types of herbs. No specifications on the *Ocimum* species
TulComm 0004	Holy Basil tea mixed with other types of herbs. No specifications on the *Ocimum* species
TulComm 0005	Holy Basil tea mixed with other types of herbs. No specifications on the *Ocimum* species
TulComm 0006	Holy Basil tea mixed with other types of herbs. No specifications on the *Ocimum* species
TulComm 0007	Holy Basil tea mixed with other types of herbs. No specifications on the *Ocimum* species
BasComm 0001	Dried basil. No specifications on the *Ocimum* species
BasComm 0002	Dried basil. No specifications on the *Ocimum* species
MenComm 0001	Mint tea. No specifications on the genus nor species

TulComm.0001, also used in the research by Jürges et al. [14], served as a positive control. All products are maintained as vouchers at the Botanical Institute of the KIT.

DNA extraction

Reference and commercial samples were ground with mortar and pestle using liquid nitrogen. DNA was extracted using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin) as described in Jürges et al. [14], and the final concentration and purity of the DNA were estimated spectrophotometrically (NanoDrop, Thermo Fisher Scientific Inc.).

Barcodes and PCR conditions

Trait-independent marker assay

The plastidic region *psbA-trnH intergenic spacer* was amplified, and the resulting PCR product subsequently used for an RFLP assay using a restriction digest with *Hinf* I (New England BioLabs Inc.) as previously described [14]. A schematic representation of the primer position for

the amplification of the *psbA-trnH igs* followed by RFLP can be seen in Supplementary Fig. S1a.

Trait-related marker assay

As marker linked with the presence of ME, the gene encoding eugenol EOMT served as trait-related marker. The putative sequence of EOMT was amplified from Krishna Tulsi (voucher ID 5751, Table 1) and Sweet Basil (voucher ID 9056, Table 1) from gDNA as template using a *Taq* polymerase with proofreading function (New England Biolabs, Frankfurt) and the forward and reverse oligonucleotide primers published by Renu et al. [32]. The thermocycling conditions were as suggested by Renu et al. [32]: an initial denaturation at 98 °C for 30 s followed by 35 cycles of 10 s denaturation at 98 °C, 30 s annealing at 56 °C, and 30 s elongation at 72 °C. The reaction terminated with a final elongation for 2 min at 72 °C. The sequences of both EOMT alleles (deposited in GenBank under the accession numbers given in Table 1) were used to design diagnostic primers to amplify a fragment of EOMT from *Ocimum*: EOMTfw 5'-TCCGGTCTATCCCTTCTGCCG-3' and EOMTrev 5'-ACCGACGGCATCTTTGCATC-3'. An additional reverse primer located outside of the fragment amplified by these primers, EOMTrev$_R$: 5'-GGATAAGCCTCTATGAGAGACC-3' taken from [32], was used for a multiplex PCR that, in case of *O. tenuiflorum*, yielded a diagnostic side band in addition to the small amplicon yielded by EOMTfw and EOMTrev. The PCR was adjusted for these EOMT based and consisted of an initial denaturation at 94 °C for 5 min. The actual amplification consisted of 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 61 °C, and 30 s elongation at 72 °C. The reaction terminated with a final elongation for 10 min at 72 °C, using a conventional *Taq* polymerase without a proofreading function (New England Biolabs, Frankfurt). All reactions proceeded in presence of 10 mg/ml of bovine serum albumin and 5 M betaine to buffer against plant phenolic compounds and unspecific amplifications. The resulting amplicons were later labelled with SYBRSafe (Invitrogen) and visualised on a 1.5% agarose gel after electrophoresis at constant voltage of 100 V, run for 30 min. A schematic representation of the primer position for the amplification of this multiplex PCR assay can be seen in Supplementary Fig. S1b.

Combined trait-related and trait-independent maker assay

As a third approach, we used the trait-related markers (*EOMT*) along with the trait-independent marker (*psbA-trnH intergenic spacer*) in a single multiplex PCR assay. To achieve that, a similar pair of EOMT targeted primers was designed, where the annealing temperature was adjusted to that of the primers targeting the *psbA-trnH intergenic spacer*. These modified primers were EOMTfw': 5'-TCCGGTCTATCCCTTCTGCC-3 and EOMTrev': 5'-CCGACGGCATCTTTGCATC-3'. The ARMS primer (EOMTrev$_R$) described for the trait-related marker assay was not used in the multiplex PCR. The conditions of this multiplex PCR were the same as those used for the *psbA-trnH* reaction described above. A schematic representation of the primer position for the amplification of the *psbA-trnH igs* together with the EOMT marker can be seen in Supplementary Fig. S1c.

