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PHYLOGENETIC STUDY OF TEN SPECIES FROM *CENTAUREA* (ASTERACEAE) IN DUHOK CITY, KURDISTAN REGION-IRAQ

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ABSTRACT

The current research aimed to estimate the evolutionary relationships of ten *Centaurea* L. species growing naturally in the Duhok City, Kurdistan region of Iraq. The combing Start Codon Targeted (SCoT) markers with Internal Transcribed Spacer (ITS) gene region barcode were performed. To detect the DNA sequence variations and phylogenetic tree reconstruction, the Dice similarity matrix, the unweighted pair group method with arithmetic mean (UPGMA) clustering and Maximum Likelihood (ML) methods were applied. 104 polymorphic bands were scored with an average of 10.4. The Polymorphic Information Content (PIC) and Resolving Power (Rp) values ranged between (0.24 to 0.36) and 3.4 in primer (SCoT1) to 12 in primer (SCoT53) with an average of 0.319 and 5.74 respectively. The lowest similarity value was 0.52 between *C. behen* L. and *C. solstitialis* L., while the highest was 0.82 between *C. balsamita* and *C. rigida*. The reconstructed polytomous dendrogram was as follows: clade one; *C. solstitialis* L.; clade 2, *C. balsamita* Lam. and *C. virgata* Lam.; clade three subdivide into two subclades: *C. iberica* Trev. ex Spreng., *C. hayalolepis* Boiss., *C. brugueriana* (DC) Hand. Mazz. and *C. gigantea* Sch. Bip. Ex Boiss., *C. regia* Boiss., *C. rigida* Banks & Sol., *C. behen* Lam. Furthermore, *C. brugueriana* (DC) Hand. Mazz., *C. iberica* Trev. ex Spreng, *C. behen* L., *C. solstiotialis* L. and *C. balsamita* Lam. were nested with National Center for Biotechnology Information (NCBI). In contrast, the remaining taxa were mixed with other closely related species. Thus, ScoT markers and ITS DNA barcode were considerably effective for investigating the evolutionary relationships of *Centaurea* taxa.

KEY WORDS: Phylogenetic Study, *Centaurea,* Asteraceae, Duhok, Kurdistan, Iraq.

1. INTRODUCTION

Asteraceae is one of the baggiest families of angiosperms, comprises of 43 tribes, 12 subgenera, 1600 genera, and up to 30000 species (Funk et al. 2009). The Cardueae tribe has been divided into 12 main subtribes including Centaureinae (Herrando-Moraira et al. 2019). Considerable studies conducted to delimit, determine, and classify the subtribe Centaureinae (Wagenitz and Hellwig, 2000; Garcia-Jacas et al., 2000; Greuter, 2003; Herrando-Moraira et al., 2019). Herrando-Moraira et al., 2019 recognized 12 monophyletic genera containing *Centaurea*. The genus *Centaurea* s.l., comprises approximately 400-700 species which considered as one of the largest genera of Daisy family. This genus is widely distributed in Mediterranean basin to Irano–Turanian areas (Hellwig, 2004).

Taxonomically, *Centaurea* regards as a problematic genus due to considerable variation on morphology, karyology, and pollen diversity (Wagenitz 1955, Uysal et al., 2017; Şirin et al., 2022). In the middle of fifteenth, Wagenitz 1955 separated the *Centaurea* species into three distinct groups based on pollen structure and diversity: *Cyanus*/*Montana*, *Acroncentron* and *Jacea*. Furthermore, Garcia-Jacas et al. (2001) approved the finding when evolutionary relationships were studied. Thirteen years later, Hilpold et al. (2014a) divided the *Centaurea* s. lat. morphologically into two distinct groups: C. sect. *Phalolepis* (Cass.) DC., and sect. *Centaurea* (Cass.) DC. On the same year, the genus *Centaurea* subdivided to 3 subgenera: *Centaurea*, *Cyanus* (Mill.) Cass. ex-Hayek and *Lopholoma* (Cass.) Dobrocz (Hilpold et al., 2014b).

This considerable variation was obvious in Iran with 70 species (Wagenitz, 1980), Turkey with 219 taxa (Özbek, 2021), and mediterranean region with 250 (Susanna & Garcia-Jacas, 2007). Consequently, the *Centaurea* as a genus represents as an outstanding model plant for evolutionary process analysis, introgression, hybridization, gene flow revealing (Hilpold et al., 2014b; Garcia-Jacas, et al., 2009).

