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ORIGINAL PAPER

Genetic authentication by RFLP versus ARMS? The case of Moldavian dragonhead (*Dracocephalum moldavica* L.)

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Abstract Moldavian dragonhead (*Dracocephalum moldavica* L.), due to its pleasant lemon scent and medical effects, has acquired increasing impact as functional food. The high diversity within the genus, limited supply not keeping pace with the growing demand, the morphological similarity with other Labiatae, and trading under the common name Turkish Melissa invite adulteration by surrogate species. We have developed several verified reference accessions of *D. moldavica* L. along with potential surrogate species to compare different approaches of authentication, also in commercial samples. We report on three strategies of authentication—a microscopic method, based on the relative size of epidermal pavement cells versus palisade cells, and two strategies of genetic authentication based on the barcoding marker large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (rbcL). We can detect single-nucleotide exchanges between *D. moldavica* L. and the potential surrogate species *Melissa officinalis* L. and *Nepeta cataria* L. by restriction fragment length polymorphism (RFLP), and we show that we can use this to verify the presence of *D. moldavica* even in dried and highly fragmented mixtures from commercial samples. We further develop a third strategy derived from the so-called amplification refractory mutation system (ARMS), based on multiplex PCR of the rbcL marker upon addition of specifically designed intermediate primers that will generate a diagnostic second band in case of *D. moldavica* L., but not for the surrogate species. We demonstrate that this ARMS approach is superior to the RFLP strategy, because it safeguards against experimental fluctuations, can unequivocally verify the presence of *D. moldavica* in commercial samples declaring its presence, and yields a clear outcome in a one-step protocol.

Keywords Amplification refractory mutation system (ARMS) · *Dracocephalum moldavica* L. · Molecular authentication · Restriction fragment length polymorphism (RFLP) · Ribulose-bisphosphate carboxylase oxygenase large subunit (rbcL)

Introduction

Globalisation extends the spectrum of plants entering the European market. Growing consumer awareness for the medical aspects of food, and a general attention on health issues in an ageing society stimulate the introduction of novel foreign plant products often derived from traditional medical or dietary use. These products are mostly located somewhere at the interphase between food supplement and phytomedicine, and only few of them are regulated by the EU Traditional Herbal Medicinal Products Directive. Moreover, only those herbal preparations, where no historical record is documented for Europe, fall under the legislation of the Novel-Food-Regulation of the European Union [1]. For most of these foreign plant products, legislation is therefore far from clear. This problem is not a merely academic: These plants are used, in their country of origin, in the context of a specific cultural and medical tradition that safeguards against undesired side effects. When isolated from this context, unforeseen problems can arise such as adverse interactions, adulteration, and even toxicity [2]. In addition, such market trends can cause short-term limitations in the supply with these plants, creating a situation,

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where producers are tempted to extend their supply by similar, but less efficient, surrogate plants, posing further challenges to the European authorities in charge of consumer protection.

Many of these novel plants fall into the taxonomically difficult family of the Labiatae, composed of more than 7,000 species that are currently grouped into more than 200 genera [3]. Since the Labiatae are rich in etheric oils (mostly monoterpenes) secreted from glandular hairs or glandular scales, they have been traditionally used as both spices and for medical purposes. The complexity of this group along with their readiness to form hybrids has led to a taxonomic situation that, even for taxonomists specialised in this group, is far from transparent. The inconsistencies in Labiatae taxonomy (a bonmot ascribed to Lindley once termed the situation even as a "disgrace of botany") have been mostly resolved in the meantime. However, even those species that are traditionally used as medical plants in Europe harbour taxonomic complexities that are far from being understood.

The Moldavian dragonhead (*Dracocephalum moldavica* L.) provides a perfect illustration for this issue. Although apparently used in phytomedical preparations already in the middle ages, it was not clearly delineated from *M. officinalis*, but traded as *Herba Melissae Turcicae* or "Foreign Melissa" (*M. peregrina*)—the first differential description of "true" Melissa from Moldavian dragonhead as its surrogate by Hayne [4] dates back to as early as 1822. Due to its strong lemon scent, the Moldavian dragonhead has become popular as component of herbal teas, often used with phytomedical connotations. In fact, dragonhead is traditionally used for medical purposes in Central Asia, the centre of diversity for this genus. In the traditional medicine of the Uigur people, *D. moldavica* is used for coronary diseases as well as for pain relief [5], and the related species *D. subcapitatum* and *D. kotschyi* from Iran even are effective against Trypanosome diseases [6]. Recently, extracts of *D. moldavica* were found to act as efficient and biologically safe insect repellent for food storage [7, 8]. Due to the aromatic nature of this plant, the chemical analysis of essential oils has been in the focus and neral, citral, geraniol, geranial, and other monoterpenes had been identified as leading components [9–11]. However, *D. moldavica* is also rich in non-volatile compounds with medical efficacy. For instance, a novel 3,4,5-trimethoxyphenylethanol *β-D*-glucopyranoside has been isolated from plants collected in Xinjiang Province and found to be highly efficient in quelling inflammation-triggered production of nitric oxide [12].

The genus *Dracocephalum* is morphologically very close to the sister genus *Nepeta* and harbours 70 morphologically very similar species inhabiting alpine and semiarid regions of Central Asia. Just for China alone, 35 *Dracocephalum* species have been described [13]. The identification of *D. moldavica* L. is therefore far from trivial. The high variability in the composition of essential oils reported for this species by different authors [9–11] is often attributed to different environmental conditions. However, it might simply reflect genetic differences (chemovars, subspecies, or even cryptic species).

Microscopic analysis as efficient traditional approach to authenticate dried herbal mixtures, as those typical for commercial samples of *D. moldavica*, relies on robust anatomical features that are, in addition, sufficiently different between species. However, only few anatomical studies are available for *D. moldavica* [14, 15]. Moreover, the traits addressed in those studies, such as shape and organisation of glandular hairs or scales, seemed to be highly variable and even discrepant between these studies as pointed out in detail in [15]. It is not clear whether the investigated specimen really represented the same species, *D. moldavica*, because those studies did neither report on taxonomic determination, nor on the deposition of botanical vouchers. Chemical authentication, for instance by thin-layer chromatography or gas chromatography, does not help either, due to the high variability of essential oils [9–11] that, in addition, vastly overlap with those found in the surrogate species *M. officinalis*.