Gas chromatography

Duplicates of the commercial samples described in Table 2 were ground to a powder in liquid nitrogen using a mortar and pestle. The powder was weighed and suspended in ethyl acetate (EtOAc) in a proportion of 1:5 (w/v). Samples were incubated in the dark overnight at 20 °C, shaking at 150 rpm. The extracts were filtered using Chromafil® PET-20/15 MS (Macherey–Nagel GmbH & Co. KG, Düren, Germany) and subsequently analysed by Gas Chromatography and Flame Ionisation Detection (7890B GC System, Agilent, Waldbronn, Germany), using an HP-5 nonpolar 30 m×0.32 mm×0.25 µm capillary column (Agilent). The carrier gas was helium at a flow rate of 1.5 ml·min^{-1}. The injected volume was 1 µl, and the split ratio 12.5:1. The run consisted of initial heating at 40 °C kept for 1 min. Then a ramp followed, where temperature rose with a pace of 5 °C·min^{-1}, to 60 °C, which was then kept for one further minute. Subsequently, a second ramp with 3 °C·min^{-1} followed to reach 170 °C, which was then kept for one min. Eventually, a third ramp with 30 °C·min^{-1} raised the temperature up to 270 °C, which was then kept for 5 min. A standard for ME (Sigma-Aldrich, Deisenhofen, Germany) was used to estimate the retention time of the compound.

Sanger sequencing and data analysis

Amplicons from reference plant material were purified using MSB® Spin PCRapace (STRATEC Molecular GmbH, Berlin) following the protocol of the provider. After suspension in 20 µl water, the DNA was sent for sequencing to Eurofins Genomics (Cologne, Germany). The raw sequence reads were edited using the software FinchTV version 1.4.0 (Geospiza Inc., Seattle, WA; Windows), BioEdit version 7.0.4.1 [33] and MEGA X: Molecular Evolutionary Genetics Analysis version 10.0.4 for larger datasets [34]. The alignment of the sequences was done using Muscle algorithm in MEGA X.

Results and discussion

Limitations using a trait-independent marker for authentication

As demonstrated in our previous work [14], it is possible to discriminate different species of *Ocimum* by a fingerprinting method, where the amplicon for the *psbA-trnH igs* marker yields different restriction patterns during a subsequent RFLP. The assay should yield a digested *psbA-trnH* amplicons of at 188 and 236 bp in *O. tenuiflorum* and the full-length *psbA-trnH* amplicon of 420 bp in other *Ocimum* sp. (Fig. 1A). To validate these results, we conducted this