In Iraq, there are four morphological groups of *Centaurea* divided into fourteen sections including 43 native species distributed all over Iraq mainly in restricted mountain areas (Ghazanfar et al., 2019).

Thus, enormous molecular studies have been conducted to solve these taxonomic problems in the genus *Centaurea*. Since the early twentieth, the genus *Centaurea* enormously subjected to molecular applications in terms of DNA barcoding, predominantly Internal Transcribed Spacer (ITS) gene regions (Garcia-Jacas et al. 2000, 2001; Font et al., 2009; Hilpold et al. 2014b; Arnelas et al. 2018; Lopez-Alvarado et al., 2020; Sirin et al., 2022), DNA markers; simple sequence repeats and Inter Simple Sequence Repeats (a López-Vinyallonga, et al., 2015; Atasagun, 2022). However, Start Codon Targeted (SCoT) is widely used on Asteraceae family and some of these researches focusing on *Centaurea* species (Feng et al., 2016; Kamińska et al., 2019; Jedrzejczyk 2020; Atia et al., 2021; Jamshidi et al., 2024). In the current study, the genetic variations and species relationships are investigated using DNA markers (SCoT) and nuclear gene (rDNA) barcodes. The achieved results might help for reconstruct concert phylogenetic tree for all remaining taxa in Iraqi flora.

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2. MATERIAL AND METHODS

2.1 Plant samples

The plant materials were samples from the different locations of the Duhok city – Kurdistan Region of Iraq. The collected samples were dried and the herbarium specimens were prepared then deposited in the Duhok Province University Herbarium (DPUH) (Table 1 and Figure 1).

Table 1: showing *Centaurea* taxa codes, names, locations, coordinates, and herbarium specimens identification numbers.

Codes	Taxa names	**Location**	**Coordinates**	**Herbarium specimens ID No**
1C	*C. solstitialis*	Zakho City / sheransh	37.23168945N 42.84625196E	3937
2C	*C. bruguierana*	Duhok Baedre	36.74798584N 43.24991194E	3949
3C	*C. balsamita*	Zakho Sere Reza	37.11914062N 42.67837174E	4257
4C	*C. behen*	Zakho Sere Reza	37.11914062N 42.67837174E	4256
5C	*C. rigida*	Duhok Zawete	36.92932129N 43.14986068E	3945
6C	C. *hyalolepis*	Zakho – Duhok Road	37.1274189N 42.67811940E	3938
7C	*C. regia*	Zakho City Gali	37.07717896N 42.65238483E	3940
8C	C. *iberica*	Zakho City Gali	37.07717896N 42.65238483E	4255
9C	*C. gigantea*	Duhok Bablo	36.87799072N 43.13043568N	3947
10C	*C. virgta*	Zakho Sheranesh	37.17327881N 42.74206720E	4258

