DNA-Based Identification of Calendula officinalis (Asteraceae)

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Protocol Note

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

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

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

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

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

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

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

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

METHODS AND RESULTS

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

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

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

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

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

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

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

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

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

Note: *n* = number of individuals.
[a] Nucleotide position is given, with diagnostic nucleotides in parentheses; the first is the species-specific nucleotide.

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

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

DISCUSSION

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

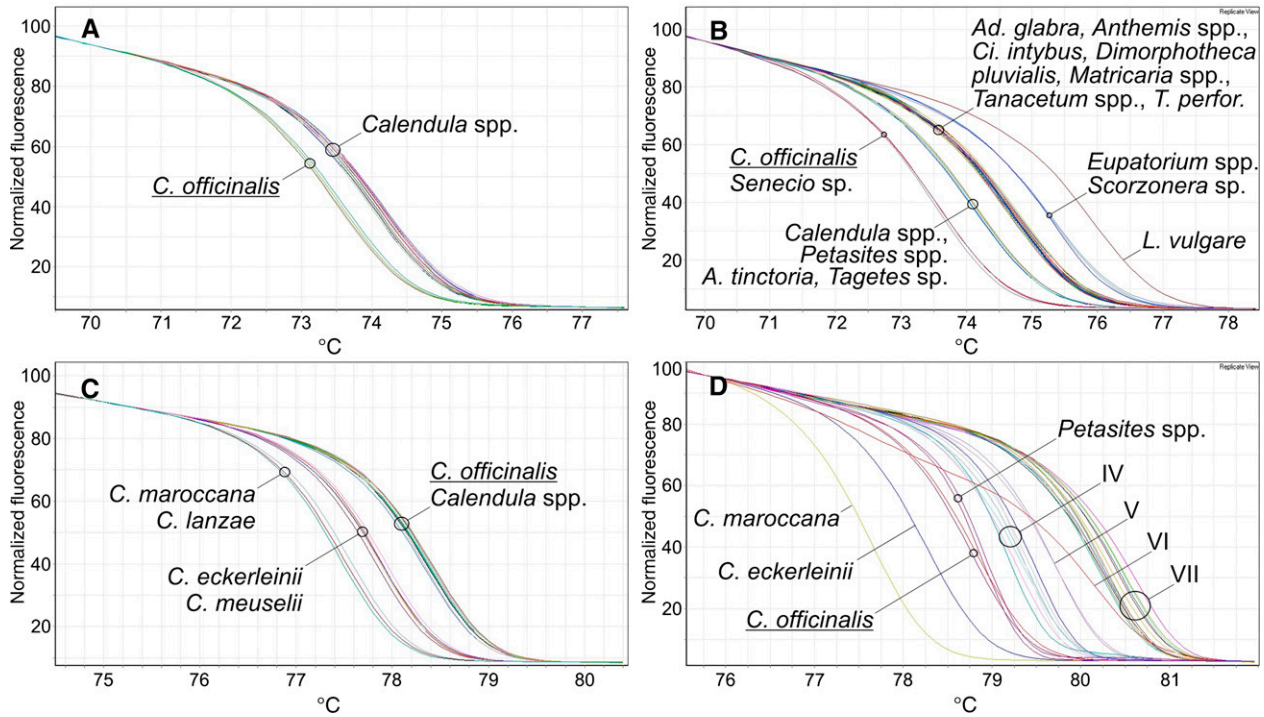

Fig. 1. HRM analysis based on two chloroplast markers. *A.* = *Anthemis*, *Ad.* = *Adenostyles*, *C.* = *Calendula*, *Ci.* = *Cichorium*, *L.* = *Leucanthemum*, *T. perfor.* = *Tripleurospermum perforatum*. (A) HRM analysis with the primer pair Cal_trnK_2F&R amplifying one species-specific SNP (A/C)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

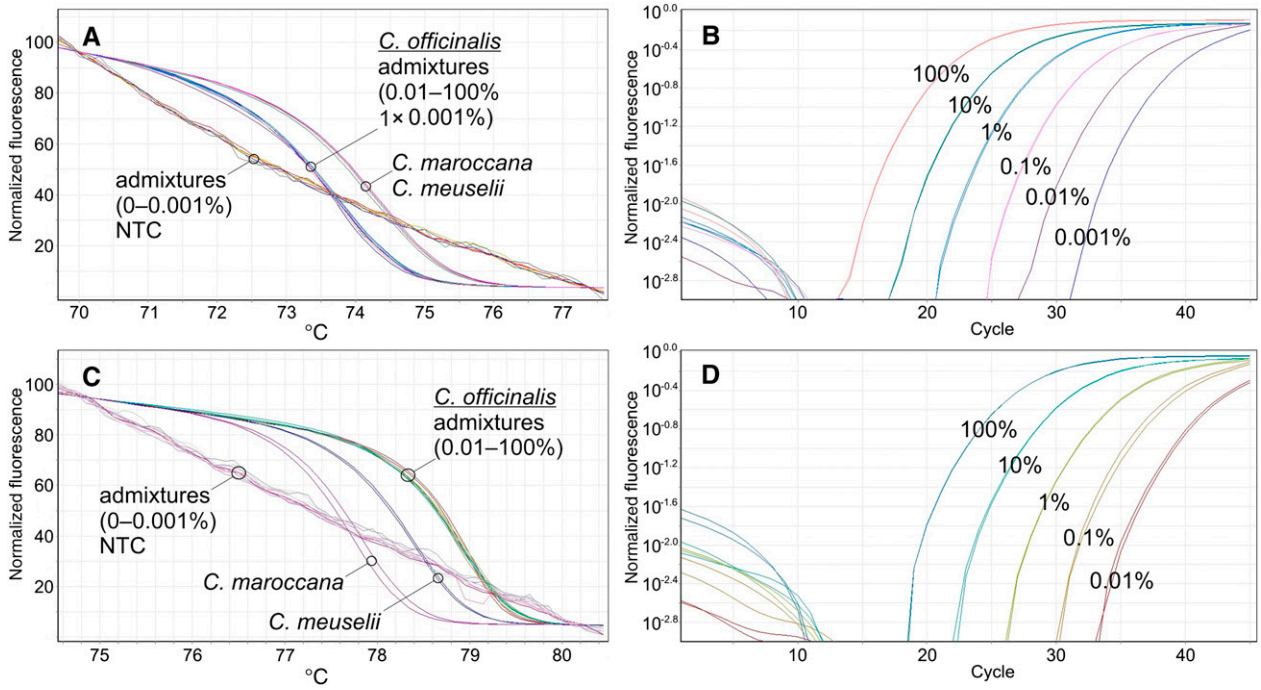

Fig. 2. Analysis of artificial admixtures of *Calendula officinalis* in saffron. All properly amplified admixture samples showed an equivalent HRM curve like the *C. officinalis* references. (A) HRM analysis with the primer pair Cal_trnK_2F&R. (B) Amplification plot of the qPCR corresponding to A. (C)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

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

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

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

Appendix 1. Continued.

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

Note: *n* = number of individuals.

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

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

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