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ORIGINAL PAPER

Development and validation of microscopical diagnostics for 'Tulsi' (*Ocimum tenuiflorum* L.) in ayurvedic preparations

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Abstract During recent years, ayurvedic plants have entered the European market as a novel food trend. This confronts food analytics with the task to assess the composition of exotic and often unknown herbal preparations in teas or spices. Using the trend plant 'Tulsi' (Holy Basil, *Ocimum tenuiflorum* L.) as model, we developed microscopical diagnostics on markers that can be reliably assessed in dried or even fragmented specimens as typically occurring in commercial ayurvedic preparations, where DNA extraction is difficult. First, a reference for 'Tulsi' was defined based on the plastidic internal transcribed spacer (*ITS*) as marker. Second, this reference was morphologically delineated from other *Ocimum* accessions potentially used as surrogates for 'Tulsi' (such as *O. basilicum* L.) leading to a microscopical assay based on the density of glandular scales and glandular hairs, the epidermis with trichomes and the cells of the palisade parenchyma. Third, this assay was statistically validated for its ability to discriminate surrogate species from true *O. tenuiflorum*. First applications of this assay on commercial 'Tulsi' products demonstrated a high frequency of surrogate additions.

Keywords Basil · Detection method · Internal transcribed spacer (*ITS*) · *Ocimum tenuiflorum* L. · 'Tulsi' · Validation

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Introduction

During recent years, ayurvedic medical and spice plants have entered the European market as functional food. A prominent example for this trend is the Holy Basil 'Tulsi' (*Ocimum tenuiflorum* L.), a plant central for Indian Ayurveda, esteemed for its beneficial effects on general constitution and health [1]. 'Tulsi' is administered against headache, rheumatic pains and arthritis, but also malaria, fever and allergies. Ancient ayurvedic scriptures describe a protective effect of 'Tulsi' against insect bites and recommend to use this herb for air-cleaning [2]. The traditional use of 'Tulsi' has been supported by scientific evidence for antioxidant and detoxifying effects [3], as well as antibacterial, antiviral, and antifungal activity of oils extracted from 'Tulsi' [4]. In addition, it seems to alleviate the symptoms of *Diabetes mellitus* [5]. These medical implications stimulated the marketing of different ayurvedic preparations that are promoted in Europe under the designation 'Tulsi' and are usually distributed as dried tea or spice mixtures.

The prices that can be achieved by such ayurvedic preparations, the limitations of supply for this exotic herb, and the difficulty to reliably address 'Tulsi' in food diagnostics provide ideal conditions for the spread of surrogate preparations mostly consisting of conventional Basil (*Ocimum basilicum* L.). The situation is even further complicated by the fact that popularized descriptions of ayurvedic cuisine designate also an East Asian accession of *O. basilicum*, the so called 'Thai Basil', as 'Tulsi'.

A central task of food monitoring is to safeguard consumers against deception and misdirection [6]. The non-standardized nomenclature in combination with the general use of 'Tulsi' as dried herbal mixture poses special challenges to food monitoring. So far, microscopic diagnostics has been the most reliable way to test multicomponent

specimens such as the typical preparations of 'Tulsi', because it is very difficult to extract DNA of sufficient amount and quality for molecular assays. There exists a wealth of technical literature that describes and illustrates food plants commonly used in Europe, e.g. [7] to assist microscopical diagnostics. However, for novel foods, such as 'Tulsi', this type of information is completely lacking. This is not surprising—diagnostic assays of 'Tulsi' have not been in the focus of ayurvedic literature that mainly deals with the medical and beneficial effects of this herb.

To bridge this gap, we ventured to develop a diagnostic assay to reliably address 'Tulsi' in commercial preparations, i.e. in dried mixtures with other plant material. For this purpose, we first had to identify reference specimens by means of molecular markers. We were then able to define morphological markers that allow discrimination between 'Tulsi' and other accessions of *Ocimum* that are potentially used as surrogates. Eventually, we were able to validate this microscopical assay statistically for its ability to discriminate surrogate species from true *O. tenuiflorum* and we could show that is sufficiently robust to be amenable for microscopical diagnostics even in commercial dried spice and tea preparations of 'Tulsi'.