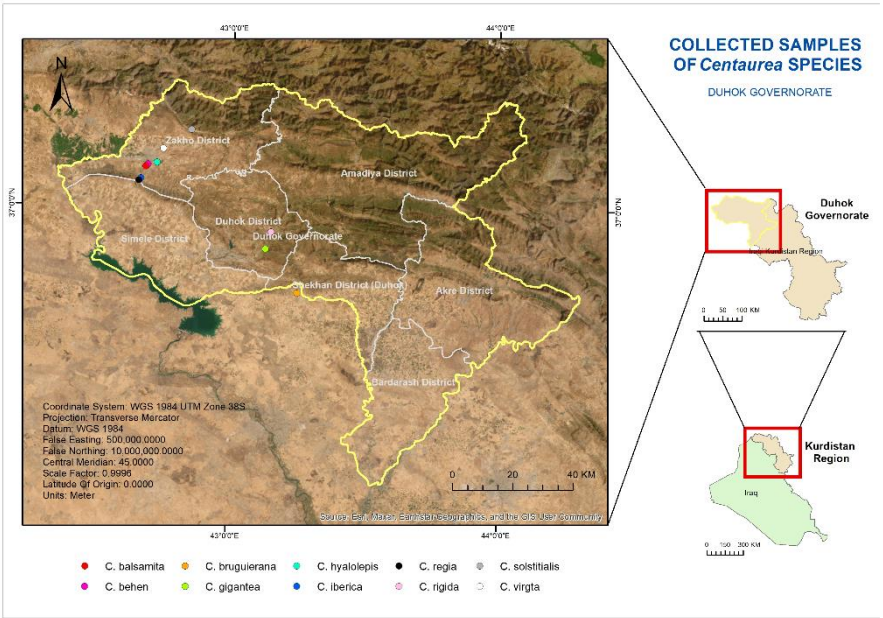

Figure 1: A map illustrates the distribution of the collected samples of the *Centaurea* plants species in the Duhok governorate. The map is designed in ArcMap software version 10.7.1.

2.2 DNA extraction from *Centaurea* taxa:

The DNA samples were isolated from the fresh leaves of 10 *Centaurea* L. species following the standard cetyl-trimethylammonium bromide (CTAB) method (Weigand et al., 1993) with minor modifications by Hussein and Jubrael (2021). The DNA quantity was estimated using the nanodrop spectrophotometer.

2.3 Polymerase Chain reaction (PCR) technique:

For the DNA barcoding of Nuclear Ribosomal DNA Internal Transcribed Spacer, ITS and (SCoT) markers amplifications, two different data set of primers were used

(Table 2 and Table 4). 20µl PCR product was prepared as follows: 2 µl of DNA sample (50 ng), 2 µl (10 pmol) primers, 10 µl of DNA Master mix (Addbio, Korea) and 4 µl of sterile water. The following parameters were set to conduct the PCR amplifications; the thermocycler conditions were adjected for the nuclear DNA barcode as follows: 5 min at 95 °C followed by 35 cycles of 95 °C for 30 as denaturation temperature, then annealing temperature was set to 30 s and 30 at 55 °C, 72 °C respectively and then 10 min at 72 °C as an extension, whereas different program was set for SCOT marker ammplification: the iniatial denaturation temperature was 94˚C for 3 min., then 35 cycles of 1 min at 94˚C, 1 min of 50˚C as annealing temperature , and the extension was 2 and 5 min at 72˚C respectively.

Table 2: shows the oligonucleotides name, sequences and banding size used in current study.

oligonucleotides name	forward primer	reverse primer	reference	banding size (bp)
ITS region	**5'TCCTCCGCTTATTGATATGC3'**	**5'TCCGTAGGTGAACCTGCGG3'**	**White *et al.*, 1990**	**700**

2.4 Capillary Standard Sequencing

The PCR products of the ITS gene region were packed and delivered to the Macrogen Inc company (Seoul, South Korea http:// dna.macrogen.com) to sequence the single direction sanger sequencing. The amplified product was purified using the illustra GFX PCR DNA and Gel Band Purification Kit from (GE healthcare) following the manufacturer's instructions. The purified product was deposit in the Macrogen; a public biotechnology company in South Korea for DNA sequencing according to the business's instructions.

2.5 Data scoring and analysis

Each band was represented as a marker. Then, the presence (1) or absence (0) used as a code to score the SCoT marker fragments manually to generate a matrix for the raw data. The data analyzed applying the NTSYS software version 2.02 (Rohlf, 1998). The power of these markers was calculated based on the Polymorphic Information Content (PIC) and Resolving power (Rp) calculations. The PIC value was estimated using bellow formula PIC = 2f (1 – f), where f= represent the bands frequency presence; and 1-f= the bands frequency absence (Roldan-Ruiz et al., 2000). On the other hand, the resolving power (Rp) applied to calculate the ability of oligonucleotides dissimilarity detection based on the Prevost and Wilkinson (1999): Rp = ΣIb where Ib (band informativeness) which is taking the following values: 1–[2|0.5–p|], where p is the bands present percentage. The dendrogram was reconstructed using the Dice similarity matrix and the unweighted pair group method with arithmetic mean (UPGMA) clustering procedures.

2.6 Phylogenetic analysis

DNA sequencing data were analyzed using the Geneious prime software (https://www.geneious.com) (Kearse et al. 2012) and Mega 11 software (Tamura et al. 2021). The DNA data was aligned using ClustalW (Thompson et al., 2003). The best model of evolution was estimated, then, Maximum Likelihood (ML) tree was conducted using Mega 11 (Tamura et al. 2021).

