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GENETIC DIVERSITY ASSESSMENT OF *Calendula officinalis* WILDING PLANTS LOCALLY GROWN IN BULGARIA, USING SRAP MARKERS

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Abstract

The pot marigold (*Calendula officinalis* L.) is a well-known garden plant cultivated also for medicinal purposes. In Bulgaria, *C. officinalis* is an annual plant, often self-seeding and generating garden escapees. The present study describes assessment of the genetic diversity of wilding *C. officinalis* plants originating from the region of the Rose Valley, Bulgaria, using SRAP (sequence-related amplified polymorphism) markers. Analysis of the data using the Structure software did not support the existence of a clear genetic structure within this set of analyzed wilding plants. Further comparative SRAP analysis of small sets of *C. officinalis* plants originating from seeds of wilding plants and seeds derived from various European seed suppliers was carried out. The results suggest that the genetic diversity of the tested wilding plants largely resembles the range of genetic diversity of the plant sets derived from commercially available *C. officinalis* seeds. The possibility of using wilding *C. officinalis* plants for selection of breeding lines and varieties, well adapted to the local conditions and suitable for organic growing and production of bioactive extracts and compounds is discussed.

Key words: *Calendula officinalis*, SRAP markers, genetic diversity, wilding plants, Bulgaria

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Introduction. Pot marigold (*Calendula officinalis* L.) is a popular garden plant, native to Southern Europe. It has been cultivated worldwide for centuries both for ornamental and medicinal purposes [1]. The aerial parts of *C. officinalis* have a wide medicinal application, including treatment of inflammations of internal organs, wounds, burns, as a diuretic, diaphoretic, antiseptic and many more, reviewed by [2]. Recently, the seeds of *C. officinalis* have received scientific attention due to the relatively high oil content – up to 20% [3]. The flower yield and the composition of *C. officinalis* extracts and seed oil depends strongly on the cultivation conditions, varieties used and harvest date [1–3]. In Bulgaria, the research on *C. officinalis* is focused mainly on its use as a medicinal plant [4, 5].

The efficient use of *C. officinalis* for production of bioactive extracts, products and compounds requires semi-industrial cultivation at organic manner of varieties with well characterized and known metabolite composition. Therefore, expanding the *C. officinalis* use as a medicinal plant will require better characterization of the *C. officinalis* genetic and metabolic diversity and development of breeding lines and varieties well adapted to the local conditions and suitable for organic cultivation and production of bioactive products. Currently, the genetic diversity of *C. officinalis* is poorly studied. Several studies employed random amplified polymorphism (RAPD) analysis to study the genetic variability among European pot marigold varieties [6, 7], and lines obtained after colchicine-induced mutagenesis [8]. Testing and using other types of molecular markers will further enhance the characterization of the existing genetic resources and their further utilization within the breeding programmes. The self-seeding of *C. officinalis* and its cross-pollination by bees results in efficient generation of garden escapees and wilding plants, well adapted to the local environmental conditions. Characterization of the genetic and metabolic diversity of the local pool of *C. officinalis* wilding plants could make possible their use for selection of lines suitable for organic cultivation and production of bioactive extracts and compounds.

Sequence-related amplified polymorphism (SRAP) markers [9] have received a broad application in genetic diversity studies, including in oil-bearing and medicinal plants [10–12].

Here we report SRAP analysis and assessment of the genetic diversity among *C. officinalis* wilding plants originating from the region of the Rose Valley, Bulgaria, as well as plants grown from seeds derived from various European seed producers. The possible utilization of wilding plants for selection of lines and organic growing of *C. officinalis* for medicinal purposes is discussed.

Material and methods. ***Plant material.*** *C. officinalis* plants originating from various seeds sources and grown in the experimental field of the AgroBioInstitute, Kostinbrod, Bulgaria were used in the study. The seeds designated Co1 were provided by the Rosa Select Ltd., Bulgaria. These seeds were collected from wilding plants from the region of Rose Valley, Bulgaria. The rest of the *C. officinalis* seeds used in the study were purchased from different European providers

including: a) seeds without designated variety: Co2 (Agrosel Srl), Co3 (OPAL ZI Ltd., Bulgaria), Co4 (Franchi Sementi S.p.a), Co5 (Lactofol), Co6 (Florian Ltd.), Co13 (Ivesto Ltd.), and b) seeds with designated variety from Muller Bloemzaden BV, Holland: Co7 (var. Ball's orange), Co8 (var. Prince orange), Co9 (var. Prince golden), Co10 (var. Prince indian), Co11 (var. Bon-bon orange) and Co12 (var. Bon-bon yellow).