Materials and methods

Plant material and samples

Habitus and ingredients of basil plants are highly variable, dependent on environmental factors such as light conditions, temperature or substrate [8]. Therefore, specimens for the different accessions were raised from seeds and cultivated in parallel under identical conditions (substrate Floraton 3, day temperature 18–25 °C, night temperature 15 °C, illumination time 10 h) in the Botanical Garden of University of Karlsruhe for the macroscopic and microscopic analysis of morphology, and for the analysis of genetic markers. The following accessions were used in this study: a commercial accession for *O. tenuiflorum* (Rühlemanns, Horstedt, Germany, *accession 1*); a commercial accession for *O. tenuiflorum* (Rühlemann, Horstedt, Germany, *accession 2*); a commercial accession for *O. basilicum* cv. 'Genoveser' (Rühlemann, Horstedt, Germany, *accession 3*); a commercial accession for *O. basilicum* × *citriodorum* (Rühlemann, Horstedt, Germany, *accession 4*); an accession termed '*O. tenuiflorum*' from the Botanical Garden Bayreuth (later identified as *O. serratum*, *accession 5*); an accession termed '*O. tenuiflorum*' from the Botanical Garden Göttingen (later identified as *O. serratum*, *accession 6*); a commercial accession for *O. gratissimum* (Rühlemann, Horstedt, Germany, *accession 7*); a commercial accession for Thai basil, *O. basilicum* cv. 'Siam Queen' (Rühlemann, Horstedt, Germany, *accession 8*); and an accession for *O. tenuiflorum* from the national crop plant collection at the Institute for Crop Plant Research in Gatersleben (accession *Oci152*). All accessions were taxonomically verified by a morphological key [9] and the molecular *ITS* marker (see below), and are kept as references in the Botanical Garden of the University of Karlsruhe.

Extraction of genomic DNA

Fresh leaf material (third leaf pair counted from the apex) was harvested from healthy plants. About 80 mg of the sample was transferred into a reaction tube (1.5-ml safe lock, Eppendorf) together with five glass beads (2 mm diameter, Roth) and shock frozen in liquid nitrogen. The frozen sample was then ground four times for 15 s using a universal dental mixer (Silamat S5, ivoclar vivadent). After each individual grinding step, the sample was returned for 30 s into liquid nitrogen to ensure that the powder did not thaw during the extraction. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hildesheim) following the protocol of the producer using an additional washing step of the columns prior to elution to ensure complete removal of the phenolics that are abundant in *Ocimum*. The concentration of the eluted DNA was determined photometrically (NanoDrop ND-100, peqlab). The E_{260}/E_{280} of the extracted DNA was between 1.7 and 1.9. The quality of the DNA-extracts was controlled by electrophoresis on a 1-% agarose gel supplemented with 5% v/v of the fluorescent dye SYBR Safe (Invitrogen).

PCR-amplification of ITS sequence

The internal transcribed sequence (*ITS*) marker was amplified by PCR following the protocol of Eckelmann [8], whereby the two *Ocimum* markers, *ITS1* and *ITS2*, were amplified separately from 20 ng of genomic DNA as template in a semi-nested PCR reaction [10] using the primer pairs Ah (F) and Ch (R) for the *ITS1* marker, and the primer pair Dh (R) and Bh(F) for the *ITS2* marker [8] with annealing at 62 °C for *ITS1* and 65 °C for *ITS2*, and 25 cycles for the first reaction, and 30 cycles for the second reaction. The amplificates were separated by electrophoresis in a 1.8% agarose gel and the correct size determined using a Eco471-AvaII digested λ DNA (Fermentas, Wilnius) and fluorescent staining with SYBR Safe (Invitrogen). The amplificate band were excised from the gel and extracted using the NucleoSpin® Extract II kit (Machery-Nagel, Karlsruhe) following the protocol of the producer, and sequenced (GATC Biotech, Konstanz). For each accession the *ITS1* and *ITS2* sequences from two independent DNA-samples were sequenced and assembled and edited for sequencing

artefacts using the SeqMan (http://www.dur.ac.uk/stat.web/Bioinformatics/seqman.htm) software.

Phylogenetic analysis of the ITS sequence

The ITS-sequences were automatically aligned using the ClustalX software (http://www.clustal.org/) and saved in the nexus-format. This automatic alignment was then edited using the SeaView software (http://pbil.univ-lyon1.fr/software/seaview.html) and the respective ITS1- and ITS2-sequences were then joined by the SubEthaEdit software (http://www.codingmonkeys.de/subethaedit/index.de.html) such that they could be merged into the pre-existing alignment for the genus *Ocimum* (Eckelmann, 2002) that was kindly provided by Eckelmann (University of Kassel). The phylogenetic trees were then calculated from this assembled alignments using the PAUP (http://paup.csit.fsu.edu/) software and visualized by the TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) software. Trees were constructed using by the neighbour-joining, the maximum parsimony, and the maximum likelihood, respectively, and were then subjected to a bootstrap analysis using the PAUP programm.

