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Evaluating the potential of a few barcode markers in identifying the species *Calluna vulgaris* (L.) Hull

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Abstract *Calluna vulgaris* is an evergreen species belonging to the Ericaceae with a wide distribution. This species is ecologically resilient and can be found in a large range of habitats. The DNA barcoding method can be used for species identification being considered a complementary tool which supports the classical taxonomy. The method is based on using one or few short standardized DNA sequences, which are species-specific. We evaluated the utility of three such sequences for the identification of *Calluna vulgaris*: *matK*, *trnH-psbA*, and *rpoC1*. The curent study investigated ten european populations of *Calluna vulgaris*, including a local population (Cliț, Sălaj). For all samples the study found a lack of intraspecific variability for the *matK* and *rpoC1* markers. The PCR amplification for trnH-psbA did not work in standard conditions. The results suggest that *Calluna vulgaris* can be consistently and correctly identified using *matK* and *rpoC1*.

Key words

Calluna vulgaris (L.) Hull., DNA barcoding, *rpoC1*, *matK*, *trnH-psbA*

Calluna vulgaris (L.) Hul*l* is a monotypic species of the Ericaceae family with a vast distribution area, from the south of Spain to the north of Scandinavia and from the Azores islands to the Ural Mountains (13). The species dominates in the heath communities throughout the north-western regions of Europe (11). Previous studies, using molecular methods (allozymes and chloroplast DNA), found variability between European populations of *Calluna vulgaris* (10, 11).

This study uses the DNA barcoding approach to see if three barcode markers differentiate between European populations of *Calluna vulgaris* or not, because DNA barcoding is considered a modern and effective molecular method (14). This method was used previously also at intra-specific level to assess variability (4). The following barcode markers were tested: *matK*, *trnH-psbA*, and *rpoC1*. *matK* and *trnH-psbA* are among the most frequently used DNA barcode markers (3) while *rpoC1* can be easily amplified through PCR with one pair of primers and it can be easily sequenced and aligned (6).

Material and Methods

Calluna vulgaris samples were collected from ten different locations across Europe including a location in Romania (Cliț, Sălaj County) (Table 1).

Total genomic DNA was extracted using a Bioline kit (Isolate Plant DNA Mini Kit), with a quick extraction protocol based on spin columns, adapted as described in Căprar et al., 2014 (2). An estimation of the quality and quantity of the DNA extracted was obtained through spectrophotometry with the Nanodrop 2000 UV-VIS Spectrophotometer (Thermo Fisher Scientific) and through agarose gel (2% w/v in TAE 1X) electrophoresis. The DNA ladder used was from Gene Direx (50 bpDNA Ladder RTU). For gel documentation a G:Box Chemi XR5 (Syngene) was used.

Table 1

Information on the location of *Calluna vulgaris* samples used in the experiment

Population code	GPS coordinates	Country
CVC	N 46° 38' 328" E 23° 00' 906"	Romania
CS	N 55° 05' E 11° 15' 08"	Sweden
CVA	N 50° 45' 50" E 13° 45' 28"	Germany
CVG	N 52° 02' 20" E 13° 44' 55"	Germany
CVF	N 60° 27' 57" E 22° 01' 30"	Finland
CVL	N 66° 32' 25" E 25° 47' 59"	Finland
CVI	N 64° 14' 15" E 12° 08' 07"	Norway
CVII	N 62° 01' 38" E 10° 48' 48"	Norway
CN	N 59° 25' 30" E 10° 42' 26"	Norway
CVX	N 57° 13' 35" E 24° 25' 05"	Latvia

PCR was used to amplify the *matK*, *trnH-psbA*, and *rpoC1* barcode markers. The sequences of the primers were taken from Kress and Erickson, 2007 (9) and are presented in Table 2. The PCR reaction was performed using 200 ng genomic DNA, 0.5 µl of each primer (20 µM), 5 µl of My Taq Reaction Buffer 5X (Bioline), 0.2 µl of My Taq DNA Polymerase (Bioline) and ddH_2O, in a total reaction volume of 25 µl. The protocol had the following steps: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 90 s and a final elongation step at 72°C for 2 min. The amplification products were visualized by using agarose gel electrophoresis as described earlier. The PCR products were purified using the Favor Prep Gel/PCR Purification kit (Favorgen) and sent for sequencing at Macrogen Europe.

Table 2

PCR primers used in the experiment

Primer	Primer sequence 5'→3'	Melting temperature (°C)
rpoC1 1f	GTGGATACACTTCTTGATAATGG	46.6
rpoC1 3r	TGAGAAAACATAAGTAAACGGGC	
matK 2.1f	CCTATCCATCTGGAAATCTTAG	46
matK 2.5r	GTTCTAGCACAAGAAAGTCG	58
trnH-psbA f	ACTGCCTTGATCCACTTGGC	62
trnH-psbA r	CGAAGCTCCATCTACAAATGG	47.3

The DNA sequences obtained were manually adjusted with the Bioedit software (7). Alignments were made using the ClustalW algorithm in MEGA 6 (12). Phylogenetic trees were generated also in MEGA 6 with the Maximum Likelihood method based on the Kimura 2 Parameter distance model (8). Bootstrap values were calculated for 1000 replicates (5). In the construction of the trees, several sequences belonging to species from Ericaceae were used as outgroups. The sequences have the following Genbank accession numbers: JF728306.1, GQ997840.1, JQ067650.2, JQ757046.1 and GG997791.1.