Genetic authentication provides alternative approaches to overcome these drawbacks. In fact, randomly amplified polymorphic DNA (RAPD) analysis has been employed to study the phylogenetic relationship between several species of *Dracocephalum* [16]. Although this approach is fast and allows even intraspecies differentiation, as shown for *M. officinalis* [17], it requires exact titration of PCR conditions to yield reliable results. For instance, depending on the brand of Taq polymerase or buffer conditions, the obtained fingerprint differed. Since the bands produced by RAPD are not known, it is therefore difficult to judge for unknown species, whether differences in the obtained fingerprints reflect species differences or are caused by fluctuations in PCR conditions. Approaches that safeguard against PCR fluctuations with an internal reference are to be preferred. In our previous work, we used restriction fragment length polymorphism (RFLP) based on the barcoding marker rbcLa to develop a diagnostic assay for the authentication of two Myrtaceae species that are both traded as Lemon Myrtle [18]. In the current work, we transfer this strategy to differentiate *D. moldavica* against adulteration with the chemically similar *M. officinalis* L., or the morphologically highly similar *N. cataria* L. A drawback of the RFLP approach is the need for a two-phase protocol, since the PCR has to be followed by a restriction digest. We therefore developed an improved strategy circumventing the need for this restriction digest by using a multiplex PCR, where the conventional rbcLa barcoding primers were accompanied by destabilized primers that will produce a second band

Table 1 Origin of the plant used in the current study

Species	Accession	Source
Dracocephalum moldavica L. DraRueh	5861	Commercial, Rühlemanns (Horstedt)
Dracocephalum moldavica L. Dra2	5862	IPK Gatersleben, BG Zürich
Dracocephalum moldavica L. Dra7	5863	IPK Gatersleben, Dr. Th. Gladis
Dracocephalum ruyschiana L.	5156	BG Karlsruhe Institute of Technology
Melissa officinalis L.	4639	BG Karlsruhe Institute of Technology
Nepeta cataria L. var. *citriodora* Ncat13	7575	IPK Gatersleben
Nepeta nuda L.	5395	BG Karlsruhe Institute of Technology

The plants are maintained under the corresponding accession code as living voucher specimens in the collection of the Botanical Garden of the KIT

Table 2 Composition of the commercial samples used in the current study

Sample	Composition
Dra_1	Dragonhead (*melissae turcicae flos*), lime blossom (*tiliae flos*), elderberry blossom (*sambuci flos*), mallow (*malvae flos*), thyme (*thymi herba*), sage (*salviae folium*), ribwort (*plantaginis lanceolatae folium*), coltsfoot (*farfarae flos*)
Dra_1*	Dragonhead sorted from sample Dra_1 based on microscopic features
Dra_2	Dragonhead (*melissae turcicae flos*), lime blossom (*tiliae flos*), elderberry blossom (*sambuci flos*), ribwort (*plantaginis lanceolatae folium*), thyme (*thymi herba*), sage (*salviae folium*), mallow (*malvae flos*)
Dra_2*	Dragonhead sorted from sample Dra_2 based on microscopic features
Moff_1	*Melissa officinalis* L.

The declared composition was verified by microscopic analysis

*Asterisks indicate that the sample has been derived from the commercial mixtures by sorting Moldavian dragonhead based on morphological features

that is diagnostic for *D. moldavica*, but not formed in *M. officinalis* or in *N. cataria*. This strategy, known as amplification refractory mutation system (ARMS), had been originally developed to detect mutations in rapid screens [19] and combines the advantages of RAPD (rapid one-step method yielding species-specific patterns) with those of RFLP (clear output that is qualitatively independent of fluctuations in PCR efficiency).

Materials and methods

Plant material and samples

Vegetative development of plants depends on the environment, especially light quantity and quality. Therefore, the reference specimens were 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 the Karlsruhe Institute of Technology. The species were redetermined using floral traits based on determination keys in current floristic literature [13, 19] and Linné's original voucher specimens deposited in the Swedish Museum of Natural History [21]. The specimens are maintained as living vouchers in the Botanical Garden of the Karlsruhe Institute of Technology. Source and identity of the specimens used in this study are given in Table 1. Two commercial samples declared to contain Moldavian dragonhead were included in this study. Both samples were tea mixtures, where Moldavian dragonhead was accompanied by lime blossoms (*tiliae flos*), elderberry blossom (*sambuci flos*), common mallow (*malvae flos*), thyme (*thymi herba*), sage (*salviae folium*), and ribwort (*plantaginis lanceolatae folium*) in different composition. One sample, in addition, contained coltsfoot (*farfarae flos*). The composition of these samples is given in Table 2 and was verified by microscopic analysis. Dried material attributed to Moldavian dragonhead was sorted from these mixtures and also included in the tests on genetic authentication.

Extraction of genomic DNA

Fresh leaf material (preferably from young leaves) was harvested from healthy plants. About 40 mg of the sample was transferred into a reaction tube (2 ml, Eppendorf) together with one stainless steel bead (diameter 5 mm) and shock frozen in liquid nitrogen. The frozen sample was then ground three times for 15 s at 20 Hz (Tissuelyser, Qiagen, Hildesheim, Germany). After each individual grinding step, the sample was returned to liquid nitrogen to ensure that the powder did not thaw during the extraction. Genomic DNA was extracted using a modified extraction method based on cetyl trimethyl ammonium bromide (CTAB, [22]) using about 25 mg ground and frozen leaf tissue. The powder was complemented with 1 ml prewarmed extraction buffer (3 % w/v CTAB) containing 8 µl/ml mercaptoethanol and

incubated for 30 min at 55 °C followed by a centrifugation step to remove debris. Subsequently, the sample was digested with proteinase K at 55 °C for 30 min, then mixed with 750 µl of chloroform/isoamylalcohol (24:1), and then spun down for 10 min with 14,000 g at 25 °C. The aqueous upper phase (containing DNA) was transferred into a fresh reaction tube, and the DNA was precipitated with 0.65 volumes of isopropanol, collected by centrifugation (10 min, 14,000 g), washed with 70 % EtOH, and dissolved in 50 µl ddH_2O. 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 2.1. 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 and RFLP of rbcL