Light microscopy

Flowers, leaves, and shoots of all specimens were documented macroscopically (Exilim Z750, Casio), and by a stereo microscope (M420, Leica; Bensheim) equipped with a digital camera (DFC 500, Leica; Bensheim) both in the fresh state and after drying. In addition, tangential hand sections from the adaxial and the abaxial surface of leaves were brightened with 60% chloral hydrate and then analysed under a light microscope (Axioskop, Zeiss; Jena) equipped with a digital image acquisition system (AxioCam, Zeiss; Jena). For quantification of microscopic traits, leaf discs from the uppermost five leaves of 12 mm diameter were obtained using a cork-borer on an elastic rubber pad. After brightening with 60% chloral hydrate under short heating, epidermal cells, glandular scales, and glandular hairs were counted using an objective with 20× magnification corresponding to a visual field of 113 mm^2.

Quantitative analysis of dried preparations

Since commercial preparations of 'Tulsi' consist of dried material, usually as mixture with other components, the quantitative analysis of glandular scale density had to be validated with respect to its performance in dried leaf material and in dependence of particle size. For this analysis, 200 mg of dried *O. basilicum* cv. 'Genoveser' were mixed with either *O. tenuiflorum* or with *O. basilicum* cv. 'Siam Queen' in various ratios and then successively sieved through standardized sieves (proof sieves, German industrial norm DIN 4188) with mesh widths of 710, 630, 250, and 180 μm. During sieving, the material was carefully ground by a pistil to enhance passage. The different fractions were then brightened by 60% chloral hydrate and then analysed by light microscopy. Due to density of glandular scales (in case of *O. tenuiflorum* as analyte) or glandular hairs (in case of *O. basilicum* cv. 'Siam Queen' as analyte), each particle could be either assigned to the analyte or to *O. basilicum* cv. 'Genoveser'. The recovered proportion of analyte particles in comparison to the weighed proportion of analyte in the initial mixture was taken as measure for the validity of the assay.

Statistical validation

The determined values for the densities of glandular scales and glandular hairs, respectively, where averaged over 20 individual leaf discs. The values for each accession were then statistically tested against *O. basilicum* cv. 'Genoveser' as reference using a *t*-test. For the analysis of commercial samples declared to contain *O. tenuiflorum*, the particles were sieved as described above, and then the ratio of epidermal cells per glandular scales were averaged over 20 independent assays. Only the epidermis cells of the adaxial leaf surfaces were counted. The differences between the accessions were first tested by the non-parametrical test of Kruskal and Wallis, and after the differences had been found to be highly significant, the differences of the individual accessions with respect to a *O. tenuiflorum* standard were tested for their significance using a *t*-test.

Results and discussion

Definition of a reference for 'Tulsi' based on molecular phylogeny

The *ITS1* and *ITS2* sequences were isolated for eight accessions from the genus *Ocimum*, among them were five accessions for 'Tulsi' (*O. tenuiflorum*), one accession for *O. basilicum* cv. 'Genoveser', one accession for *O. basilicum* cv. 'Thai', one accession for *O. basilicum* × *citriodorum*, and one accession for *O. gratissimum* (Fig. 1). These accessions were merged with the pre-existing phylogenetic data for the genus [8]. Irrespective of the approach to construct the phylogeny (neighbour-joining, maximum parsimony, or maximum likelihood), both cultivars of *O. basilicum*, as well as *O. basilicum* × *citriodorum*, and *O. gratissimum*, were found to be located at the position predicted by the phylogenetic tree constructed by Eckelmann [8]. In contrast, the four accessions for 'Tulsi' were split into two groups—whereas the two commercial *accessions 1* and *2*