Results and Discussions

The results of the DNA extraction, PCR and sequencing are synthetized in Table 3. All three markers were amplified with a single pair of primers using the same PCR conditions. Only the *matK* and *rpoC1* markers were sequenced. The sequencing was made using the forward primer. After processing, the sequences had a length of 786 bp (*matK*) and 503 bp (*rpoC1*). The *trnH-psbA* marker was not sent for sequencing due to its low PCR amplification efficiency. Although *matK* is known to present low

amplification and sequencing rates with the available primers (15), in the present experiment it was successfully amplified and sequenced. The results obtained with *rpoC1* are comparable to the results obtained for *matK*.

Table 3

Experimental results regarding the *rpoC1*, *matK* and *trnH-psbA* barcode markers in percents

DNA Isolation efficiency	Marker	PCR efficiency	Sequencing efficiency
93.3%	*rpoC1*	89.2%	90.9%
	matK	89.2%	95.2%
	trnH-psbA	35.7%	-

The sequences for the *rpoC1* and *matK* markers yielded perfect alignments which means there is no sequence variability in the *Calluna vulgaris* populations at the level of these markers. Although a local population was taken in consideration (CVC), it does not vary from the other nine populations collected from remote geographical areas (north-western Europe). Phylogenetic trees were generated using outgroups in order to reveal the homogeneity of the studied populations in what regards the *rpoC1* and *matK* barcodes. The outgroups were chosen, as available, from the Ericaceae family based on the comparison of the obtained *Calluna vulgaris* sequences with homologue sequences from Genbank using Nucleotide BLAST (1). It was found that there are no *rpoC1* sequences for *Calluna vulgaris* in the Genbank Database.

Although there are studies made with allozymes and PCR-RFLP methods which found differentiation between European *Calluna vulgaris* populations (10, 11), the DNA barcoding method used in this experiment did not find any differentiation.

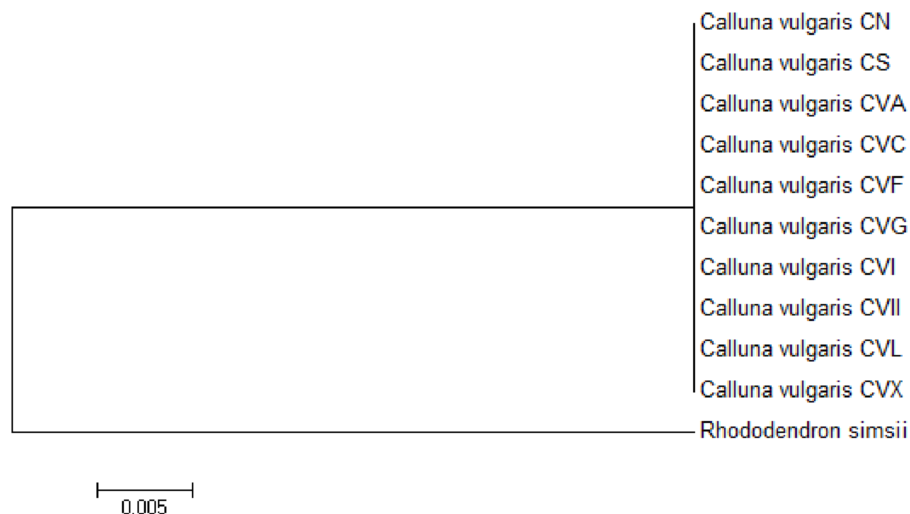

Fig. 1. Phylogenetic tree based on the *matK* barcode for ten *Calluna vulgaris* populations and one outgroup

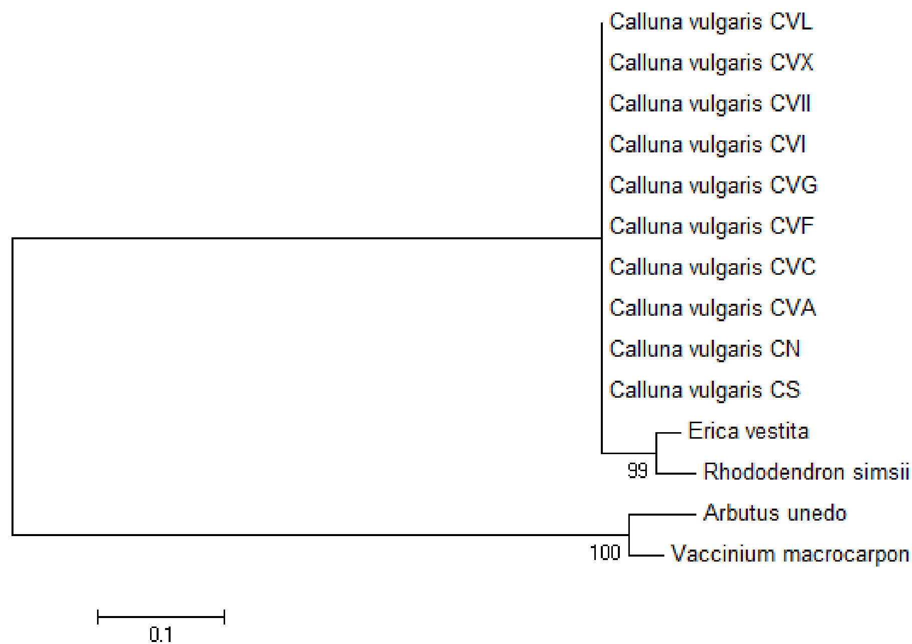

Fig. 2. Phylogenetic tree based on the *rpoC1* barcode for ten *Calluna vulgaris* populations and several outgroups

Conclusions

1.In the present experiment, the *matK* and *rpoC1* barcode markers were easy to amplify and sequence.
2.The *trnH-psbA* barcode marker, although reccommended as a suitable marker due to its high variability could not be satisfactory amplified, with the available primers, in this experiment.
3.The *matK* and *rpoC1* sequences from the *Calluna vulgaris* populations studied did not present variability which would make them suitable barcode markers for identifying this species.

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