A partial sequence of the large subunit of the ribulose-bisphosphate carboxylase gene (rbcL), rbcLa, was amplified by PCR in a 10-µl reaction using 50 ng of genomic DNA as template and a reaction mix containing single-strength buffer (thermopol, NEB), 200 µM mixed dNTPs (NEB), 200 nM of each primer (rbcLa_F/rbcLa_R, Invitrogen), 0.4 units of Taq polymerase (NEB), and 10 mg/ml bovine serum albumin (Sigma-Aldrich, Deisenhofen, Germany). The amplificates were separated by electrophoresis in a 1.5 % agarose gel, and their size was verified using a 100-bp DNA ladder (NEB) after fluorescent staining with SYBR Safe (Invitrogen). The amplificates were extracted from the gel using the Nucleo-Spin® Extract II kit (Macherey–Nagel, Karlsruhe), following the protocol of the producer, and then sequenced (GATC Biotech, Konstanz). The sequences were verified by BLAST search and aligned with related rbcL sequences (ClustalX, http://www.clustal.org) and are deposited in GenBank under the accession numbers KF307351 (*D. moldavica* accession Dra2), KF307352 (*D. moldavica* accession Dra7), KF307353 (*D. moldavica* accession DraRueh), KF307354 (*D. ruyschiana*), KF307355 (*M. officinalis*), and KF307356 (*N. cataria*). To discriminate *Dracocephalum* from *Melissa* and *Nepeta*, 6 µl of rbcLa PCR amplificate was digested overnight at 37 °C in a 25-µl reaction volume consisting of 2.5 µl 10× enzyme buffer (NEB, No. 1), 2.0 µl *BamH* I or *EcoR* I enzyme (NEB), and 14.5 µl bidistilled water. The digested amplificates were separated by electrophoresis in a 1.5 % agarose gel along with a 100-bp DNA ladder as size marker (NEB).

ARMS diagnostics

For the analysis by ARMS, rbcLa was amplified according to the previously described protocol except addition of 200 nM of the diagnostic primer DC4: 5′-TTTCCAAGGCCCACCTCATAGT-3′. The primer was designed to anneal with its 3′-prime end at position 462 where a thymine is present in the rbcLa sequence from *Dracocephalum*, which is replaced by a guanine in *Melissa* and *Nepeta*. To introduce a destabilisation of the 3′-end as prerequisite of ARMS [19], the primer was designed to contain an adenine at this position. PCR and separation of products were conducted as described above.

Phylogenetic analysis of the rbcL sequence

The rbcL sequences were used as input for a BLAST search for Labiatae rbcL, the retrieved 22 sequences were automatically aligned using the ClustalX algorithm in MEGA 4.0 [23], and the evolutionary relationships were inferred by means of the neighbour-joining algorithm [24] with bootstrap values based on 500 replicates [25] using *Stachys sylvatica* L. and *Ocimum basilicum* L. as outgroups. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. All positions (both coding and non-coding) were included; gaps and missing data were eliminated from the data set.

Light microscopy

Leaves, shoots, and flowers of all specimens were documented macroscopically (Exilim Z750, Casio) and by stereomicroscopy (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 completely developed leaves were brightened with 60 % chloral hydrate and then analysed under a light microscope (Axioskop, Zeiss; Jena) equipped with a digital image acquisition system (Axio-Cam, Zeiss; Jena).

Results and discussion

Morphological variability within Moldavian dragonhead

To test for morphological and anatomical traits that can be used for microscopic authentication, three verified specimens of *D. moldavica* L. (DraRueh, Dra2, and Dra7) were compared (Fig. 1). Shoots are quadrangular in cross section, but the specimens differed with respect to shape and coloration (Fig. 1a): Dra2 displayed conspicuous incarnations at the flanks not observed in the other two specimens. Similar incarnations have also been documented for *M. officinalis* L. [26]. Dra2 showed also a deeply purple pigmentation by anthocyanins in the periphery, most pronounced in the epidermis and less pronounced in the