2.7 Results and discussion

The preliminary results of testing the total of 15 SCoT primers, only 10 of them were work properly and generate reproducible and clear polymorphic profiles (Figure 2). Consequently, the generated data of these primers, as shown in Table (3), then implemented to estimate the genetic relationships of the ten *Centaurea* species. The total of 104 amplified bands was scored as polymorphic bands, whereas the polymorphic bands ranged from 5 to 16 with an average of 10.4. The percentage of the polymorphism was 100% in all tested primers. Thus, the SCOT markers were indicated as an efficient and applicable marker on genetic diversity analysis for the *Centaurea* species. Similar finding has been approved on work of Mirzaei and Salari (2022).

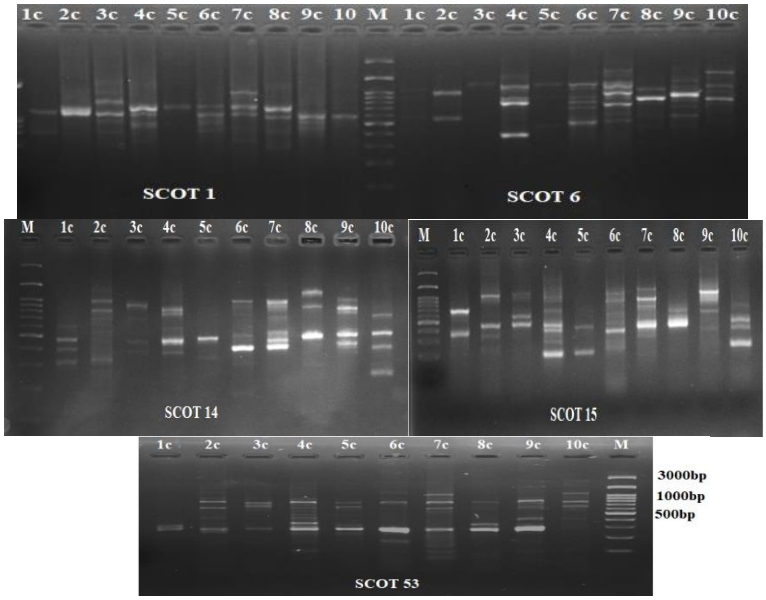

Figure 2: Amplified DNA fragments of SCoT marker: The codes on the top of pictures refer to the *Centaurea* accession numbers whereas the bottom codes are primer names (see Table 1)

The power of the oligonucleotides was estimated to differentiate the *Centaurea* species using Rp and PIC values. The Rp values was ranged from 3.4 to 12 in (SCoT1and SCoT53) primers respectively with an average of 5.74. Similarly, the PIC value was ranged from 0.24 to 0.36 with an average of 0.319 (Table 3). The PIC values are considerably variable depending on the genotype variation (Manimekalai and Nagarajan, 2006).

The genetic relationships of 10 tested *Centaurea* species based on the Dice similarity coefficient matrix shown in the (Table, 3 and Figure 3). The lowest similarity was between *C. solstitialis* and *C. behen* with (0.52) and the highest value was 0.82 between *C. balsamita* and *C. rigida*. On the other hand, the Dice similarity matrix and the UPGMA methods exhibited the clusters that were identified at the 0.83 similarity level.

Table 3: shows the genetic similarity matrix of *Centaurea* species.

C. Solstitialis	*C. bruguierana*	*C. balsamita*	*C. behen*	*C. rigida*	*C. hyalolepis*	*C. regia*	*C. iberica*	*C. gigantea*	*C. virgata*
1.0000000									
0.6767677	1.0000000								
0.7878788	0.7272727	1.0000000							
0.5252525	0.5656566	0.5555556	1.0000000						
0.7373737	0.6767677	0.8282828	0.5656566	1.0000000					
0.7171717	0.6565657	0.7474747	0.5454545	0.7373737	1.0000000				
0.5757576	0.5757576	0.6060606	0.5050505	0.6363636	0.5959596	1.0000000			
0.7171717	0.6767677	0.7676768	0.5858586	0.7373737	0.6161616	0.5353535	1.0000000		
0.6565657	0.6161616	0.6868687	0.4646465	0.6161616	0.6969697	0.5555556	0.6161616	1.0000000	
0.6363636	0.5959596	0.6464646	0.5656566	0.5757576	0.6363636	0.5151515	0.5757576	0.5959596	1.0000000