assay with different accessions (Fig. 1B). In fact, the two tested accessions of *O. tenuiflorum* yielded the double band at around 200 bp inferred from the presence of a specific single-nucleotide polymorphism creating a restriction site for *Hinf* I. In contrast, this enzyme did not cut the amplicons from *O. basilicum*, *O. x africanum*, and *O. kilimandscharicum*, lacking this single-nucleotide polymorphism. In the latter case, a band at around 400 bp reported the non-digested amplicon. Likewise, an amplicon obtained from *Mentha x piperita*, was not digested by *Hinf* I. Thus, this fingerprinting method based on RFLP of a trait-independent marker (*psbA-trnH igs*) allows to differentiate between *O. tenuiflorum* and three other species of Basil that belong to the so-called haplotype I as defined in [14]. However, this protocol does not
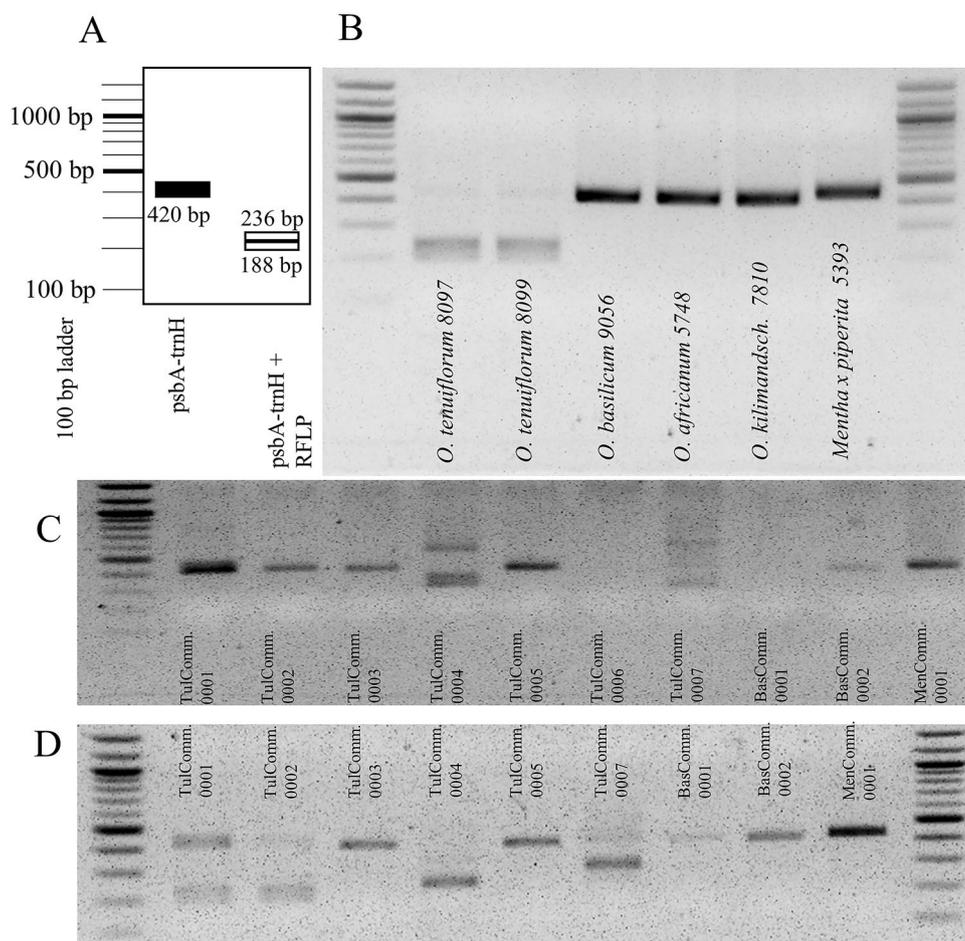
Fig. 1 Discrimination of *Ocimum tenuiflorum* based on RFLP of the *psbA-trnH* DNA barcode. **A** Banding RFLP pattern predicted from the sequence of the *psbA-trnH* marker in *O. tenuiflorum*. **B** Representative gel showing amplicates for *psbA-trnH* DNA barcode followed by digestion with *Hinf* I on different validated reference plants of *Ocimum* and *Mentha*. **C** Representative gel showing amplicates for *psbA-trnH* DNA barcode on commercial samples. **D** Representative gel showing digestion of amplicates for *psbA-trnH* DNA barcode with *Hinf* I on commercial samples shown in c sample TulComm.0006 was omitted in this assay. TulComm.0001: mix of different Holy Basils, tea; TulComm.0002: mix of different Holy Basils, tea; TulComm.0003: Holy Basil tea; TulComm.0004: Holy Basil mixed with other herbs, tea; TulComm.0005: Holy Basil mixed with other herbs, tea; TulComm.0006: Holy Basil mixed with other herbs, tea; TulComm.0007: Holy Basil mixed with other herbs, tea; BasComm.0001: dried basil; BasComm.0002: dried basil; MenComm.0001: mint tea. For the details of the declarations on these samples refer to Table 2

allow for discrimination in a single PCR. Moreover, while it allows to discriminate samples, identification still requires subsequent sequencing of undigested amplicons.

As a further drawback, this differentiation does not work, when *O. tenuiflorum* (haplotype II) is compared to species belonging to the same haplotype or to haplotype III [14]. For instance, the species *O. campechianum* and *O. gratissimum* produced the same pattern as *O. tenuiflorum*.

In the next step, we applied this RFLP approach to commercial samples (Fig. 1D). Only for samples TulComm.0001 and TulComm.0002 declared to contain different types of Tulsi as herbal tea, the *psbA-trnH* region was digested by the enzyme *Hinf* I and, thus, turned out to contain the content that was declared. All remaining commercial samples, supposedly containing Holy Basil, were not digested. Samples TulComm.0004 and TulComm.0007, declared as Tulsi tea, produced a band, which was smaller, but was not the double band characteristic for RFLP of *O. tenuiflorum*, and also a band that was significantly higher as the expected 420 bp (Fig. 1C). Commercial samples TulComm.0003, TulComm.0005 and TulComm.0007 were not digested in the RFLP, although the declaration claimed that they harboured Holy Basil. Sample TulComm.0006 produced no band at all, indicating potential DNA degradation problems due to excessive processing (Fig. 1C). Samples BasComm.0001 and BasComm.0002, declared as Sweet Basil, were not digested as expected. As to be expected, sample MinComm.0001, containing Mint tea as negative control for the RFLP produced only the uncut amplicon, as it should, since it lacks a restriction site for *Hinf* I. Thus, using RFLP, we could infer that samples TulComm.0003, TulComm.0004, TulComm.0005 and TulComm.0007 did not correspond to *O. tenuiflorum*. Although this technique allows us to discern *O. tenuiflorum* from other species in commercial samples, there are two main issues remaining. When handling a large amount of samples, a two-step protocol is time consuming, limiting economic feasibility. Therefore, one-step alternatives would be desirable, such as the Amplification-Refractory Mutation System (ARMS) approach. Here, a third primer would target the diagnostic Single Nucleotide Polymorphism (SNP), separating *O. tenuiflorum* from other species of *Ocimum*, yielding a second, smaller, amplicon for one species, but not for the other [25]. In the current case of the *psbA-trnH* region for *O. tenuiflorum*, when compared to other *Ocimum* species, this strategy was not amenable, since the diagnostic SNP localised to very AT-rich area, such that design of a destabilised ARMS primer is not possible. For these reasons, we have looked for alternatives, using a trait-related marker. The trait of interest here is the abundance of ME as potentially genotoxic compound.

