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Original scientific article

SPECIES IDENTIFICATION AND GENETIC DIVERSITY OF *Alcea* (Malvaceae) USING SCOT MOLECULAR MARKERS: MEDICINAL PLANT

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The genus *Alcea* that is a member of Malvaceae family, is a Mediterranean perennial herb with major diversity centers in the Middle East and Western Mediterranean Basin. *Alcea* is a unique genus with several endemic taxa, and many of these endemic taxa from the Irano-Turanian phytogeographical area are particularly interesting. The genus contains 34 species in Iran, 15 of which are endemic. As a result of the importance of *Alcea* species, molecular data were collected for this genus in this research. For this study, 83 plants were used, which were randomly obtained from 5 species found in 6 provinces. Genomic DNA Amplification with 5 primers resulted in 75 bands, 66 of which were polymorphic (88.12%). The high average MI and PIC values indicated that SCoT primers have a high capacity for detecting polymorphic loci among the species of *Alcea*. The range of genetic similarities between 5 collected species was estimated to be between 0.77 and 0.89. The SCoT markers analysis revealed that the species *Alcea angulata* and *Alcea popovii* had the least similarity, while *Alcea loftusii* and *Alcea sulphurea* had the most similarity. The current study's objectives are as follows: 1) is it possible to identify *Alcea* species through SCoT markers, 2) what genetic structure do these species have in Iran, and 3) what is the species inter-relationship? The current research found that SCoT markers can be used to identify the species.

Keywords: *Alcea*; Iran, Species Identification; Structure, SCoT **(**Start Codon Targeted)

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INTRODUCTION

Identifying the accurate boundaries of a species is critical to have a better perspective of any biological studies. Therefore, species delimitation is a subject of extensive part of studies in the framework of biology (ZHANG, *et al*., 2021). **T**he genus *Alcea* L., a member of Malvaceae family, is perennial herb of Mediterranean with main centers of diversity in the Western Mediterranean Basin and the Middle East. According to RIEDL (1976), 39 species are found in Iran, however, the amount has since been shrunk to 34 because of rearranging taxonomies among them, 15 species are endemic (PAKRAVAN, 2008b; YIN *et al*., 2021; JIA *et al.*, 2020). *Alcea* species are mainly yearly, biyearly or perennial, mainly tall-growing hemicryptophytes. The stem is erect and rarely branched from the base or acaulescent in a few cases. The leaves are variable in shape from simple to lobed, palmatipartite or palmatisect. Carbohydrates are found in the mucilage of plants in the Malvaceae family, which has medical usages (AZIZOV *et al*., 2007; BI *et al*, 2021; CHENG *et al*., 2021). The family's species, particularly *Alcea rosea*, have been utilized as demulcents, aperients, diuretics, emollients, and to treat burning skin disease, sensations, and constipation (SHAHEEN *et al*., 2010).

Alcea exhibits a considerable taxonomic complexity. So far, two infrageneric classifications have been proposed in *Alcea*, each classified into some informal groups (BENTHAM and HOOKER, 1862; CANDOLLE, 1837; BAKER, 1890; EDLIN, 1935). Even so, differences in fruit characteristics and staminal column led to consider *Althaea* and *Alcea* as two separate taxa (ALEFELED, 1862; BOISSIER, 1867).

Many molecular marker methods have been developed and widely employed in assessing genetic diversity, phylogenetic relationships, and population structure as plant molecular biology progresses. In recent years, progressions in genomic tools have offered a variety of new marker methods including gene targeted and functional markers and the development of several innovative DNA-based marker systems. One of the new, simple and dependable gene-targeted marker systems is start codon-targeted (SCoT) polymorphism. This molecular marker provides a simple and reproducible DNA-based marker substitute based on the short conserved region in the plant genes surrounding the ATG (COLLARD and MACKILL, 2009) translation start codon (COLLARD and MACKILL, 2009).

The current study was conducted to assess the relationships and genetic diversity among various *Alcea* species by employing novel gene-targeted molecular markers, namely SCoT. Due to being the first research using SCoT markers in *Alcea* genus, a molecular study was conducted on 83 collected samples from 5 species of *Alcea*.