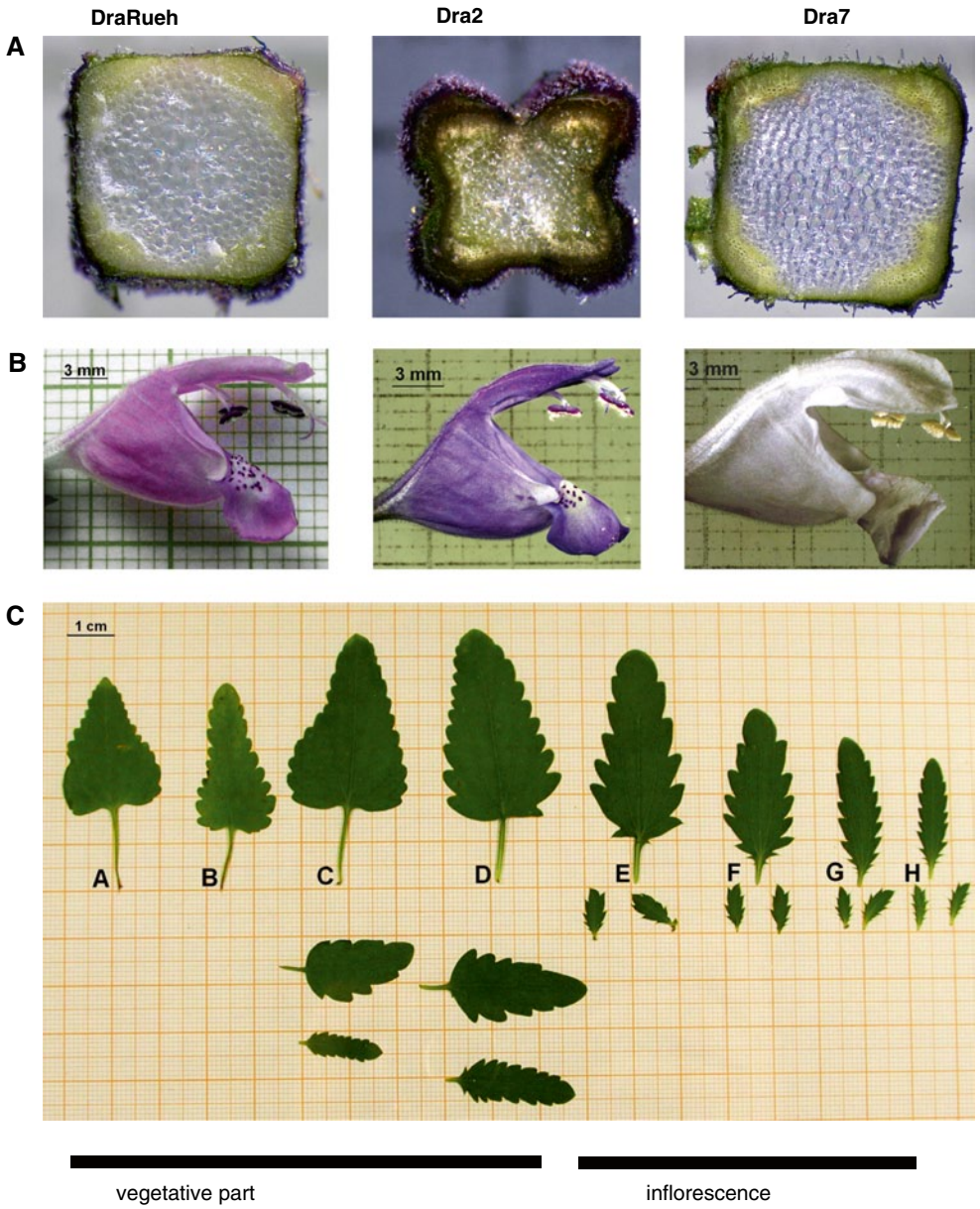


Fig. 1 Morphological variability of *Dracocephalum moldavica* L. **a** Stem cross sections for the accessions DraRueh, Dra2, and Dra7 investigated in the current study. **b** Flower morphology of DraRueh, Dra2, and Dra7. Note the absence of pigmentation in Dra7. **c** Developmental variability of leaf morphology illustrated by a leaf series from the base (*left*) to the *top* (*right*) of a flowering shoot from Dra7

subcortical parenchyma layer. A prominent pigmentation was also observed in DraRueh, but confined to the epidermis. In contrast, Dra7 was only weakly colorated in the epidermal layer. These differences in colour intensity were also mirrored in the flowers (Fig. 1b): Flowers of Dra2 showed up a deep violet characteristic for high concentrations of anthocyanins, whereas flowers of DraRueh were more pink and of Dra2 were even completely white (similar to the flowers of *M. officinalis* L.). Although all three genotypes showed the characteristic set-up of the typical Labiatae flower, there were clear differences in the details of the upper lip: whereas in Dra2 the upper lip was well extended over the stamina in a helmet-like manner, lip extension was reduced in DraRueh, and in Dra2, the upper lip was so short that the stamina protruded out of the flower—a trait that is also observed in *M. officinalis* L. [26]. Also, the lower lip was shaped differently between Dra7 and the other two accessions. This variability of stem and flower traits is accompanied by a high developmental variability of leaf shape during development that changes from triangular at the base of the inflorescence to serrate-ovoid at the apex (Fig. 1c). Thus, the plant parts to be expected in

Table 3 Microscopic features of leaves of *Dracocephalum moldavica* versus *Melissa officinalis*

	Dracocephalum moldavica	*Melissa officinalis*
Type of leaf	Bifacial	Bifacial
E-ad	Puzzle-shaped, weakly indented cell walls	Puzzle-shaped weakly indented cell walls
E-ab	Puzzle-shaped strongly indented cell walls	Puzzle-shaped strongly indented cell walls
Stomata E-ad	Diacytic	Absent
Stomata E-ab	Diacytic	Diacytic
Trichomes		
E-ad	Short, unbranched trichomes (1–3 cells, mostly 1–2 cells)	Short, unbranched trichomes (1 cell), and a few long unbranched trichomes (2–8 cells)
Trichomes E-ab	Short, unbranched trichomes (1–3 cells, mostly 2–3 cells)	Like E-ad
G-hairs E-ad	Short hairs with 2 G-cells, and long hairs with 1 G-cell	Short hairs with 2 G-cells, and few long hairs with 1 G-cell
G-hairs E-ab	Like E-ad	Like E-ad
G-scales E-ad	Absent	Absent
G-scales E-ab	Scales with 14–24 G-cells	Scales with 8 G-cells
Palisades/E-cell	Mostly 5–7	Mostly 10–20

Abbreviations Epidermal cells adaxial E-ad, Epidermal cells abaxial E-ab, Glandular G

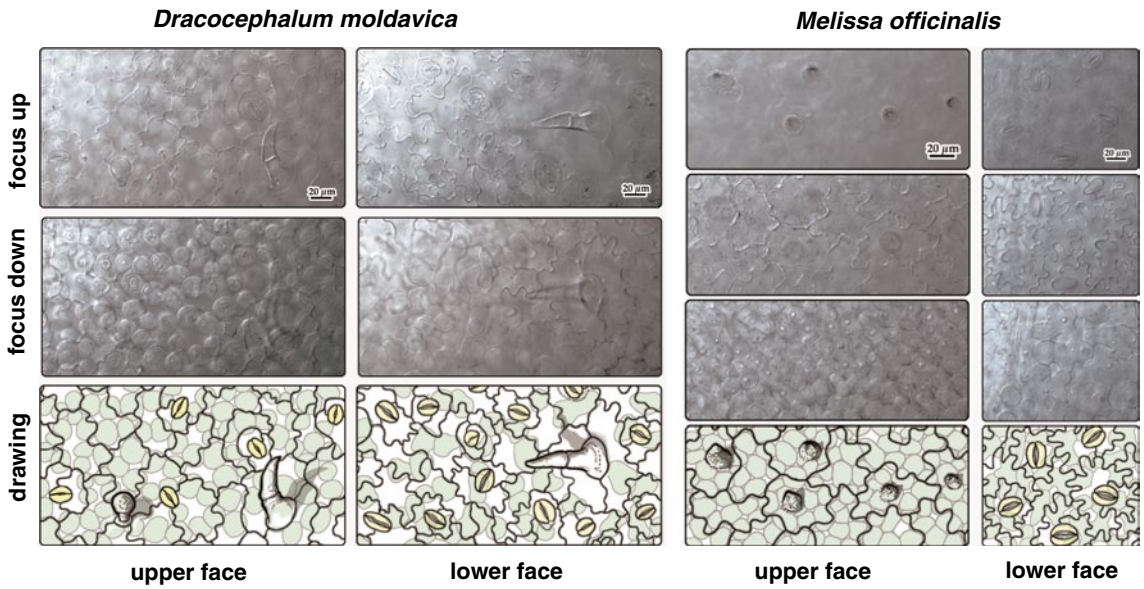


Fig. 2 Microscopic differentiation of *D. moldavica* L. from *M. officinalis* L based on epidermal morphology and relative cell size of parenchymatic versus epidermal cells

commercial samples of dragonhead vary in morphology and coloration. Moreover, these traits overlap with *M. officinalis* L. and change during development and thus are not suitable for differential diagnostics.