DNA extraction. Total DNA was extracted from fresh leaves (100 mg), ground to fine powder in liquid nitrogen using TissueLyserII laboratory mill (QIAGEN GmbH), at 30 Hz for 2 min. Further, a modified CTAB protocol [13] was used. The purified total DNA was quantified spectrophotometrically using Nanodrop 2000 (Thermo Scientific), equalized to 25 ng μL^{-1} and stored at – 20 °C until further use.

SRAP analysis. The SRAP primers were designed according to Li and Quiros [9]. The forward 'ME' primers were labelled at the 5' end with FAM dye. PCR reactions were carried out in a volume of 25 µl containing: 2 µL of DNA templates (25 ng μL^{-1}), 0.1 µM of forward and reverse primer, 12.5 µL of MyTaq HS DNA Polymerase (Bioline Ltd., UK). Fragments were amplified using the following PCR programme: 5 min at 94 °C; 3× cycles of 1 min at 94 °C, 1 min at 35 °C and 1 min at 72 °C; 35× cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C; and a final elongation step of 3 min at 72 °C [12]. PCR products were diluted 10-fold. For electrophoresis, 1 µL of the diluted PCR amplified product was mixed with 0.5 µL of the GeneScan 600 LIZ Size Standard and 9 µL of the Hi-Di Formamide (Thermo Fisher Scientific Inc). The mixture was denatured, separated and analyzed on the 3130 Genetic Analyzer using 3130 POP-7 Polymer and a 36 cm capillary array (Thermo Fisher Scientific Inc.).

Statistical analysis of experimental data. FAMD 1.31 [14] was used to build a neighbour-joining dendrogram based on Jaccard indices. GDdom [15] was used to calculate gene diversity/polymorphic information content (PIC) for each marker (primer pair) applying the formula according to Roldàn-Ruiz et al. [16]. Structure v 2.3.4 [17] was used for analysis of genetic structure while the method of Evanno et al. [18] was used to estimate the most probable K. The 10 different iterations at each K value were combined using Clumpak [19].

Results and discussion. Three SRAP primer pairs, namely EM3 × ME5, EM5 × ME7 and EM7 × ME1, were used in the present study. The primer pairs were selected for their ability to prime PCR amplification from DNAs of the tested *C. officinalis* plants and to generate electropherograms and SRAP patterns with low noise and distinct and well-observed SRAP peaks. In the first round of the study, the selected SRAP primer pairs were used for SRAP analysis of a set of 91 plants derived from seeds collected from wilding plants from the region of the Rose Valley, Bulgaria. Analysis of the data using the Structure software with 10 iterations for each K from K=1 to K=30 did not support the existence of a clear genetic structure within this set of analysed plants, (data not