Occurrence of methyleugenol in *Ocimum* sp.

ME is a phenylpropanoid naturally found in different plant species [35]. A literature review on the occurrence of this compound in *Ocimum* species (Table 3) reveals a large variation in content, even within a given species. Whether these differences derive from genetic factors (chemotypes), or from different environmental conditions during cultivation, is unknown in most cases. We had addressed this question in a previous study, where we raised different accessions of *Ocimum* under equal fluence rates of different light qualities [14]. When we investigated the resulting essential oils by high-performance thin-layer chromatography along with standards for eugenol and ME, it turned out that light quality did, indeed, modulate the abundance of compounds. However, the overall pattern was clearly under genetic control of the particular genotype. Since it is the genotype that defines the pattern of accumulated compounds (although the amplitude to which this pattern is expressed depends

Table 3 Literature data for methyleugenol (ME) contents in different species of *Ocimum*

Ocimum species	ME content[a] (%)	References
O. tenuiflorum L.	(–)	[36, 37]
	0.005	[38]
	0.5–3.1	[35, 36]
	20.1	[36]
	52	[35]
	56.18	[39]
	72.5–73	[36, 40]
	78–81	[35]
	92.4	[41]
O. basilicum L.	(–)	[36, 37, 40, 42]
	0.1–1.1	[40]
	0.29–0.3	[36, 39]
	5.6–12.3	[35]
	15.53	[37]
O. x africanum Lour.	(–)	[36, 37]
	0.1	[36]
O. americanum L.	(–)	[36, 37]
	0.02	[39]
O. gratissimum	(–)	[36, 37, 40, 41, 43]
	0.1–0.28	[36, 39]
	14.54	[44]
O. campechianum Mill.	(–)	[43]
	0.2–0.3	[43]
	9.5	[43]
	12	[44]
	60.6–69.5	[43]
	80–87	[45]
O. kilimandscharicum Gürke.	(–)	[37, 42]
	0.1	[40]
	53.9	[46]

[a]ME content: methyleugenol content from leaves oil/extracts

(–) Methyleugenol not present in the sample

also on environmental conditions), it is feasible to use DNA sequences to infer the presence of different amounts or even the absence of a particular compound. In our case, the enzyme responsible for the synthesis of ME, the EOMT, qualifies as a trait-related marker.

Using EOMT as trait-related marker for authentication

As shown in Table 3, ME is present in several *Ocimum* species, a compound produced by eugenol-*O*-methyl transferase (EOMT). We, therefore, used the sequence of the EOMT enzyme as trait-related marker and, thus, to get a better resolution beyond that of the neutral markers, such as *psbA-trnH igs* (Fig. 1D). The idea was to develop an ARMS assay, where more than one SNP would allow for a more robust differentiation of the diagnostic ARMS primer. This should yield a diagnostic second band at 197 bp in addition to the full-length amplicon of 418 bp (Fig. 2A). This second band would then confirm the presence of *O. tenuiflorum* in the sample. When we probed 13 validated reference accessions for *Ocimum* (Fig. 2A), we could detect this second band in all five tested accessions of *O. tenuiflorum*, while all *Ocimum* accessions belonging to haplotype I in sensu [14], produced only the full-length amplicon. However, *O.*

gratissimum and *O. campechianum* (representing haplotypes III and II clustering into the neighbourhood of *O. tenuiflorum*) also showed the smaller band at 197 bp.