Epidermal patterning as diagnostic feature

Since morphology was found to be too variable, we investigated anatomical features that can be used to discriminate *D. moldavica* L. from *M. officinalis* L. These features must remain constant, even when the size of the plant changes due to age or environment. Epidermal patterning is brought about by an iterative mechanism based on inhibitory signals emitted by the forming meristemoid. These signals are perceived by the neighbouring cells that, in response to these signals, are prevented from meristemoid commitment [27]. This regulatory loop is very robust and, since it is iterative, independent from leaf size. We therefore recorded anatomical features of the upper and lower epidermis in the different accessions of *D. moldavica* L. versus *M. officinalis* L. The results of this analysis are summarised in Table 3: The adaxial, upper epidermis differs, with diacytic stomata in

D. moldavica L., whereas stomata are completely lacking in the upper epidermis of *M. officinalis* L. (Fig. 2). Trichomes are short and unbranched in *D. moldavica* and consist mostly of one to two cells in the upper and two to three cells in the lower epidermis. In *M. officinalis*, trichomes are mostly unicellular and short on both faces of the leaf. In addition, there exist longer, unbranched trichomes not seen in *D. moldavica* L. Glandular hairs are similar between both species, and glandular scales are composed of more cells (14–24 cells) in *D. moldavica* L. as compared to *M. officinalis* L. (8 cells). Whether this can be used as discriminative marker is questionable, because the number, although generally higher in *D. moldavica* L., shows high variation consistent with published records on the anatomical variability of glandular hairs and scales described in the previous publications [15]. The most robust diagnostic marker was the ratio of epidermal cells to the subtending palisade cells. In *D. moldavica*, one epidermal pavement cell spanned five to seven cells of the palisade parenchyma, whereas in *M. officinalis* L. it spanned 10–20 palisade cells. This trait is conspicuous (Fig. 2) and can be easily assessed even in dried specimens as characteristic of commercial samples.