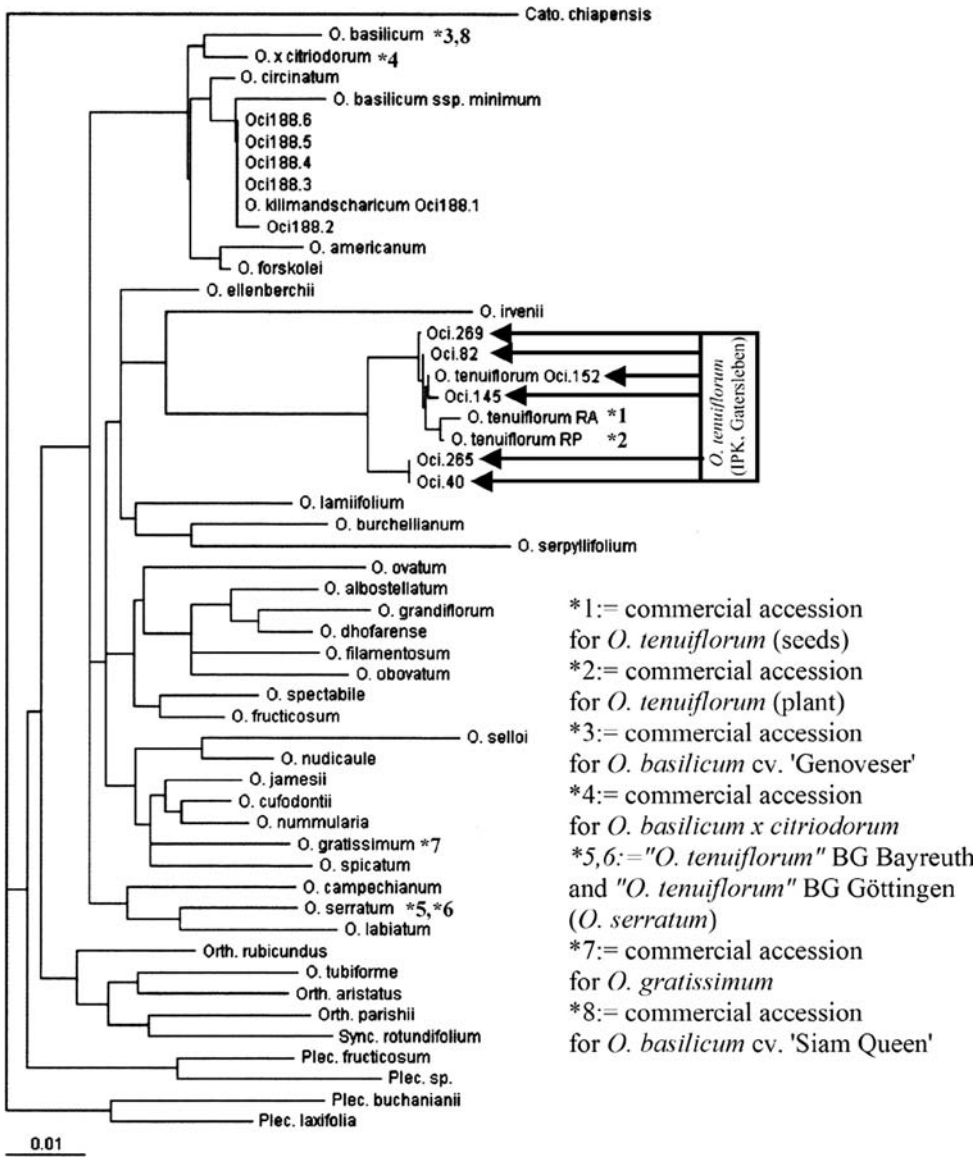

Fig. 1 Phylogenetic position of the plant accessions used in this study inserted into a neighbour-joining tree for the *Ocimum ITS1* and *ITS2* markers calculated from a merged alignment from the data by Eckelmann [8] with the sequences obtained from the accessions listed in the figure

clustered together with the reference from the Gatersleben gene bank (Oci152) into the *O. tenuiflorum* group, the *accessions 5* and *6* that had been obtained as '*O. tenuiflorum*' from the Botanical Gardens of the Universities of Bayreuth and Göttingen were found to contain *ITS* sequences identical to those of *O. serratum*. Moreover, the morphology of *accessions 5* and *6* differed from those of *accessions 1* and *2*, and the reference accession *Oci152*, and was later determined to be identical to that of *O. serratum* using a morphological key [9]. This means that of the five available accessions of *O. tenuiflorum*, three (*accessions 1*, *2*, and *Oci152*) could be confirmed to be true *O. tenuiflorum*, whereas two (*accessions 5* and *6*) had been incorrectly defined and represented accessions of a different species, namely *O. serratum*. For the further analysis we therefore used the *accessions 1*, *2*, and *Oci152* as reference lines for 'Tulsi'.