When commercial samples declared to contain Holy Basil were analysed by this fingerprinting assay based on the trait-related marker EOMT (Fig. 2C), the results were less clear as for the reference material. Samples containing Holy Basil should have two amplicons, one at around 400 bp representing the full-length amplicon, and one at around 200 bp, representing the side band resulting from the binding of the diagnostic ARMS primer. However, only four of the tested ten samples produced a clear band at all. In those, the resulting band was around 200 bp, presumably corresponding to the diagnostic side band reporting binding of the ARMS primer. In contrast to the prediction, we failed to detect the large band of around 400 bp in any of the samples, which is limiting the validity of the assay. The most straightforward explanation is the degradation of the DNA during processing of the commercial products, such that the abundance of full-length template is not sufficient to drive the amplification by PCR. The shorter diagnostic ARMS fragment should be less prone to this problem, such that in some samples the corresponding diagnostic band is amplified, while the full-length band is absent. Thus, the EOMT
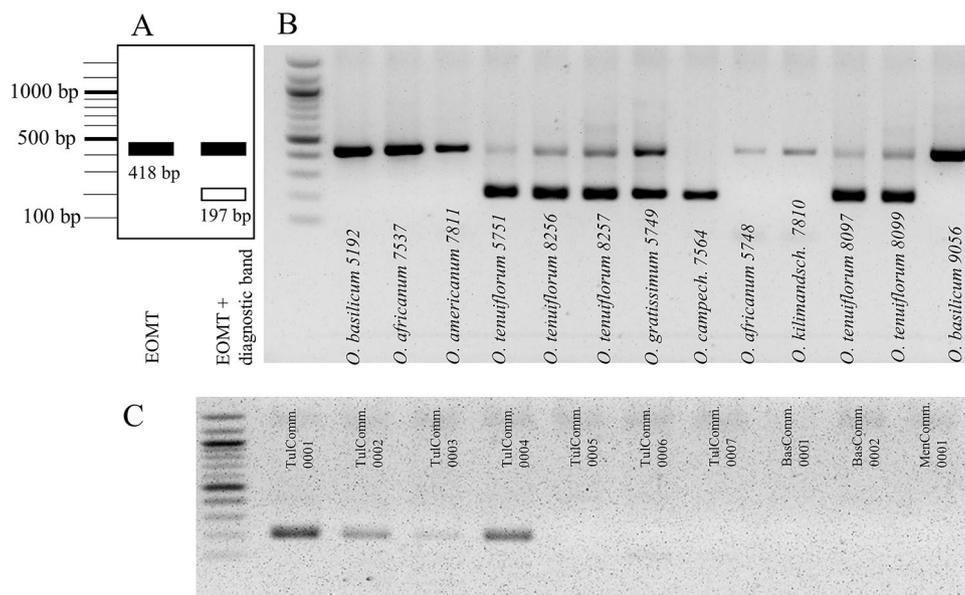
Fig. 2 Discrimination of *Ocimum tenuiflorum* based on the trait-related eugenol *O*-methyltransferase (EOMT) using a duplex ARMS strategy. **A** Predicted pattern for *O. tenuiflorum*. **B** Representative gel showing amplificates obtained for a core-collection of validated reference plants for *Ocimum*, where several informative SNPs in *O. tenuiflorum* allow for binding of a diagnostic primer, such that a smaller band at around 200 bp appears. **C** Representative gel showing amplificates for the trait-related marker EOMT for commercial Basil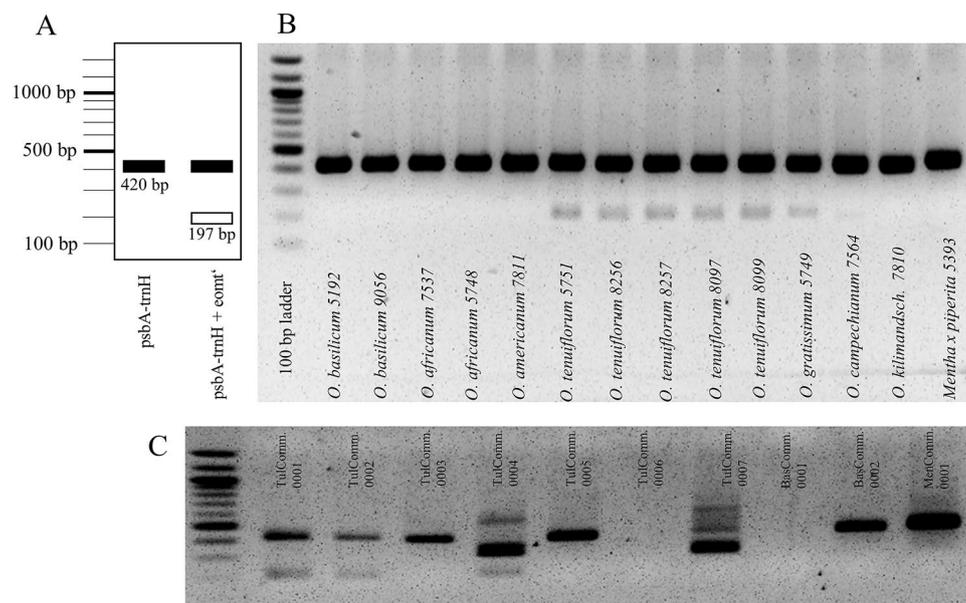 products and Mint as outgroup. TulComm.0001: mix of differ-ent Holy Basils, tea; TulComm.0002: mix of different Holy Basils, tea; TulComm.0003: Holy Basil tea; TulComm.0004: Holy Basil mixed with other herbs, tea; TulComm.0005: Holy Basil mixed with other herbs, tea; TulComm.0006: Holy Basil mixed with other herbs, tea; TulComm.0007: Holy Basil mixed with other herbs, tea; BasComm.0001: dried basil; BasComm.0002: dried basil; MenComm.0001: mint tea. For the details of the declarations on these samples refer to Table 2