MATERIALS AND METHODS

Plant materials

During July-August 2018-2019, 83 individuals were surveyed from 5 geographical populations belonging to 5 *Alcea* species Iran's provinces of in East Azerbaijan, Kermanshah, Esfahan, Tehran, Hamadan, Semnan and Fars (Table 1). For SCoT analysis, 83 plant accessions (5 to 12 specimens from each population) from 5 various populations with varying eco-geographic features were collected and kept in -20 until further usage. Table 1 contains more information about the accessions' geographical distribution.

Table 1. List of the studied taxa, including origin of voucher specimens.

Taxa	Locality	Collector & Voucher
Alcea popovii Iljin F	Tehran, Damavand Semnan, 20 km NW of Shahrud	Salehi 9988 Golabi 8546
Alcea loftusii (Baker) Zohary	Kermanshah, Islamabad Tehran, road of Firozkuh	Laleh 9876 Fatemi 3569
Alcea striata (DC.) Alef	Fars, 7km from Evaj to Lar	Fatemi 3567
Alcea angulata Freyn & Sint.	Hamedan, 20 km S of Nahavand Azarbaiejan, 48 km from Tabriz to Marand	Fatemi 3568 Fatemi 3569
Alcea sulphurea (Boiss.& Hohen.) Alef.	Azarbaiejan, Kaleiybar, Arasbaran	Fatemi 3579

Morphological studies

Morphometry was performed on five to twelve specimens from each species. Totally, 32 morphological parameters (22 qualitative and 10 quantitative) were investigated. The collected data were standardized (variance = 1, Mean= 0) and then used to calculate Euclidean distance for ordination and clustering analyses (PODANI, 2000; ZOHARY, 1963a, b; HUTCHINSON, 1973; RIEDL, 1976; HEYWOOD *et al*. 1978). These characters are: calyx length, calyx width, corolla length, corolla shape, corolla color, leaf shape, leaf length, seed surface ornamentation, stamens position, style position, leaf margin and disc, and sepal indumentums.

SCoT Assay and DNA Extraction

In each of the populations studied, Fresh leaves were collected at random from one to twelve plants. Silica gel powder was used to dry them. Genomic DNA was extracted using the CTAB activated charcoal protocol. The extracted DNA's quality was assessed using a 0.8% agarose gel. COLLARD and MACKILL (2009) developed 22 SCoT primers, 5 of which had enlarged, clear, and rich polymorphism bands (Table 2). PCR reactions were conducted in a 25µl volume mixture containing the following component: 0.2 mM of each dNTP; 1.5 mM $MgCl_2$; 50 mM KCl; 50 mM KCl; 10 mM Tris-HCl buffer, pH 8; (Bioron, Germany); 20 ng genomic DNA; 0.2 µM of one primer and 3 U of *Taq* DNA polymerase (Bioron, Germany). In Techne thermocycler (Germany) the amplifying reactions were conducted with the PCR settings as follows: 5 minute denaturation phase at 94°C, proceeded by 40 cycles of 1 minute at 94° C; 1 minute cycles at 52-57°C and 2 minute cycles at 72°C. The reaction was finished by the final 7-10-minute extension phase at 72°C. The PCR amplified items were identified on a 1% agarose gel after staining with ethidium bromide. The size of the fragment was measured using a ladder with a molecular size of 100 bp (Fermentas, Germany).

Data Analysis

Morphological Researches

Morphological trait (Variance = 1, Mean = 0) were initially standardized and employed for determine the Pythagorean distance between the pairs of taxa (PODANI, 2000). We used the ordination methods of UPGMA for classifying the plant samples (PODANI, 2000). In order to

demonstrate morphological variation between populations, ANOVA was carried out, while PCA biplot was used for classifying the most erratic morphological features among the studied populations (PODANI, 2000). PAST version 2.17 (HAMMER *et al*., 2012) was employed for multivariate statistical analysis of morphological findings.