Morphological and histological characteristics of 'Tulsi' as compared to other Basil species

The accessions 1 and 2 that had been verified to be true *O. tenuiflorum* were characterized macro- and microscopically. The plants reached 30-50 cm in height and were strongly branched, shoots were erect, hairy and lignified at their base, phyllotaxis was opposite–alternate, petioles 10–20 mm in length, leaves ovoid in shape, about 50 mm in length, and 30 mm in width. The whole leaf lamina as well as the leaf veins was covered by soft hairs, the leaf base was triangulate and slightly rounded, the leaf margin slightly serrated, the leaf tip slightly rounded. The inflorescence was organized in hexafloral pseudo-whorls, about 60–100 mm in length, with acropetally progressively stunted internodes. The stalk of individual flowers were 3 mm in length, and accompanied by ovoid bracts of

4–10 mm length, and 3 mm width. Petals were bell-shaped, bilabiate and about 3 mm in length with a distinct dorsiventrality. Their colour was light violet or even white. The calyx was 4 mm in length and 2–4 mm in width, stamina were protruding 2–4 mm, and the rear filaments were covered with hairs at their base. Seeds were light brown, ovoid, 0.6 mm in length, and 0.4 mm in width, their surface appeared glandulate.

On the adaxial and abaxial leaf surfaces of all *Ocimum* accessions glandular scales and hairs could be observed. Depending on the expansion of the leaf, these scales could differ in size, but maintained their characteristic pattern of four cells (Fig. 2b). In addition, glandular hairs were present (Fig. 2a) mostly consisting of two cells. As compared to *O. basilicum* cv. 'Genoveser' (Fig. 2f), the size of palisade or spongy parenchyma cells in relation to the epidermal cells was distinctly smaller in *O. tenuiflorum* (Fig. 2e). In that respect, *O. tenuiflorum* resembled *O. gratissimum* (Fig. 2g). However, *O. gratissimum* could be easily discriminated from *O. tenuiflorum* by its pronouncedly lobate epidermal cells (compare Fig. 2g, e). In addition, *O. tenuiflorum* produced long unbranched trichomes with 3–5 cells on both the upper and lower leaf surfaces (Fig. 2c, d), similar to *O. gratissimum.* In contrast, in *O. basilicum* cv. 'Genoveser' and *O. basilicum* cv. 'Siam Queen' only a few short trichomes were observed along the leaf margins. A survey of diagnostic characteristics discernible by light microscopy is shown in Table 1.

The most prominent trait of *O. tenuiflorum* that clearly separated it from all other accessions investigated in this study with exception of *O. gratissimum*, was the high density of glandular scales that became already manifest by inspection under a stereo microscope (Fig. 3). Whereas in the other accessions the ratio of adaxial epidermal cells per individual glandular scale ranged between 150 (*O. serratum*) till 280 (*O. basilicum* cv. 'Siam Queen'), it was less then 60 in all three true accessions of *O. tenuiflorum*. We tested also other cultivars of *O. basilicum* and observed values ranging up to 345 (in case of *O. basilicum* cv. 'Dark Opal', data not shown). The only other accession with such a low ratio was *O. gratissimum*, but as pointed out above, it is histology distinct by the pronounced lobation of epidermal cells (compare Fig. 2g, e).

A statistical analysis of the differences using the test by Kruskal and Wallis (the non-parametrical version of ANOVA) showed that the variance was completely attributable to taxonomic differences, whereas intraspecific variance (between the three accessions of *O. tenuiflorum*) was negligible (data not shown). When the individual accessions were then compared to *O. tenuiflorum* by pairwise *t*-tests (data not shown), the values for *O. gratissimum* were found not to be significantly different from those for *O. tenuiflorum*, whereas the values for all other accessions were different from those for *O. tenuiflorum* at the $P > 0.99$ level.