marker performs less efficiently in commercial samples, which is a drawback with respect to the trait-independent *psbA-trnH igs* marker, where most tested samples produced a result (compare Fig. 1D and Fig. 2C), although the size of the amplicons is comparable (around 400 bp). The reason for this poor performance might be the fact that EOMT as nuclear gene is present only in two copies, while the plastidic *psbA-trnH igs* occurs in numerous copies, such that sufficient template is available even under conditions of partial degradation due to product processing. The absence of the full-length amplicon leads to a fundamental problem, though: the absence of any bands might either imply that the respective sample does not contain EOMT at all, or not a EOMT with the informative SNP reporting the *tenuiflorum*, or that the integrity of the DNA is not sufficient to even yield the smaller side band. Thus, the absence of the diagnostic amplicon does not allow the conclusion that the sample is not Holy Basil. The DNA degradation obviously hampering the analysis in commercial samples bears the risk of numerous false-negative results. In other words, the use of EOMT as trait-related marker creates a problem of "over-specificity". Thus, this trait-related marker, although showing a clear outcome for reference plants, is misleading and does not offer a proper identification when used in commercial samples. To overcome this shortcoming, we asked in the next step, whether combining the trait-independent plastidic *psbA-trnH igs* marker with the trait-related EOMT marker in a multiplex PCR would render the assay more robust against the impact of DNA degradation.

Multiplexing trait-related and trait-independent makers

We kept using the plastidic DNA barcode as trait-independent marker and tailored the previous trait-related marker primers (EOMTfw' and EOMTrev'), to ensure that the annealing temperature was fitting all set of primers. All reference plants listed in Table 1 were analysed to verify, whether, even when a sample does not possess EOMT, in this case Mint, the plastidic marker can report at least the success of the amplification as such. This should yield the full-length *psbA-trnH* amplicon of 420 bp and a diagnostic second band representing the EOMT' marker at 197 bp (Fig. 3A). As shown in Fig. 3B, all samples produced a distinct band at around 400 bp, corresponding to the expected size of the *psbA-trnH* amplicon. In addition, a second band of around 200 bp, corresponding to the expected size of the diagnostic EOMT amplicon, became visible in all tested *O. tenuiflorum* accessions along with *O. gratissimum*. This

Fig. 3 Discrimination of *Ocimum tenuiflorum* based on the enzyme eugenol *O*-methyltransferase as trait-related marker (EOMT') and the trait-independent marker *psbA-trnH*. **A** Predicted pattern for *O. tenuiflorum*. **B** Representative gel showing amplificates obtained for a core-collection of validated reference plants for *Ocimum* along with *Mentha x piperita* as outgroup. **C** Representative gel showing amplificates of the trait-related EOMT and the trait-independent marker *psbA-trnH* for commercial Basil products and Mint as outgroup. TulComm.0001: mix of different Holy Basils, tea; TulComm.0002: mix of different Holy Basils, tea; TulComm.0003: Holy Basil tea; TulComm.0004: Holy Basil mixed with other herbs, tea; TulComm.0005: Holy Basil mixed with other herbs, tea; TulComm.0006: Holy Basil mixed with other herbs, tea; TulComm.0007: Holy Basil mixed with other herbs, tea; BasComm.0001: dried basil; BasComm.0002: dried basil; MenComm.0001: mint tea. For the details of the declarations on these samples refer to Table 2

band was also detectable, however very faintly, in *O. campechianum*. The outgroup *M. x piperita*, supposedly void of ME, only yielded the 400-bp band. Thus, the result from this multiplex approach was consistent with the results from RFLP (Fig. 1B) and from ARMS on the EOMT marker (Fig. 3B) since it was delineating *O. tenuiflorum*, *O. gratissimum*, and *O. campechianum* from the other Basils, especially *O. basilicum*.

When transferring this duplex PCR strategy to commercial samples (Fig. 3C), we could see the putative *psbA-trnH* amplicon at around 400 bp in the majority of samples, although sample TulComm.0006 and BasComm.0001 did not produce an amplicon, as already predicted from Fig. 1C. In sample TulComm.0004, supposedly containing Holy Basil in a mixture with other herbs, several bands were observed in this range, one strong band that was significantly smaller as 400 bp, some weaker of a size larger than 400 bp. These might stem from the other, non-declared herbs that overlay with the amplicon from Holy Basil. However, sample Tulcomm.0007 has a similar case and the band at around 400 bp is well observed. The smaller band at around 200 bp, reporting the presence of *O. tenuiflorum* appeared for samples TulComm.0001 and TulComm.0002, matching the RFLP results (Fig. 1D). These samples seem to contain, indeed, *O. tenuiflorum*. Likewise, sample TulComm.0004 produced this diagnostic band, which in this case was inconsistent with the RFLP result (Fig. 1D). One might speculate that an unspecific amplification occurred given the presence of other herbs that are ME producers. This band was absent from the other samples, although TulComm.0003, TulComm.0005 and TulComm.0007 were declared to contain Holy Basil. Also for the samples that just contained a