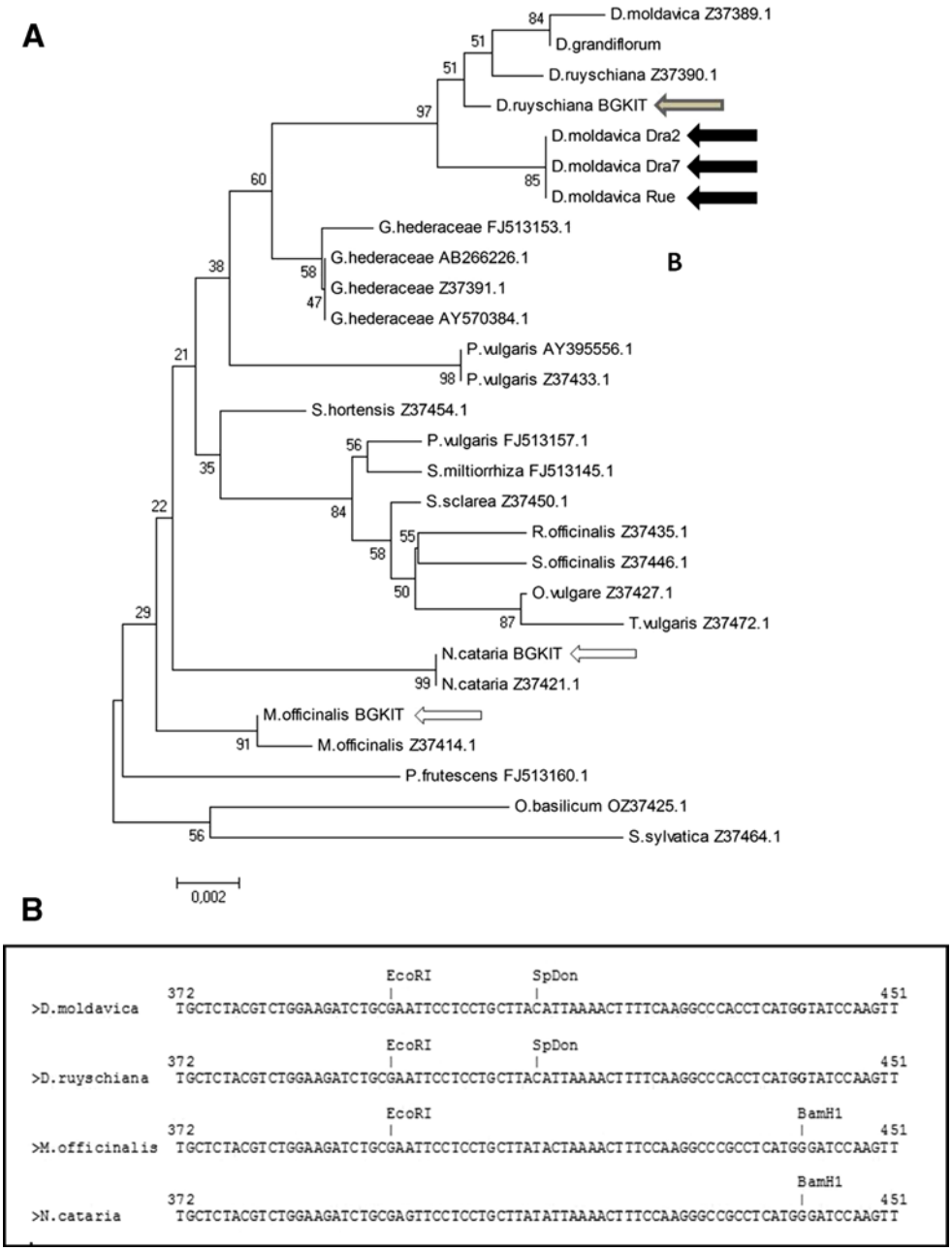
RFLP based on the rbcLa marker

Since the morphological traits were too variable to serve for diagnostic purposes and the microscopic authentication based on the ratio between epidermal pavement cells and subtending palisade cells is somewhat cumbersome, we explored the rbcLa marker as base for genetic authentication. By genomic PCR, we amplified rbcLa and obtained valid sequences for three accessions of *D. moldavica* L., one accession of *D. ruyschiana*, one accession of *M. officinalis* L., and one accession of *N. cataria* L. All these sequences were checked by a second run, and the taxonomic identity of all accessions had been verified before extraction of DNA. As to be expected, these sequences were highly similar, but several base exchanges were detectable, especially between the *Dracocephalum* accessions and *M. officinalis* and *N. cataria*. To evaluate these differences, we located the sequences with respect to other members of the Nepetoideae subfamily drawn from GenBank using *S. sylvatica* and *O. basilicum* as outgroup based on the neighbour-joining algorithm [24]. All three accessions of *D. moldavica* L. yielded identical sequences and clustered together with *D. ruyschiana* L. in the *Dracocephalum* clade (Fig. 3a). There were two sequences deposited in GenBank for both species. Whereas the sequence for *D. ruyschiana* L. was very similar, the three sequences isolated by us significantly differed from the sequence Z37389.1 deposited in GenBank and derived from a phylogenetic study on the Nepetoideae subfamily [28]. Whether these differences represent intraspecies variation or whether different species of the genus are involved is unclear, because no information on the identity or voucher references had been reported in that study. Irrespective of this minor detail, the reconstructed phylogeny shows a clear separation from *Dracocephalum* from its surrogate species *M. officinalis* and *N. cataria*. For both of these species, sequences were already available in the database and found to be identical with those isolated in the current work.

In the next step, the sequences were analysed for differential restriction sites (Fig. 3b). In fact, two of such sites could be identified. A base exchange of A for G at position 397 of rbcLa in *N. cataria* L. eliminated a recognition site for *EcoR* I present in both *M. officinalis* and *Dracocephalum* (and also in all other Nepetoideae sequences analysed). Second, a base exchange of G for T at position 442 of rbcLa in all *Dracocephalum* accessions analysed eliminated a recognition site for *BamH* I present in all other available Nepetoideae sequences, including *M. officinalis* and *N. cataria*.

Based on these two diagnostic differences in restriction sites, an RFLP assay was designed. Digestion of the rbcLa fragment with *BamH* I was predicted to generate one band in *Dracocephalum* corresponding to the uncut fragment. In contrast, restriction should yield two smaller bands of 445 bp and 155 bp for *Nepeta* and *Melissa* (Fig. 4a). This prediction was tested experimentally using pure and commercial samples of dragonhead and its potential surrogates (Fig. 4b). As predicted from the sequence analysis, restriction of the rbcLa fragment yielded one band of around 600 bp for samples from *D. moldavica* L. or commercial samples declaring dragonhead, whereas two bands of the predicted size (445 and 155 bp) were observed for the surrogate species *M. officinalis* L. and *N. cataria* L as well as *N. nuda*. To corroborate the validity of the approach, the commercial samples were sorted using the microscopic features given in Table 3, confirming the result obtained for pure samples of dragonhead. Thus, RFLP based on restriction with *BamH* I allows us to detect adulterations of dragonhead by other Labiatae in commercial samples. However, in case of adulteration, it would not be possible to tell whether the surrogate is *M. officinalis* L. or a member of the genus *Nepeta*. A second drawback of this approach is that a failure of the restriction (for instance due to degradation of the *BamH* I enzyme) would also produce one uncut band indicative of dragonhead, which would leave adulterations gone unnoticed.

We therefore tested RFLP based on restriction of the rbcLa fragment with *Eco R* I predicted to yield one band of around 600 bp in members of the genus *Nepeta* (corresponding to the uncut rbcLa fragment), whereas both *D. moldavica* L. and *M. officinalis* L. should exhibit two bands of 395 and 205 bp (Fig. 4c). This prediction was verified and confirmed experimentally (Fig. 4d). Thus, RFLP based on restriction with EcoR I allows us to unequivocally detect



adulteration by *Nepeta* species. However, the two bands at 395 and 205 bp would not be unequivocal proof for the presence of dragonhead, but would also be produced in case of adulteration with *M. officinalis* L. The advantage of that strategy over RFLP using *BamH* I is that the indication of dragonhead in the sample is safeguarded against a failure of the restriction digest, since the characteristic double band is observed only for successful restriction.

ARMS based on the rbcLa marker

Both RFLP-based strategies described above have their specific advantages and drawbacks: in one case (*BamH* I), dragonhead can be clearly discriminated against adulteration with either *Melissa* or *Nepeta*, but failure of restriction digest would leave adulterations gone unnoticed. In the alternative strategy (*EcoR* I), the detection of dragonhead is safeguarded against restriction failure, but the presence of the characteristic double band would also be obtained in case of adulteration with *Melissa*. A general drawback of the RFLP approach is that it requires a two-step protocol: first, the rbcLa marker has to be amplified by PCR, and the amplificate has then to be digested overnight.

To overcome these drawbacks of these RFLP-based strategies, we employed an alternative approach termed ARMS. This method is based on a multiplex PCR, whereby