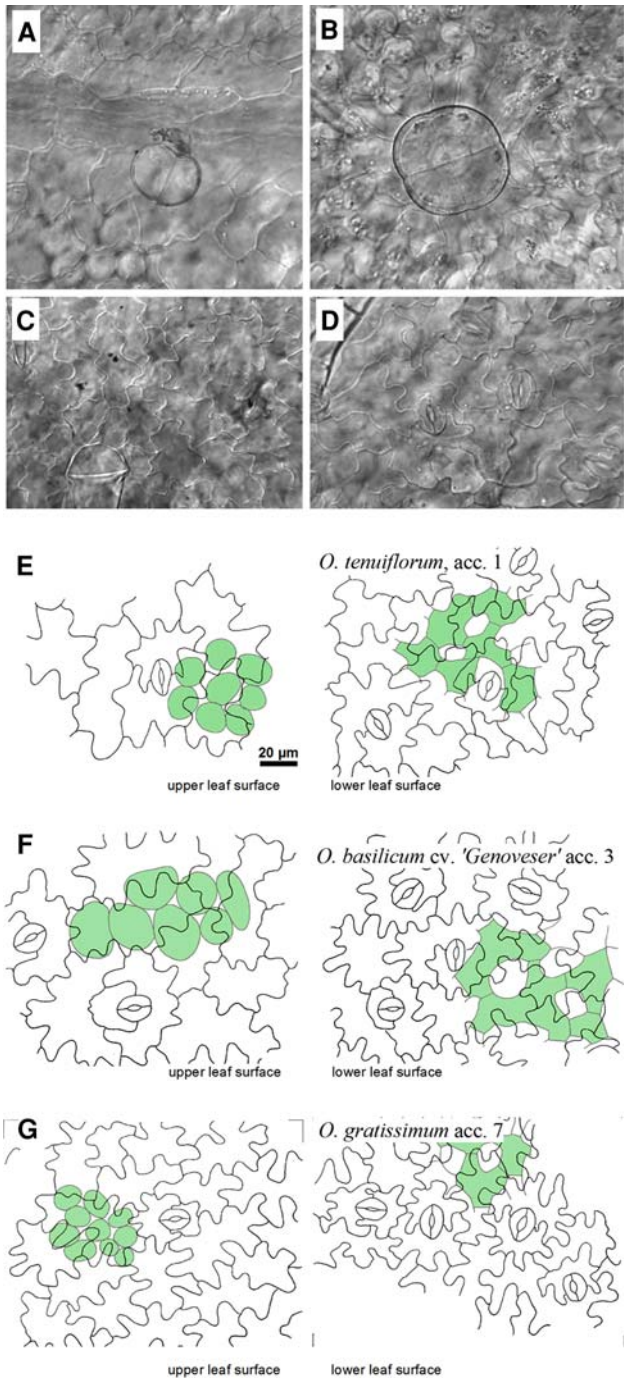

Fig. 2 Morphological traits relevant for the identification of *O. tenuiflorum* as compared to other Basil species. Glandular hair (**a**) and glandular scale (**b**) of *Ocimum* spp., adaxial epidermis (**c**), abaxial epidermis (**d**) of a dried leaf of *O. tenuiflorum* (600×). **e**–**g** Relation between epidermal pavement cells and subtending mesophyll at the upper (*left-hand column*) and the lower (*right-hand column*) surface of the leaf in *O. tenuiflorum* (**e**), *O. basilicum* cv. 'Genoveser' (**f**), and *O. gratissimum* (**g**)

In parallel, the density of glandular hairs was investigated. Here, the so called 'Thai' basil (*O. basilicum* cv. 'Siam Queen') was found to be endowed with a higher

Table 1 Diagnostical features of different *Ocimum* accessions amenable to light microscopy of dried leaves

	O. tenuiflorum	*O. basilicum* cv. Genoveser	*O. basilicum* cv. Siam Queen	*O. gratissimum*
E-ad	Puzzle-shaped, weakly lobed cell walls	Puzzle-shaped, weakly lobed cell walls	Puzzle-shaped, weakly lobed cell walls	Puzzle-shaped, strongly lobed cell walls
E-ab	Puzzle-shaped, weakly lobed cell walls	Puzzle-shaped, strongly lobed cell walls	Puzzle-shaped, strongly lobed cell walls	Puzzle-shaped, strongly lobed cell walls
Stomata E-ad	Diacytic	Diacytic	Diacytic	Diacytic
Stomata E-ab	Diacytic more than in E-ad	Diacytic more than in E-ad	Diacytic more than in E-ad	Diacytic more than in E-ad
Trichomes E-ad	Long, un-branched (3–5 cells) and short (1–2 cells)	Only new, short trichomes (1–2 cells) at the leaf margin	Only few short trichomes (1–2 cells) at the leaf margin	Long unbranched trichomes (4–5 cells)
Trichomes E-ab	Like E-ad	Like E-ad	Like E-ad	Like E-ad
Glandular hairs E-ad	Short (2 cells)	Short (2 cells)	Short (2 cells)	Numerous, short (2 cells)
Glandular hairs E-ab	Short (2 cells)	Short (2 cells)	Short (2 cells)	Numerous, short (2 cells)
Glandular scales E-ad	Numerous scales (4 cells)	Few scales (mostly 4 cells)	Few scales (mostly 4 cells)	Numerous scales (4 cells)
Glandular scales E-ab	Numerous scales (4 cells)	Some scales (4 cells)	Some scales (4 cells)	Numerous scales (4 cells)
Palisades/E-cell	Around 6	Around 3	Around 3	Around 8