non-specified type of Basil, and in the RFLP had turned out to be from haplotype I, as well as for the Peppermint sample, the diagnostic 200-bp band was absent. Hence, the coupled trait-independent marker *psbA-trnH*, together with the trait-dependent marker based on the EOMT in a multiplex PCR, presents a plausible alternative for *O. tenuiflorum* identification. The results of this extended assay were consistent with those for the RFLP (Fig. 1D) but did not require an additional restriction step. Moreover, many samples that had not yielded any results for the *EOMT* marker alone (Fig. 2C), became accessible by this multiplex PCR approach.

Methyleugenol in commercial samples: the EOMT marker is only for authentication

Our trait-related marker, based on the EOMT, targets *O. tenuiflorum*, which is mainly a ME producer. However, ME occurrence and accumulation vary among and within *Ocimum* species (Table 3). Moreover, processing of plant material can contribute to metabolite degradation. Therefore, we analysed the commercial samples for their content of ME (Table 4, Supplementary Fig. S2). From the commercial samples that were declared to contain Holy Basil, all samples contained ME, though only TulComm.0001 and TulComm.0002 exhibited more than trace amounts (Table 4, Fig. 4). The same samples were positive for the trait-related *EOMT* molecular marker and in the RFLP had displayed the presence of the SNP diagnostic for *O. tenuiflorum*. These results suggest that this trait-related marker reliably identifies ME chemotypes of *O. tenuiflorum*, even in dried processed plant material sold as tea/herbal mixtures. Our study showed as well that samples declared to

Table 4 Comparison of three DNA-based authentication assays results from commercial samples and the methyleugenol (ME) content in the samples' extracts detected by Gas Chromatography

Samples	Trait-independent marker assay	Trait-related marker assay	Combined trait-independent and trait-related marker assay	ME content[a] %
TulComm.0001	+	/	+	11.58%
TulComm.0002	+	/	+	10.20%
TulComm.0003	−	/	−	t.a
TulComm.0004	−	/	−	t.a
TulComm.0005	−	/	−	t.a
TulComm.0006	/	/	/	t.a
TulComm.0007	−	/	−	t.a
BasComm.0001	−	/	/	5.36%
BasComm.0002	−	/	−	6.66%
MenComm.0001	−	/	−	(−)

The DNA-based authentication results from the trait-independent marker assay, trait-related marker assay and combined trait-independent and trait-related marker assay are considered positive (+) when authentication patterns in commercial samples correspond with *O. tenuiflorum*, negative (−) when the authentication pattern does not correspond with *O. tenuiflorum*, and deficient (/) when a proper result interpretation was not possible. For the details of the declarations on the samples refer to Table 2

[a]ME content: percentage from total chromatogram area. *t.a* trace amounts

(−) Methyleugenol not present in the sample
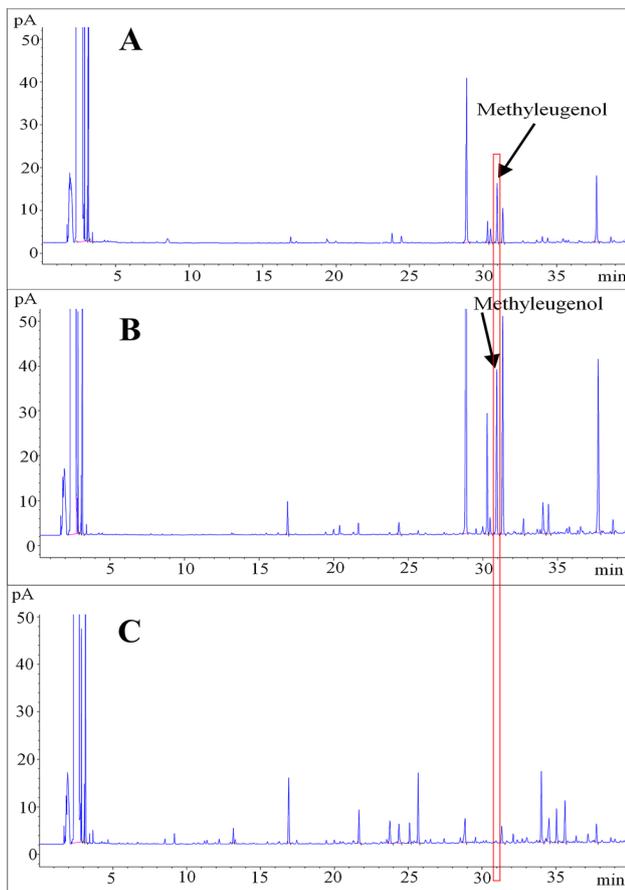
Fig. 4 Representative Gas Chromatogram of selected commercial samples aiming to the detection of methyleugenol (ME). **A** Chromatogram showing the peak of ME in sample TulComm.0001: mix of different Holy Basil, tea. **B** Chromatogram showing the peak of ME in sample TulComm.0002: mix of different Holy Basil, tea. **C** Chromatogram showing trace amounts of ME in sample TulComm.0005: Holy Basil mixed with other herbs, tea. For the details of the declarations on these samples refer to Table 2