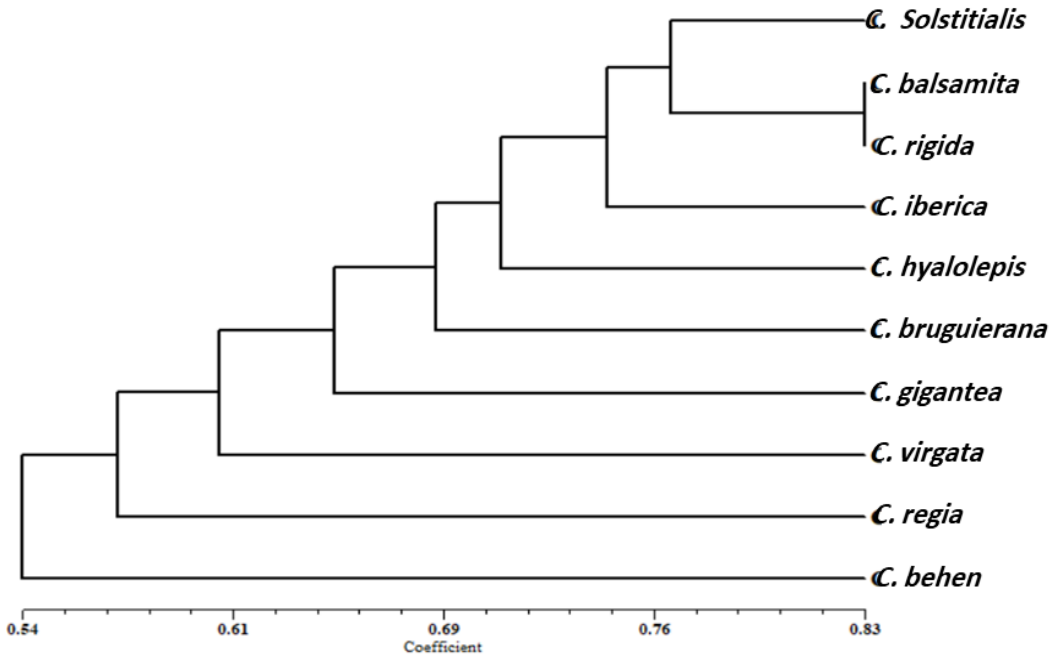

Figure 3: The illustrated dendrogram of cluster analysis of ten *Centaurea* species

Table 4: oligonucleotides names, sequences, number of bands, polymorphic bands proportions, polymorphism information contents (PIC), resolving powers (Rp) in the *Centaurea* species.

oligonucleotides names	oligonucleotides sequences (5′- 3′)	Namber of bands	Polymorphic bands	polymorphic bands proportion	PIC	RP
SCOT 1	CAACAATGGCTACCACCA	5	5	100	0.24	3.4
SCOT 6	CAACAATGGTCACCACGC	13	13	100	0.33	6.2
SCOT 11	AAGCAATGGCTACCACCA	7	7	100	0.30	3.6
SCOT 14	ACGACATGGCGACCACGC	13	13	100	0.34	7.2
SCOT 15	ACGACATGGCGACCGCGA	9	9	100	0.36	5.2
SCOT 32	CCATGGCTACCACCGCAC	8	8	100	0.32	3.6
SCOT 33	CCATGGCTACCACCGCAG	13	13	100	0.31	5.4
SCOT 34	ACCATGGCTACCACCGCA	10	10	100	0.32	6
SCOT 47	ACAATGGCTACCACTGCC	10	10	100	0.35	4.8
SCOT 53	ACAATGGCTACCACCGAC	16	16	100	0.32	12
Total		**104**			**3.19**	**57.4**
Mean		**10.4**			**0.319**	**5.74**

Phylogenetic analysis

The Nuclear ITS gene region, as a barcode, shows considerable amplifications with an approximate of 700 bp sequencing length (Figure 4). In plants, barcoding is considerably applied to resolve the evolutionary relationships of closely related species suggesting that this technique regarded as the significant tools for species differentiation (Alvarez & Wendel, 2003; Hollingsworth et al. 2011)