◂**Fig. 3** Characteristics of the rbcLa marker. **a** Evolutionary relationship for the investigated taxa among 28 taxa from the Labiatae inferred by the neighbour-joining (NJ) algorithm based on the rbcLa marker. Numbers next to the branches represent the bootstrap values from 500 replicates. Evolutionary distances are given in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set. D. moldavica_Dra2, *Dracocephalum moldavica* accession Dra2, KIT voucher 5862 (GenBank accession KF307351); D. moldavica_Dra7, *Dracocephalum moldavica* accession Dra7, KIT voucher 5863 (GenBank accession KF307352); D. moldavica_Rue *Dracocephalum moldavica* accession Rue, KIT voucher 5861 (GenBank accession KF307353); D. moldavica_Z37389.1, *Dracocephalum moldavica* (GenBank Z37389.1); D. ruyschiana_BGKIT, *Dracocephalum ruyschiana* KIT voucher 5156 (GenBank accession KF307354); D. ruyschiana_Z37390.1, *Dracocephalum ruyschiana* (GenBank Z37390.1); D. grandiflorum, *Dracocephalum grandiflorum* (Z37388.1); M. officinalis_ *Melissa officinalis* KIT voucher 4639 (GenBank accession KF307355); M. officinalis_Z37414.1, *Melissa officinalis* (GenBank Z37414.1); G. hederaceae_Z37391.1, *Glechoma hederaceae* (GenBank Z37391.1); G. hederaceae_ AB266226.1, *Glechoma hederaceae* (GenBank AB266226.1); G. hederaceae_ AY570384.1, *Glechoma hederaceae* (GenBank AY570384.1); G. hederaceae_FJ513153.1, *Glechoma hederaceae* (GenBank FJ513153.1); P. vulgaris_AY395556.1, *Prunella vulgaris* (GenBank AY395556.1); P. vulgaris_Z37433.1, *Prunella vulgaris* (GenBank Z37433.1); P. vulgaris_FJ513157.1, *Prunella vulgaris* (GenBank FJ513157.1); O. basilicum_OZ37425.1, *Ocimum basilicum* (GenBank OZ37425.1); R. officinalis_Z37435.1, *Rosmarinus officinalis* (GenBank Z37435.1) O. vulgare_Z37427.,1 *Origanum vulgare* (GenBank Z37427.1); S. miltiorrhiza_FJ513145.1, *Salvia miltiorrhiza* (GenBank FJ513145.1); S. officinalis_Z37446.1, *Salvia officinalis* (GenBank Z37446.1); S. rutilans_Z37449.1, Salvia rutilans (GenBank Z37449.1); S. sclarea_Z37450.1, *Salvia sclarea* (GenBank Z37450.1); S. uliginosa_Z37451.1, *Salvia uliginosa* (GenBank Z37451.1); S. hortensis_Z37454.1, *Salvia hortensis* (GenBank Z37454.1); T. vulgaris_Z37472.1, *Thymus vulgaris* (GenBank Z37472.1); N. cataria_BGKIT, *Nepeta cataria* voucher 7575 (GenBank accession KF307356); N. cataria_Z37421.1, *Nepeta cataria* (GenBank Z37421.1); P. frutescens_FJ513160.1, *Perilla frutescens* (GenBank FJ513160.1) S. sylvatica_Z37464.1, *Stachys sylvatica* (GenBank Z37464.1). **b** Differential restriction sites in rbcLa from *D. moldavica, D. ruyschiana, M. officinalis,* and *N. cataria*

one intermediate primer will generate a second, smaller band in addition to the complete fragment. This intermediate primer is designed such that annealing is destabilised by introducing bases that are illegitimate with respect to the target sequence. In case of mutations in the target sequence, this destabilisation will prevent the intermediate primer from annealing such that the side band will not be observed. This strategy had been originally developed to rapidly screen populations for mutations in specific target sequences [19]. However, if the sequence in the species of interest (in our case dragonhead) is used as template for the ARMS design, any adulterant with even minor changes in the target motif would become detectable by its failure of producing the side band. A major advantage of the ARMS strategy over RFLP is that any failure of amplification itself would be immediately detected by the absence of the full-length band. The second major advantage is that no second restriction step is required—the result is obtained immediately after PCR.

We therefore designed a diagnostic ARMS primer for the rbcLa sequence of dragonhead that should anneal 159 bp upstream of the rcbLa reverse primer. To introduce destabilisation of the 3′-end, a base exchange from G to A was introduced into the diagnostic primer (DC4) compared to the dragonhead target sequence. A multiplex PCR using this primer in combination with the two conventional rbcLa primers (Fig. 5b) should produce, in addition to the full-length amplificate at 599 bp, a second band at 159 bp in a situation where the ARMS primer annealed to its target sequence (Fig. 5c). When this was tested experimentally, the multiplex PCR using this primer, DC4 (Fig. 5d), produced the predicted diagnostic second band at 159 bp for both pure and commercial samples of *D. moldavica*, whereas for *M. officinalis* and two tested species of *Nepeta,* only single band at 599 bp was observed. Thus, the ARMS strategy delivered a single-step protocol that by the presence of the rbcLa band simultaneously reports on the success of the PCR and the presence of dragonhead in the sample.

It should be kept in mind that both ARMS and RFLP probe for specific species and thus are based on a hypothesis on the nature of potential adulterations and sequence information from potential adulterants. Moreover, the presence of more than one adulteration will create complex outcomes that are difficult to interpret. Thus, fields of application for the ARMS strategy might be large sets of similar samples have to be screened for specific adulterations.

Conclusions and outlook

Consumer protection is challenged by globalisation shifting numerous new plant products from their traditional context into the European market. Authentication of these products, especially in processed samples, is a challenge, especially for plant families that are rich in species and therefore taxonomically difficult. We have used Moldavian dragonhead, *D. moldavica* L., as case study to assess traditional microscopic analysis versus genetic authentication. Although we succeeded to define anatomical markers that allow us to discriminate *Dracocephalum* from surrogation by *M. officinalis* L., this approach requires considerable experience and time, since the relative number of palisade cells subtending epidermal pavement cells has to be assessed. Moreover, morphological traits in *Dracocephalum* seem to be variable, which is also reflected in discrepancies in earlier publications on anatomical features. We therefore used the genetic barcoding marker rbcLa to develop a diagnostic assay and compared two strategies: RFLP and ARMS in both pure and commercial samples. Although RFLP using *EcoR* I was able to discriminate *D. moldavica* L. from *M.*