E-ad epidermal cells from the adaxial leaf surface, *E-ab* epidermal cells from the abaxial leaf surface

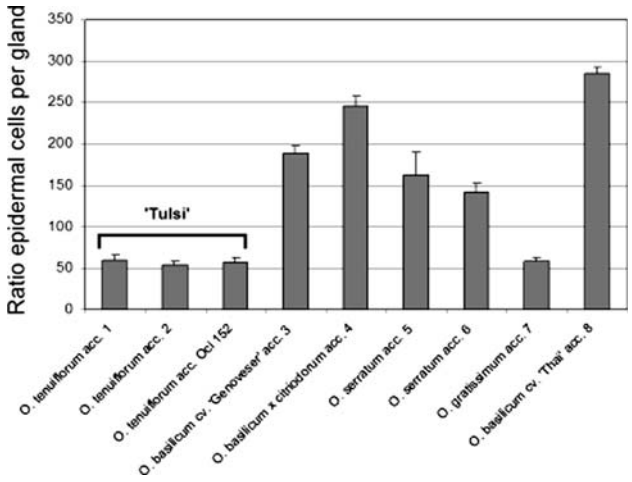

Fig. 3 Relative densities of glandular scales in different accessions of the genus *Ocimum*. Density is scored as average ratio of epidermal cells per glandular scale and represents a population of 20 leaf discs per accession. *Error bars* show the standard error

density of glandular hairs (Table 2), whereas the density of glandular scales was low and statistically not different from *O. basilicum* cv. 'Genoveser'. Here, a Kruskal–Wallis test showed that the difference between upper and lower surface of the leaf was irrelevant (data not shown), whereas the difference between the two cultivars of *O. basilicum* was significant at the $P > 0.95$ level.

Therefore, the density of glandular scales can be used as diagnostic marker to identify *O. tenuiflorum* in preparations declared to contain 'Tulsi', whereas the density of glandular hairs can be used as diagnostic markers to discriminate between *O. basilicum* cv. 'Siam Queen' and *O. basilicum* cv. 'Genoveser'.

Validation of the microscopical assay to detect 'Tulsi' and 'Thai' basil

To validate the use of glandular-scale density (for *O. tenuiflorum*) or glandular-hair density (for *O. basilicum* cv. 'Siam Queen') as diagnostic marker under the conditions that are relevant for practical use, we produced mixtures consisting of 25% of dried, ground *O. tenuiflorum* or *O. basilicum* cv. 'Siam Queen' (as analyte 1) that had to be discriminated against *O. basilicum* cv. 'Genoveser' (as analyte 2). The sample was ground and sieved through different mesh widths to test, whether recovery rate was dependent on particle size. The particles were assigned to analytes 1 and 2 using glandular-scale or glandular-hair densities as diagnostic traits (Table 3). When *O. tenuiflorum* was challenged by *O. basilicum* cv. 'Genoveser', the recovery was almost complete, i.e. almost all particles of *O. tenuiflorum* were correctly attributed. The difference with the 25% introduced into the assay was tested statistically and found not to be significant. This was valid down to a particle size of 100 µm (when the particles from *O. tenuiflorum* still harboured one or more glandular scales), which means that the assay for the detection of *O. tenuiflorum* is very robust even for the use in dried, mixed preparations. The identification of *O. basilicum* cv. 'Siam Queen' if challenged by *O. basilicum* cv. 'Genoveser' was found to be more difficult. Here, the minimal particle size was 180 µm, because at that size most particles from *O. basilicum* cv. 'Siam Queen' still contained at least one glandular hair, whereas at smaller sizes most particles were void of glandular hairs such that it was not possible to define them unequivocally. Interestingly, the recovery was significantly higher ($P > 0.99$) than the 25% value (Table 3), which means that this assay is

Table 2 Density of glandular hairs in the two *Ocimum basilicum* cultivars 'Genoveser' versus 'Thai Siam'

Cultivar	Mean density (hairs mm^{-2})	s_e	n
'Genoveser', E-ad	0.59	0.014	10
'Genoveser', E-ab	0.59	0.029	10
'Thai Siam', E-ad	1.26	0.045	10
'Thai Siam', E-ab	1.04	0.073	10

n Number of leaf discs, *E-ad* epidermis cells from the adaxial leaf surface, *E-ab* epidermis cells from the abaxial leaf surface

prone to false positive results. This limits the use of this assay to qualitative applications.