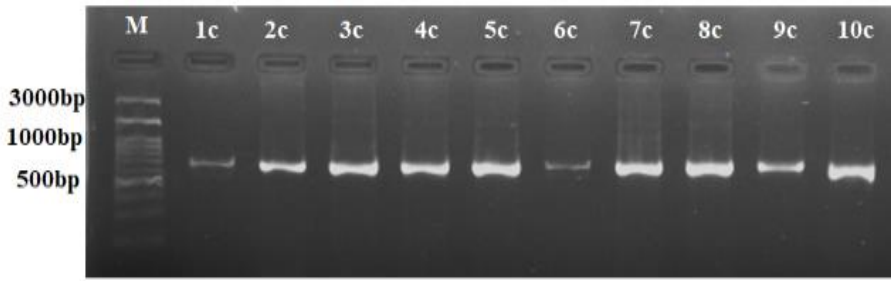

Figure 4: Amplified nuclear DNA fragments of ITS gene region: The codes on the top of pictures refer to the *Centaurea* accession numbers (see Table 1)

Despite that, the amplified gene length was 700 bp, only 630 bp was sequenced due to a single direction primer sequencing. The 10 *Centuarea* species were aligned using *ClustalW*. The best fitting substitution model of molecular evolution was chosen based on the Akaike information criteria (AIC) and Bayesian information criterion (BIC) using Modeltest v3.7 (Posada & Crandall 1998). The Tamura 3-parameter model by Tamura (1992) was selected as best. Consequently, polytomous phylogenetic trees was constructed as follows: clade one; *C. solstitialis*; clade 2, *C. balsamita and C. virgata*; clade three subdivide into two subclades: *C. iberica, C. hayalolepis, C. brugueriana* and *C. gigantea, C. regia, C. rigida, C. behen* (Figure 5). Although the current taxa are much restricted to Iraq and surrounding area, some of current results has been approved by (Sirin et al., 2022).

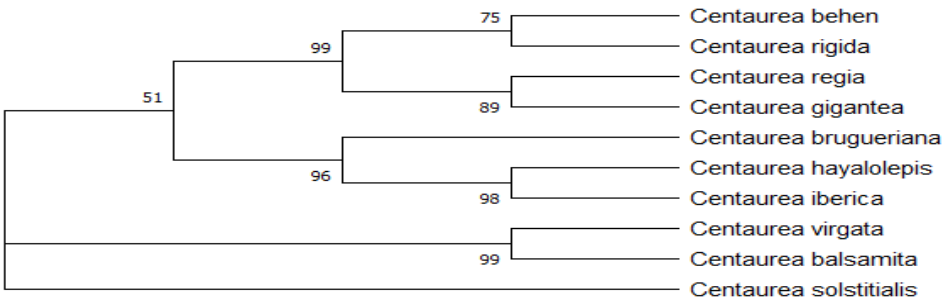

Figure 5: Maximum likelihood tree of 10 *Centaurea* species using the nuclear ITS gene region. The number above each branch is the bootstrap values if it has > 50% support.

Further investigations were carried out to find out the taxonomic position as well as checking the correct names of the studied taxa. Accordingly, 34 taxa including the current 10 investigated data were aligned and phylogenetic trees were reconstructed. Subsequently, the current taxa were positioned all around tree and clustered with either same name taxa or different closely related taxa; *C. brugueriana, C. iberica, C. behen, C. solstiotialis* and *C. balsamita* are clustered with the same names accession from gene bank, whereas C. *regia, C. rigida,* and *C. gigantea* were nested and mixed with other closely related species (Figure 6). Suggesting that genetic introgression or hybridization might occur among these taxa, this result significantly agrees with previous findings (Garcea, 1992; Font et al., 2009; Hilopd, 2014)