Molecular Analysis

SCoT bands were utilized for investigating genetic diversity and were coded as binary characters (absence = 0, presence = 1). The primers' discriminatory capabilities used was evaluated through two parameters, marker index (MI), and polymorphism information content (PIC), to characterize each primer's ability to identify polymorphic loci among the genotypes. MI was determined as MI = PIC × EMR For each primer, in which EMR is obtained by the polymorphic fragment fraction (β) and the polymorphic loci number per primer (n). For each primer, both the effective multiplex ratio (EMR) and the number of polymorphic bands (NPB) were determined. Parameters such as the number of effective alleles, Shannon information index (I), the gene diversity of Nei (H), and polymorphism percentage (P% =number of total loci or number of polymorphic loci) were determined (FREELAND *et al.*, 2011). The formula for calculation of Shannon's index was: H' = -Σpiln pi. Rp is characterized per primer as: Rp = ∑ Ib, in which "Ib" is the band informativeness, which ranges between 1-(2x [0.5-p]), and "p" is the proportion of each genotype that contains the band. GenAlEx 6.4 software was used to determine the percentage of PCA, UHe polymorphic loci, and H' (PEAKALL and SMOUSE, 2006). Nei's genetic distance between populations was utilized for Neighbor-Net networking and Neighbor Joining (NJ) clustering (FREELAND *et al*., 2011; HUSON and BRYANT, 2006). The relationships between the populations' geographical and genetic distances were determined by the Mantel test (PODANI, 2000). DARwin software version 5 and PAST version 2.17 were used for these analyses (2012). The AMOVA test (with 1000 permutations) was employed for assessing population genetic variations in GenAlex 6.4. Nm, a gene flow estimate from Gst in PopGene version 1.32 (1997), was calculated as follows: Nm = 0.5 (1 - Gst)/Gst. This technique takes into account the same amount of gene flow between all populations.

RESULTS

Identifying Species and inter-relationship

Morphometry

ANOVArevealed significant differences (P <0.01) in quantitative morphological traits among the investigated species. PCA analysis was used for determining the most varied characteristics among the studied taxa. It was discovered that the initial three factors accounted for more than 82% of the total variation. With 65% of total variation in the first PCA axis, such characteristics like leaf length, leaf shape, corolla color, and seed surface ornamentation had the highest correlation (>0.7)m while corolla length, stem length, leaf width influenced PCA axes 2 and 3 respectively. Because various ordination and clustering methods yielded comparable results, morphological PCA plots are presented here (Figs 1). Plant specimens from each species were generally classified together and made separate classifications. This finding indicates that both qualitative and quantitative morphological characteristics distinguished the studied species. No intermediate forms were found in the specimens studied.

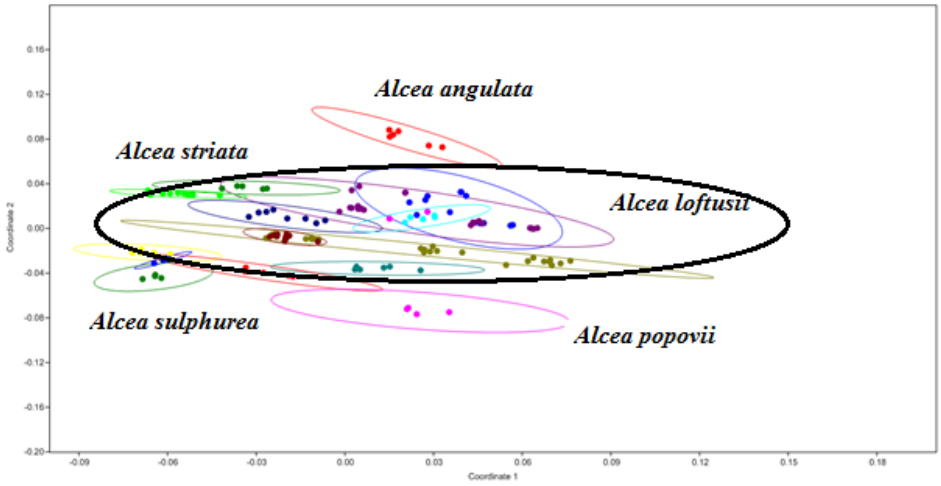

Figure 1. PCA plots of morphological characters revealing species delimitation in the *Alcea* species