contain an unspecified species of Basil (BasComm.0001 and BasComm.0002) contained also substantial amounts of ME, although these samples did not exhibit digestion in the RFLP and, thus, were not clustering with *O. tenuiflorum* (Fig. 1D). Thus, the absence of the diagnostic band of the EOMT marker is no guarantee that the respective sample is void of ME. This false-negative result shows that, while the EOMT can be used to authenticate *O. tenuiflorum*, it cannot be used to safeguard against ME. Likewise, the authentication of a sample as *O. tenuiflorum* can be false positive with respect to an inferred presence of ME as shown by the samples TulComm.0001-0007. Thus, the association of the EOMT marker with the trait (accumulation of ME) is not sufficiently tight to use this marker as predictor for ME content. However, it is well suited for authentication of *O. tenuiflorum*.

Optimisation and outlook: multiplexing trait-related and trait-independent makers

For the identification of Holy Basil in commercial samples, our starting point was a method based on a trait-independent marker largely used in several species, the *psbA-trnH igs,* coupled with a restriction enzyme step. This led us to optimise the identification process to both maximise resources and to trace the toxicologically relevant compound ME. The criteria for optimising DNA-barcoding was established by answering three questions: (1) Does this method has a "one-step" reaction? (i.e. PCR). (2) Does this method include a positive control that will allow us to detect technical problems? (3) Does the new method discriminate samples with a resolution comparable to those used previously? and (4) Does this method allow to trace specific chemotypes? Based on these questions, we have developed the multiplexing trait-related and trait-independent marker method for Holy Basil.

Further, even though this process was targeted to authenticate Holy Basil in the food industry, the primers developed here can be similarly used in other scenarios. Here we present three:

Holy basil in ayurveda

As mentioned before, *Vitex negundo* can also be a surrogate for *O. tenuiflorum*, although admitted only for pharmaceutical application, not for use as food product. This surrogate species does not contain ME [47–50]. Therefore, the multiplex PCR developed here can discriminate *O. tenuiflorum* from *V. negundo*.

Oregano vs *Thyme*

Oregano adulteration by Thyme and other herbs has become an issue in food fraud [51]. As the Basils, both taxa harbour ME. We, therefore, applied the multiplexing PCR to Oregano and Thyme (Supplementary Fig. S3). We analysed reference plants from the KIT Botanical Gardens of *Origanum* sp. (Oregano), along with *Thymus* sp. (Thyme)*,* and *Satureja* sp. (Savory)*,* reported to be used as surrogates of Oregano. All reference material produced a *psbA-trnH igs* band between 400 and 500 bp, and a band for EOMT at around 800 bp. This result does not allow the discrimination between the named species, however, highlights the potential for developing new primers based on the EOMT amplicons. Subsequently, commercial samples that were declared to contain either Oregano or Thyme were tested. Here, in most cases both bands were present, with one exception of a commercial sample presenting a band at around 200 bp, suggesting that this sample consists of plant material of completely different identity. Since the success of the PCR depends on the size of the amplicon, especially in processed samples,

where DNA is often partially degraded, this multiplex PCR should be tailored in future studies for diagnostic amplicons of smaller size, based on the informative region of the diagnostic EOMT band. However, even without this optimisation, the results show the potential to develop a simple assay for a more reliable discrimination between Oregano and Thyme.

Real-time PCR

The combined trait-independent and trait-related assay could be adapted to be used in real-time PCR. This might allow a more well-defined result than gel visualization, potential for quantification via calibration curve of DNA concentration and Ct values, and assessment of mixture samples by analysing the melting curve [52].

Conclusions

We were able to obtain a DNA-based authentication method to verify commercial samples declared as Holy Basil or Tulsi (*O. tenuiflorum*) based on a duplex PCR combining a specific enzyme, EOMT, with the plastidic marker *psbA-trnH igs*. This assay allows to pre-screen large numbers of samples in a single step to narrow down the population to be authenticated by sequencing, for the purpose to verify the declared content. The method has the potential to be extended to other herbal compounds and facilitates systematic surveillance of commercial products as a contribution to improved consumer safety. However, the assay cannot be used to infer statements on the content of ME.

Declarations

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