Analysis of commercial samples

We tested the performance of this assay in commercial samples that declared to contain 'Tulsi' (Table 4). The incidence of glandular scales in relation to the number of epidermal cells was determined from 20, in one case from 43, independent samples. For all four tested commercial samples, this value was differed by a factor of 4–6 from that obtained for *O. tenuiflorum*. The statistical probability (determined by a *t*-test) that the values observed for these respective commercial samples would be obtained with a *O. tenuiflorum* reference is $P < 0.01$. Whether these samples contain cultivars of *O. basilicum* instead of *O. tenuiflorum*, was not clear, the determined incidences (ranging between 205 and 321 in contrast to 57 for *O. tenuiflorum*) are compatible with the values observed in different cultivars of *O. basilicum* (*O. basilicum* cv. 'Genoveser': 188, *O. basilicum* cv. 'Dark Opal': 345). However, the samples could also contain other, unknown species of *Ocimum*. Irrespective of this limitation, this exploratory experiment shows that a control of commercial 'Tulsi' preparations might be necessary.

Conclusion

As exemplary case study for other ayurvedic preparations, we have developed a microscopical assay for the 'Holy Basil' *O. tenuiflorum* ('Tulsi'). Ayurvedic preparations pose special challenges to diagnostics—since they usually come in mixtures of dried powders or fragmented material, it is in most cases very difficult or even impossible to extract DNA for the use of molecular markers. Therefore, microscopical diagnostics is the only approach that allows verifying the declared analytes. A second challenge is the difference in terminology between ayurvedic tradition and scientific botany. Although 'Tulsi' or 'Holy Basil' is the general term for *O. tenuiflorum*, this term seems to be used in some regions of India for other species of *Ocimum* as well, in everyday language, 'Tulsi' is even the designation for any kind of basil. Thus, depending on the origin of the preparation, 'Tulsi' may mean different things. Nevertheless, our exploratory of commercial products showed clearly that in most of these preparations there was not any *O. tenuiflorum*. Our diagnostic method is based on the density of glandular scales and can be easily adapted to routine

Table 3 Validation of the microscopical assay for the identification of *Ocimum tenuiflorum* and *Ocimum basilicum* cv. 'Thai Siam' in a mixture with *Ocimum basilicum* cv. 'Genoveser'

Analyte 1	Analyte 2	Determined for analyte 1	Determined for analyte 2	Mesh size (μm)	n
O. tenuiflorum 25%	*O. basilicum* cv. 'Genoveser' 75%	23.37 ± 0.82	76.63 ± 0.82	180–250	5
O. tenuiflorum 25%	*O. basilicum* cv. 'Genoveser' 75%	23.59 ± 1.28	76.41 ± 1.28	100–180	5
O. basilicum cv. 'Thai Siam' 25%	*O. basilicum* cv. 'Genoveser' 75%	33.55 ± 1.25	66.65 ± 1.25	250–630	7
O. basilicum cv. 'Thai Siam' 25%	*O. basilicum* cv. 'Genoveser' 75%	31.12 ± 1.62	68.84 ± 1.62	180–250	7

Table 4 Analysis of *Ocimum* spec. leaf fragments in commercial samples declared to contain *Ocimum tenuiflorum*

Sample	Declared	E-ad/glandular scales	s_e	n	P
Reference	*Ocimum tenuiflorum*	57	2.1	94	
1	'Tulsi', Indian Basil, ground fine tea	321	40.3	20	<0.01
2	'Tulsi' as herbal tea mixture with orange and ginger	318	26.1	20	<0.01
3	'Tulsi' as herbal tea mixture with cinnamon and coconut	318	39.5	20	<0.01
4	*Folia Basilici sancti* 'Tulsi' as cut leaf fragments	205	20.4	43	<0.01

P statistical probability (*t*-test) that the value observed for the respective commercial sample would be obtained with a *Ocimum tenuiflorum* reference

assay even on a semiquantitative level, because it is sufficient to score particles containing glandular scales to obtain a valid estimate for the content of *O. tenuiflorum*. In the long term, it might become even possible to automatize this assay by image-analysis systems by developing algorithms that allow recognition of the characteristic cross-like set up of glandular scales.

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