Species Identification and Genetic Diversity

For investigating genetic relationships among the species of *Alcea*, five SCoT primers were tested; all of which generate reproducible polymorphic bands in all five species of *Alcea*. Across five *Alcea* species, 66 amplified polymorphic bands were produced. The amplified fragments were between 100 and 3000 bp in size. SCoT-16 had the most polymorphic bands (22), while SCoT-18 had the fewest (9), and each primer had an average of 12.7 polymorphic bands. The range of the five SCoT primers PIC was between 0.24 (SCoT-11) and 0.55 (SCoT-17), and the average was 0.40 per primer. The range of the primers' MI was between 3.11 (SCoT-18) and 5.47 (SCoT-11), and the average was 4.9 per primer. The range of SCoT primers' EMR was between 7.23 (SCoT-11) and 12.55 (SCoT-16), and the average was 9.6 per primer (Table 2). The primers that had the highest EMR values were regarded as more informative for identifying the genotypes.

All the five *Alcea* species that were amplified with SCoT primers had their genetic parameters calculated (Table 3). The range of unbiased expected heterozygosity (*H*) was between 0.15 (*Alcea sulphurea*) and 0.29 (*Alcea striata*), and the mean was 0.22. Shannon's information index (*I)* followed a similar pattern. The highest value of Shannon's information index was 0.26 as observed in (*Alcea striata*), while the lowest value of it was 0.17 as observed in (*Alcea sulphurea*), and the mean was 0.23. *Alcea loftusii* had 0.234 observed number of alleles (*N*a), while *Alcea striata* had 1.345 one. The number of effective alleles (*N*e) varied from 1.011 (*Alcea loftusii*) to 1.677 (*Alcea striata*).

Table 2. Scot primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
SCoT-6	CAACAATGGCTACCACGC	14	14	100.00%	0.44	5.12	11.56	4.85
SCoT-11	AAGCAATGGCTACCACCA	11	11	100.00%	0.24	2.44	7.23	5.47
SCoT-16	CCATGGCTACCACCGGCC	22	22	100.00%	0.34	3.55	12.55	5.11
SCoT-17	CATGGCTACCACCGGCCC	16	10	66.00%	0.55	3.63	8.56	4.76
SCoT-18	ACCATGGCTACCACCGCG	12	9	90.22%	0.35	4.11	7.56	3.11
Mean		15	12.7	88.12%	0.40	4.1	9.6	4.9
Total		75	66					

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers

Table 3. Genetic diversity parameters in the studied Alcea species.

SP	N	Na	Ne	I	He	UHe	%P
Alcea popovii Iljin	18.000	0.477	1.187	0.256	0.233	0.248	41.26%
Alcea loftusii (Baker) Zohary	13.000	0.234	1.011	0.244	0.23	0.26	49.23%
Alcea striata (DC.) Alef	20.000	1.345	1.677	0.26	0.284	0.292	50.91%
Alcea angulata Freyn & Sint.	15.000	0.358	1.117	0.23	0.25	0.22	44.30%
Alcea sulphurea (Boiss.& Hohen.) Alef.	16.000	0.458	1.039	0.17	0.12	0.15	39.38%

Abbreviations: N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations.

A significant genetic difference (P = 0.001) is revealed among studied species by the AMOVA test. This test showed that 25% of total variation was within species and 75% was among species (Table 4). Furthermore, significant D_est values (0.932, P = 0.001) and Nei's GST (0.55, P = 0.001) were found to demonstrate genetic differentiation between these species. These findings indicated that genetic diversity was distributed more evenly among *Alcea* species than within species.

Table 4. Analysis of molecular variance (amova) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	37	1234.364	76.711	15.122	75%	
Within Pops	110	222.443	12.844	6.866	25%	75%
Total	147	1456.80		21.033	100%	

Because various ordination and clustering methods yielded comparable results, NJ clustering is displayed here (Figure 2). Plant specimens from each species were generally grouped together and created separate cluster. According to these findings, the studied molecular characteristics can classify *Alcea* species into two main groups or clusters. We found no

intermediate forms in the studied specimens. Two major groups were generally formed in NJ tree (Figure. 2). Populations of *Alcea popovii* and *Alcea striata* were positioned in the first major group and were separated from the other species. The second major group had two sub-clusters.