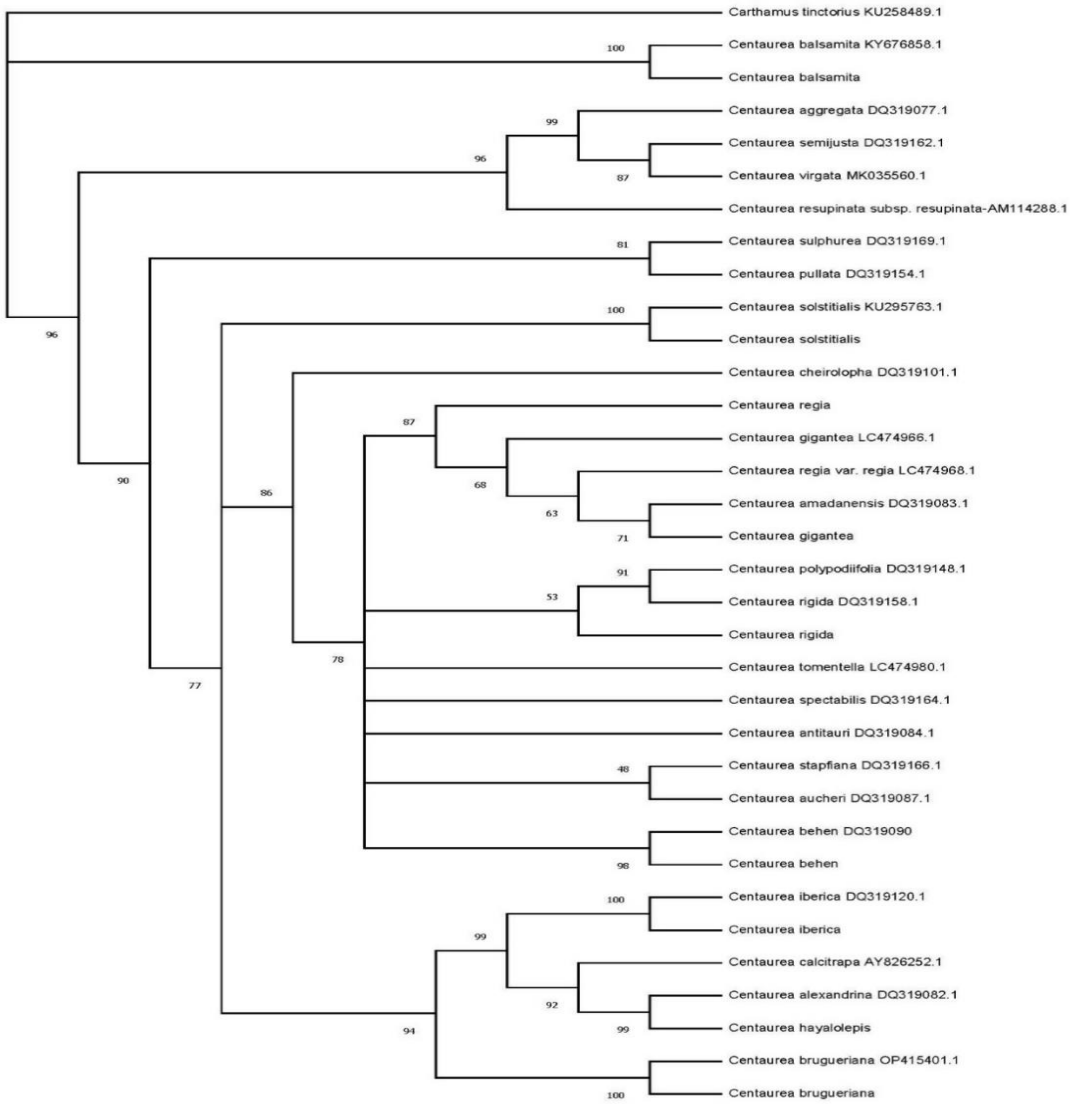

Figure 6: Maximum likelihood tree of 34 *Centaurea* species using the nuclear ITS gene region. The number above each branch is the bootstrap value if it has > 50% support.

CONCLUSIONS

The current study clarifies a new insight into the genetic diversity and species identification of some *Centaurea* species. The combination of the nr DNA region barcode with SCoT-PCR marker considers an effective method for reconstructing the evolutionary relationships and species identification of various *Centaurea* species. The SCoT marker approved that as a powerful tool for genetic variation determination. On the other hand, the internal transcriber spacer region barcoding confirms the marker results and further investigated the taxonomic position of closely related species on a phylogenetic tree. These findings will help to discover and build up the phylogenetic relationships of remaining *Centaurea s*pecies in Iraq as well as recommend applying rDNA barcoding and SCoT marker for interspecific and intraspecific identification.

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