DNA-Based Identification of Calendula officinalis (Asteraceae)

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Protocol Note

**DNA-based identification of *Calendula officinalis* (Asteraceae)**

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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 the orange cultivars (Talhouk et al., 2007), and as feed additives to improve the color of the orange cultivars (Talhouk et al., 2007). 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...
(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 stigma (*Crocos 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 μM 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), 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 alignment 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 *psbA-trnH* intergenic spacer, and the *psbA-trnF* intergenic spacer were sequenced from 22 samples of 10 *Calendula* species and two *Dimorphotheca pluriloba* (L.) Moench samples (GenBank accession no.: KM365075–KM365196, 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 MgCl₂, 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 10/20/20 s. The melting analysis was performed by increasing the temperature from 68°C to 82°C by 0.1°C. 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 [c/o (2^ct)] – 1. The straight calibration line included five measuring points for each primer combination. The efficiency of the *trnK* primers was 93.0% (R² = 0.9994), and the efficiency of the *trnL-trnF* primers was 78.5% (R² = 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. lancea Maire, group II consisted of samples of *C. eckerleini* Ohle and *C. mesulei* 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](http://www.bioone.org/loi/apps)

| PCR and Sequencing | Reverse primer | Reference |
|--------------------|----------------|-----------|
| ITS*               | TCTCTCCGGCTTTGATGAC         | White et al., 1990 |
| Cal_trnK_2F*       | 12 matK-1506R               | Johnson and Solitis, 1994 |
| Cal_trnK_2F*       | 13 matK-1848R               | Johnson and Solitis, 1994 |
| Cal_trnL-F_1F*     | 5′ AUGUCUGUUGAGGAAAAGTTG    | Heinzen, 2007 (matK11); Weising and Gardner, 1999 (ccmp1) Taberlet et al., 1991 |
| rbcLa_F            | 5′ TATGACAATTGGCAGG         | Sang et al., 1997 (psbA3); Tate and Simpson, 2003 (rbcH) Levin et al., 2003 (rbcL_B); Fazekas et al., 2008 (rbcL_aj634R) |
| HRM Analysis       | TCTAGCCCTAAATGGTGTTT           | This study |
| Cal_trnK_2F*       | 5′ TACACGCTGCGCTGCAATG       | This study |

* Amplicon size: Cal_trnK_2F&R = 71 bp; Cal_trnL-F_1F&R = 126 bp.

http://www.bioone.org/loi/apps
Table 2. Diagnostic nucleotide candidates to distinguish individual species.

| 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) 0 | 0    | 0         |
| C. incana subsp. . microphylla | 1 | 513 (T/C) | 254 (C/A) | 1190 (T/G) | 0 | 0 |

Note: n = number of individuals.

*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/mL. 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]; C. cartwrightianus Herb. [JX903624.1]; Iris pseudacorus L. [KC118962.1]; trnL-trnF: Iris luevigata 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 Crocus 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 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...
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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| Species                                  | n | Herbarium ID no. (Laboratory code) | Collection locality (Collection date) |
|------------------------------------------|---|-----------------------------------|---------------------------------------|
| 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)                 | 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. eckerleitii Ohle                      | 12 | IPK-CAL 9                         | Morocco, ACCID: 49196                 |
| C. incana Wild. (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. lancae 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. meuseli 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 L. 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 | WU0327733 (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)* | Collection locality (Collection date)‡ |
|---------|---|-----------------------------------|---------------------------------------|
| C. triplinerve | 1 | IPK-CAL 49 | Morocco, ACCID: 50047 |
| C. triplinerve | 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 alissima L. | 1 | IPK-ANTHE 18 | Cultivated, ACCID: 49159 |
| A. arvensis L. | 1 | IPK-ANTHE 7 | Cultivated, ACCID: 49145 |
| A. austriaca Jaq. | 1 | Anh 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 |
| T. tinctoria L. | 1 | IPK-ANTHE 25 | Armenia, ACCID: 57847 |
| A. tinctoria | 1 | IPK-ANTHE 33 | Cultivated, ACCID: 236444 |
| A. tinctoria | 2 | Rühl-Ant05x | Trade sample |
| A. tinctoria | 3 | Ast 03-5 | Austria, V, Baumann Gasse; 48°15’15”N, 16°25’54”E (23.6.2011) |
| 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. perforatum L. | 1 | Rühl-Eup02 | Trade sample |
| E. purpureum L. | 1 | Rühl-Eup03 | Trade sample |
| Helianthus annuus L. | 1 | Cal111 | Cultivated, V, Siebensterngasse |
| T. perforatum | 1 | Rühl-Chr02 | Trade sample |
| Tripleurospermum perforatum (Mérat) M. Laínz | 1 | Anh 04 | Austria, LA, Kamptal; 48°37’51”N, 15°36’51”E (6.8.2011) |

Note: n = number of individuals.

*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.

†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.

‡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.