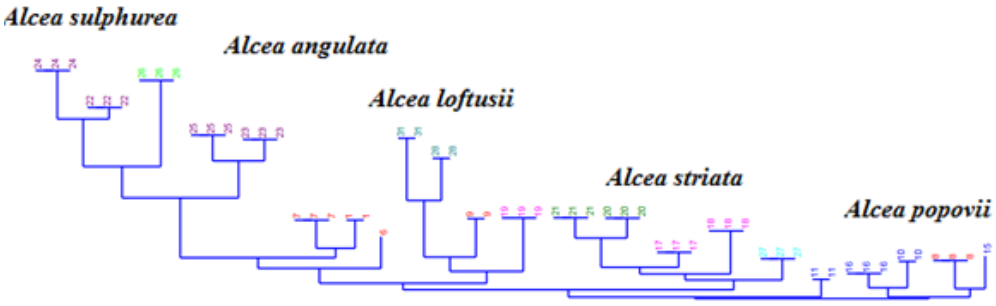

Figure 2. NJ tree of SCoT data revealing species delimitation in the *Alcea* species

The first sub-cluster was made up of *Alcea angulata* plants, whereas the second was made up of *Alcea loftusii* and *Alcea sulphurea* plants.

In general, SCoT-derived relationships agree well with morphological-derived species relationship. This is consistent with the previously presented genetic diversity parameters and AMOVA. Genetically, the species are distinct. These findings suggest that SCoT molecular markers are applicable in classifying *Alcea* species. Popgene software's Nm analysis produced a mean Nm= 0.422, which is regarded to be a low value for gene flow among the species studied.

A significant correlation (r = 0.44, p=0.0002) was revealed between geographical distance and genetic distance by Mantel test with 5000 permutations, indicating that isolation by distance (IBD) existed among the studied *Alcea* species.

The genetic identity of Nei's and also the genetic distance among the species under consideration were determined (Table not included). According to the findings, *Alcea loftusii* and *Alcea sulphurea* had the highest level of genetic similarity (0.89). *Alcea angulata* and *Alcea popovii* had the least amount of genetic similarity (0.77). The low Nm value (0.422) shows ancestrally shared alleles among the studied species or limited gene flow, as well as high genetic differentiation among and within *Alcea* species.

DISCUSSION

Genetic diversity plays an important role in the long-term evolution of a population or a taxon in biology, and it is the foundation of taxon's evolution, growth, and existence. As a result, studying taxon genetic diversity is crucial for understanding the taxon's origin, evolution, and

taxonomy. Furthermore, such studies shall offer a theoretical foundation for the utilization, breeding, conservation, and development of the germplasm resource (LUBBERS *et al.*, 1991).

In our study morphology and genetic diversity in 5 taxa of *Alcea* are given in detail for the first time. The current study sought to identify diagnostic characteristics among *Alcea* species in Iran. As previously indicated (ESCOBAR GARCIA *et al*., 2012), Morphological characteristics are regarded as an effective method for species identification. Morphological studies of the studied species of *Alcea* showed that qualitative characters and quantitative measures (the result of ANOVA test) are well distinguished from each other (The result of the PCA plot). Furthermore, PCA analysis indicated that morphological characteristics such as leaves size and shape , calyx size and indumentum, and corolla shape and color may be used in the delimitation of species groups.