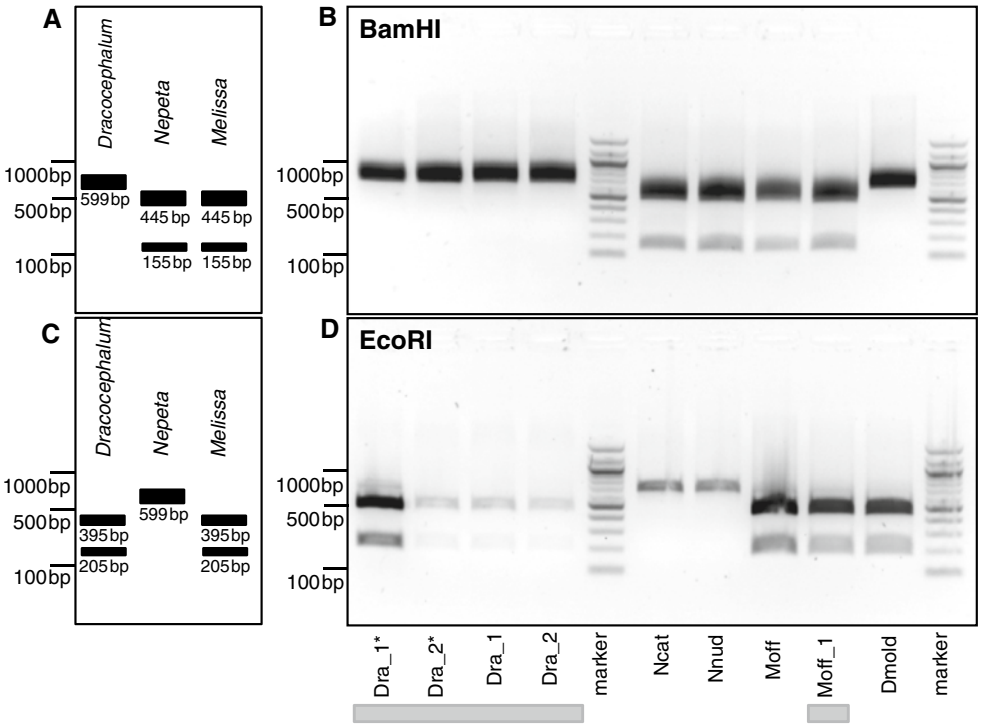


Fig. 4 Discrimination of *Dracocephalum moldavica* L. from its surrogate species *M. officinalis* and *N. cataria* based on RFLP of the rbcLa marker in pure samples and commercial tea mixtures. **b** RFLP produced by restriction with *BamH* I. **c**, **d** RFLP produced restriction *EcoR* I. **a**, **c** Banding patterns predicted from the sequence of the rbcLa marker. **b**, **d** Representative electrophoretic patterns observed for pure samples of *N. cataria* L. (Ncat), *Nepeta nuda* L. (Nnud), *M. officinalis* L. (Moff), and *D. moldavica* L. accession Dra2 (Dmold), along with commercial samples Dra_1, Dra_2, and Moff_1 along with samples Dra_1* and Dra_2* derived from the respective commercial sample by selecting putative dragonhead material based on microscopic features described in Table 3

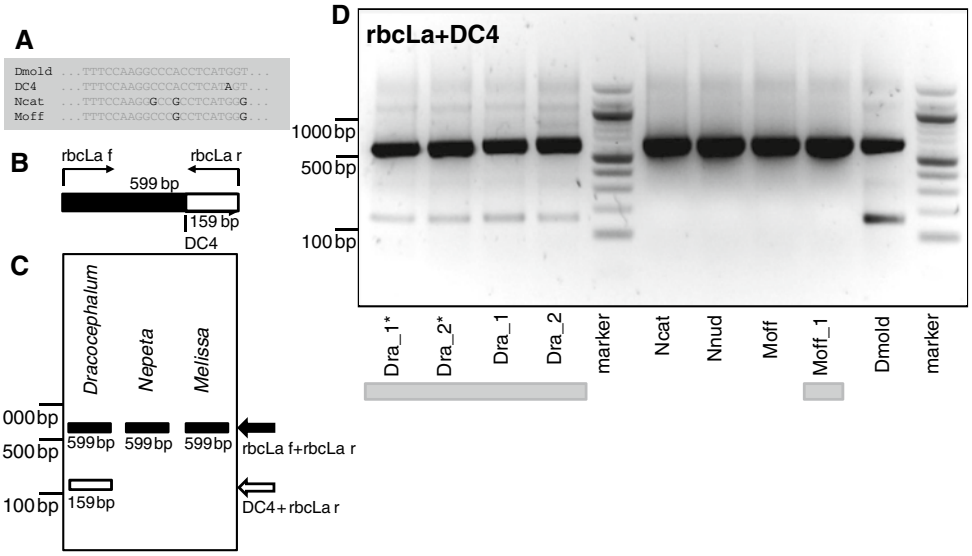


Fig. 5 Discrimination of *Dracocephalum moldavica* L. from its surrogate species *M. officinalis* and *N. cataria* based on ARMS of the rbcLa marker in pure samples and commercial tea mixtures. Design of ARMS primer DC4 in relation to the target sequence from *Dracocephalum moldavica* L. (Dmol) versus *M. officinalis* (Moff) and *N. cataria* (Ncat). Bold letters indicate base exchanges relevant to annealing stability of the primers. **b** Position of the ARMS primer in the rbcLa fragment; **c** banding patterns predicted for successful differential annealing during multiplex PCR using the primer set-up given in **b**. **d** Representative electrophoretic patterns observed for multiplex PCR using primer DC4. For details on the samples, refer to Fig. 4

officinalis L. and *N. cataria* L., this method has the drawback that failure of digestion would pretend the presence of dragonhead in samples from the surrogates (false-positive result). In contrast, RFLP using BamH I will produce a positive result (two smaller bands) only in case of successful restriction digest, but cannot discriminate dragonhead from *Melissa*. Moreover, RFLP requires two steps (PCR, followed by restriction digest overnight).

We therefore used the ARMS strategy based on multiplex PCR, where intermediate primers are added that are designed such that through specific nucleotide exchanges relative to the target sequence they will only anneal in case of *D. moldavica* L., but not in case of the surrogates. This approach leads to a diagnostic smaller band in addition to the full-length rbcLa amplificate. The ARMS strategy offers three advantages over the RFLP strategy: (1) the diagnostic band is only produced for *D. moldavica* L., not for the surrogates, (2) the presence of the full-length rbcLa amplificate safeguards against experimental failure such as problems with the Taq polymerase, and (3) a clear diagnostic result is obtained in a one-step protocol.

Similar to other Labiatae genera, the genus *Dracocephalum* is composed of numerous species with 35 species reported alone for China. The published record on anatomy and chemical compounds in *D. moldavica* L. is highly variable, in many cases even discrepant [9–11, 14, 15]. A closer look revealed that in none of these studies the taxonomic identity of the investigated specimen had been verified nor were any vouchers deposited. This suggests that species identity is obviously relevant, but has been almost completely ignored, so far. We are therefore exploring the genetic variability within the genus *Dracocephalum* and the possibility of refined genetic authentication strategies with a discriminative power beyond the genus level. This will be highly relevant for the booming market for the often expensive phytotherapeutical products used in Traditional Chinese Medicine, where ARMS and RFLP have already been used for molecular authentication based on nuclear genes [29], however, without deposition of voucher plants.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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