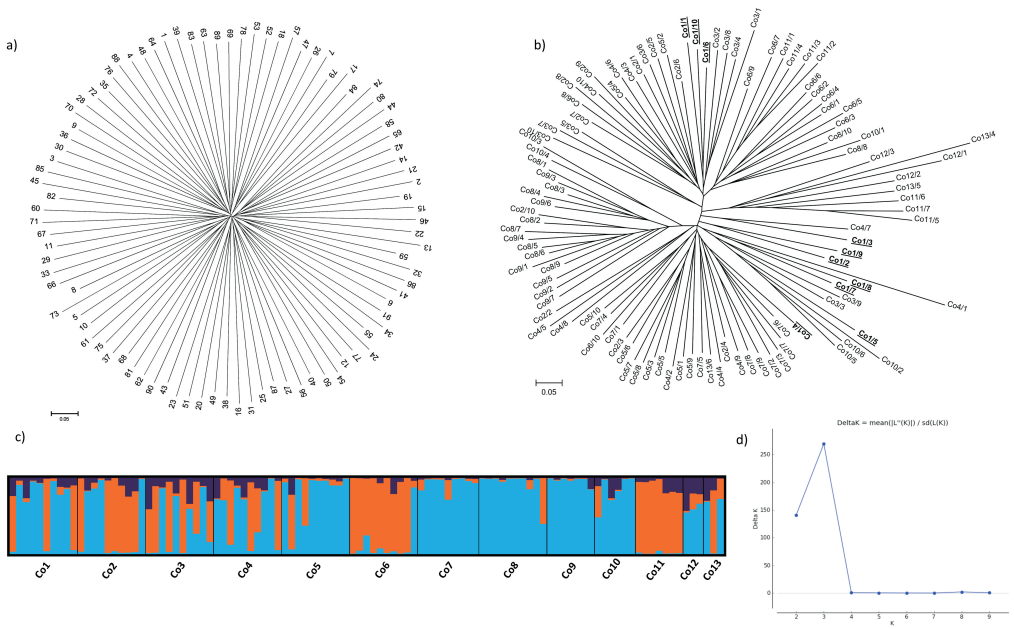


Fig. 1. (a, b) Neighbour-joining clustering based on Jaccard indices of: a) plants from seeds of wilding plants from the region of the Rose Valley, Bulgaria, and b) plants from different seed sets including seeds from wilding plants (Co1) and seeds from various commercial sources (Co2 to Co13) described in the 'Plant material' section. The last number in each plant designation corresponds to the number of the individual plant in the seed set. (c,d) Genetic structure of the analyzed plant sets described in 'b', based on SRAP marker data; c) Bar plot representing the genetic structure at K=3. d) Estimation of the most probable K using the delta K method by Evano et al. [18]

shown). Further construction of phylogenetic tree did not show distinct clustering of the analyzed plants (Fig. 1a). The second round of the study involved parallel SRAP analysis of a total of 108 plants including small subsets of plants derived from batch of seeds from different seed producers. The SRAP data showed that the application of the three primer pairs used in the study in combination with fragment analysis by capilary sequencer resulted in scoring of a relatively large number of SRAP fragments, ranging from 89 to 168 per primer pair and a total of 405 SRAP fragments for the entire plant set. Furthermore, all of the scored SRAP fragments were polymorphic among the tested plants. The analysis of the results also showed that the obtained SRAP data allowed to resolve all tested plants as the calculated PIC (polymorphic information content) ranged between 0.12 and 0.13, which is comparable with the results in the earlier reported similar studies of natural populations of *Hyssopus officinalis* [11] or genetic resources of tree species [20]. Further data analysis using chierarchical clustering showed a complex pattern with the formation of multiple clusters (Fig. 1b). Whereas some of the clusters involved plants from the same sources, the majority of the formed

clusters involved plants derived from different seed providers, or from different varieties of the same seed provider. This was also confirmed by the results for genetic structure of the entire set of tested plants shown in Fig. 1c, with delta K maximising at K = 3 (Fig. 1d). The genetic structure data also revealed that the tested set of wilding plants involved plants with genetic makeup resembling that of plants derived from the different seed suppliers.

The overal data from the present study demonstrated that SRAP markers could be efficiently applied for characterization of *C. officinalis* genetic resources providing high resolution capacity. The obtained results further suggest that the genetic diversity of the tested wilding plants well resembles the range of genetic diversity of comercially available *C. officinalis* seeds, some of which are rather genetically heterogenous as well. On the other hand, the wilding plants are better adapted to the local environment, which makes them suitable as a base for semi-industrial organic growing of *C. officinalis* for medicinal purposes. The described above, rises an attractive opportunity for utilizing the locally growing wilding *C. officinalis* plants as a source for selection of lines with high content of bioactive compounds and suitable for semi-industrial organic growing, which is essential for production of various medicinal, cosmetic and personal hygiene phytoproducts and food additives. Accordinglly, such selection will require additional characterization of the metabolic diversity and composition of bioactive compounds, as well as subsequent comparative evaluation of the biological activity of extracts from pre-selected *C. officinalis* plant lines.

Conclusions. The results of the present study demonstrated that SRAP markers could be efficiently applied for characterization of *C. officinalis* genetic resources. The obtained data suggest that the analyzed wilding *C. officinalis* plants possess genetic diversity well resembling the range of genetic diversity of the tested *C. officinalis* plants originating from seeds from various comercial sources. This makes wilding *C. officinalis* plants a promising source for selection of lines and varieties well adapted to the local conditions and suitable for semi-industrial organic growing, which is essential for their application for production of bioactive extracts and compounds.

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