Genetic Structure and Gene Flow

Within *A. rosea* populations in Iran, 93% polymorphism level was found by KAZEMI *et al*. (2011), which had a high variation in genetic similarity(0.31 to 0.75), based on RAPD markers analysis. ÖZTÜRK *et al*. (2009) used RAPD markers to examine the genetic profiles of 18 *Alcea* species and found wide differentiation (0.13 to 0.69) among them. BADRKHANI *et al.* (2014) used a SRAP marker to evaluate the genetic similarity and genetic diversity relationships among14 *Alcea* species obtained from northwest of Iran. 104 fragments were yielded by 17 SRAP primer combinations; 97 of 104 fragments (93%) were polymorphic, and on average, 5.7 polymorphic fragments were generated per primer. The range of polymorphism percentage was between 50% (ME2-EM6) and a maximum of 100%, with a mean polymorphism information content value of 0.3. *A. sophiae* and *A. flavovirens* had the least genetic similarity (0.17), whereas *A. digitata* and *A. longipedicellata* had the highest (0.68). UPGMA detected two major clusters that did not correspond to species's geographical origin. According to their findings, SRAP markers may be suitable for evaluating genetic variation in *Alcea*. So far, morphological data has been used to characterize Iranian *Alcea* species. Because of the small number of characters, the genus has a complicated classification. According to PAKRAVAN (2008) research on *Alcea*, only the leaf sequence and carpels configuration examination would be examined. A. fl avovirens and A. glabrata, for example, are only different only in the carpel size and wing width (PAKRAVAN, 2008). Our findings classified these two species into two distinct clusters.

ESCOBAR GARCIA *et al*. (2012) demonstrated a phylogeny of *Alcea* and examined earlier infrageneric taxonomic hypotheses, using three molecular markers. He also tested *Alcea*'s monophyly concerning *Althaea*. *Althaea* is a genus which is frequently merged with *Alcea*. They also deal with the morphological variation and the use of morphological characters as phylogenetic predictors. Their findings indicate that while morphological circumscription of *Alcea* is unambiguously supported by molecular data, yet, they have little use in resolving interspecific relationships, implying that *Alcea*'s high species diversity could be attributed to fast and recent radiation. Their research provides *Alcea'*s first phylogeny and intends to lay the groundwork for future research into the processes underlying the radiation of species in the Irano-Turanian region. The current research took advantage of the SCoT method abundance and ubiquity in plant genomes and their importance in genomic diversification for applying and developing retrotransposon markers to *Alcea* for the first time.

Finally, the findings of the present research indicated that the SCoT-derived primers were more efficient than other molecular markers in assessing the genetic diversity of the *Alcea*. In addition, the dendrogram and PCA clearly separated *Alcea* species, implying that SCoT method is more efficient in identifying *Alcea* species.

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IDENTIFIKACIJA VRSTA I STRUKTURA POPULACIJA *Alcea* (Malvaceae) PRIMENOM SCOT MOLEKULARNIH MARKERA: MEDICINSKE BILJKE

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Izvod

Rod *Alcea* koji je član familije Malvaceae je mediteranska višegodišnja biljka sa glavnim centrima diverziteta na Bliskom istoku i zapadnom mediteranskom basenu. *Alcea* je jedinstven rod sa nekoliko endemičnih taksona, a mnoge od ovih endemskih taksona iz iransko-turanskog fitogeografskog područja su posebno zanimljive. Rod sadrži 34 vrste u Iranu, od kojih je 15 endemskih. Zbog značaja vrste *Alcea*, u ovom istraživanju su prikupljeni molekularni podaci za ovaj rod. Za ovu studiju korišćene su 83 biljke, koje su nasumično dobijene od 5 vrsta pronađenih u 6 provincija. Amplifikacija genomske DNK sa 5 prajmera je rezultirala sa 75 traka, od kojih je 66 bilo polimorfno (88,12%). Visoke prosečne vrednosti MI i PIC su pokazale da SCoT prajmeri imaju visok kapacitet za detekciju polimorfnih lokusa među vrstama *Alcea*. Opseg genetskih sličnosti 5 sakupljenih vrsta je procenjen na između 0,77 i 0,89. Analiza SCoT markera pokazala je da najmanje sličnosti imaju vrste *Alcea angulata* i *Alcea popovii*, dok najviše sličnosti imaju *Alcea loftusii* i *Alcea sulphurea*. Ciljevi ovog rada su ispitivanja: 1) da li je moguće identifikovati vrste *Alcea* pomoću SCoT markera, 2) kakvu genetsku strukturu ove vrste imaju u Iranu, i 3) kakav je međusobni odnos vrsta? Ovo istraživanje je pokazalo da se SCoT markeri mogu koristiti za identifikaciju vrste.

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