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A SYSTEMATIC REVISION ON *CARALLUMA* SPECIES OF SAUDI ARABIA BASED ON KARYOLOGICAL AND MOLECULAR DATA

S.M. SHARAWY[1,2*], E.A. KAMEL[3,5] , E.A.K. KARAKISH[1,4] AND M.H.A. LOUTFY[5]

[1]*Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt*
[2]*Biology Department, Faculty of Science, Hail University, Hail, Saudi Arabia*
[3]*Biology Department, University College, Umm Al-Qura University, Makkah, Saudi Arabia*
[4]*Biology Department, Faculty of Applied Science, Umm Al-Qura University, Makkah, Saudi Arabia*
[5]*Biological Sciences & Geology Department, Faculty of Education, Ain Shams University, Cairo, Egypt*
**Corresponding author e-mail: sherifsharaawy@yahoo.com*

Abstract

A systematic revision of 13 species of the genus *Caralluma* (Apocynaceae – Asclepiadoideae – Ceropegieae) from the flora of Saudi Arabia based on the evaluation of karyological and molecular data was carried out. Karyotype criteria and polyploid variations were discussed. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Random Amplified Polymorphic DNA (RAPD-PCR) techniques were also used in this study to distinguish these species. Karyotype features of the studied species and molecular data were used to reassess the relationships of the 13 species of *Caralluma* in the light of the current systems of classification.

Key words: *Ceropegieae*, Karyotype analysis, SDS-PAGE, Protein analysis, DNA (RAPD-PCR), Systematic relationships.

Introduction

Caralluma R. Br. (Apocynaceae - Asclepiadoideae - Ceropegieae) is an important medicinal and widely distributed succulent taxon found in dry regions of the world (Deepak *et al*., 1997; Ramesh *et al*., 1999; Zakaria *et al*., 2001; Mahmood *et al*., 2010). The *Caralluma s.l.* comprises about 120 species distributed in Africa, Spain, Saudi Arabia, Middle East, Pakistan and India (Mabberley, 1987). In Egypt it is represented by five species (Täckholm, 1974; Boulos, 2000), while in Saudi Arabia it is represented by 14 species. These are namely *C. commutata* A.Berger., *C. deflersiana* Lavranos., *C. edulis* (Edgew.) Benth. ex Hook.f., *C. eremastrum* O.Schwartz., *C. penicillata* White et Sloane., *C. petraea* Lavranos, *C. plicatiloba* Lavranos, *C. quadrangula* (Forssk.) N.E.Br., *C. retrospiciens* (Ehrenb.) N.E.Br, *C. russeliana* (Brongn.) Cufod. = *C. acutangula* (Decne.) N.E.Br., *C. shadhbana* Lavranos, *C. sinaica* (Decne.) A.Berger., *C. subulata* (Forssk.) Decne., *C. wissmanii* Schwartz (Migahid, 1973 & 1996; Chaudhary & Al-Jowaid, 1999; Collenette, 1999; Chaudhary, 2000 and Al-Massarani, 2011). The delimitation of *Caramulla* remains problematic. The genus remains morphologically difficult as regarding its identification and nomenclature worldwide. This is mainly due to the fact that many of *Caralluma* species are exhibiting more intermediate forms in their habitat due to their inter hybridiable potency (Mahmood *et al*., 2010). Gravely & Mayuranathan (1931) had described five varieties under *C. adscendens* in India. Recently, one of these varieties has been elevated to the species level [*Caralluma geniculata* (Gravely & Mayur.) Meve & Liede] with help of molecular systematics (Meve & Liede, 2002 and Mahmood, *et al*., 2010).

Gilbert (1990) recognizes only 56 species in the genus. Moreover, he divided *Caralluma* into four subgenera on the basis of stem and flower morphology: *C.* subg. *Boucerosia* (Wight & Arn.) M. Gilbert (with 27 species), *C.* subg. *Caralluma* (with 21 species), *C.* subg. *Desmidorchis* (Ehrenb.) M. Gilbert (with five species) and *C.* subg. *Urmalcala* M. Gilbert (with three species). *Caramulla* was divided by Plowes (1995) into 17 genera, including six monotypic ones but his classification was not widely accepted in any recent regional accounts of the genus (Gilbert, 2003 and Lavranos, 2006). Cullen *et al*. (2000) describes *Caralluma* as an extremely variable genus of about 60 species. Albers & Meve (2002) stated that the genus included about 53 species.

Meve *et al*. (2001) divided the genus *Caralluma* in Asclepiadoideae tribe Ceropegieae into seven subgenera, although their flowers are so similar in many cases that they cannot be keyed out morphologically (Meve, 2002). Meve & Leide (2002) recognize only 21 species in *Caralluma s.st.* (included one subspecies and five varieties). Moreover, they stated that the species accepted in *Caralluma* sensu Gilbert (1990) fell in six distinct genera including *Apteranthes* Mikan., *Boucerosia* Wight & Arn., *Caralluma*, *Caudanthera* Plowes, *Desmidorchis* Ehrenb. and *Monolluma* Plowes.

Bruyns *et al*. (2010) stated that the total number of accepted *Caralluma s. st.* species was raised to 58 after discovering six new species and reducing four of Gilbert (1990) species to synonymy. Recently, morphological characters supported with various kinds of biochemical and molecular data, mostly protein characters, RAPD, ISSR, RFLP etc. were used to study interspecific and intraspecific divergence in *Caralluma* species (Meve & Liede, 2002; Meve *et al*., 2001 and Bruyns *et al*., 2010).

Chromosomes have been considered as sources of valid taxonomic criteria since decades and considered to be systematically informative. The classification of a number of families has been either aided or substantiated by information from chromosome criteria or features as for example, chromosome size, the position of the centromere and special banding patterns (Moore, 1978; Jackson, 1984 and Judd *et al*., 1999 & 2007).

Karyotype studies were principally based on the bases that symmetrical karyotypes are more primitive than asymmetrical ones, longer chromosomes than shorter ones, median centromeres with chromosome arms of equal length were more primitive than chromosomes with arms of unequal length, low basic numbers had given rise to higher ones (Stebbins, 1971). These features are based on the comparison between karyotypes of known relative antiquity, as determined through classical taxonomy (Sharma, 1990).

Random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers and thus do not require prior knowledge of a DNA sequence. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate (Bardakci, 2001; Haq *et al*., 2013).

Although the value of RAPD markers in taxonomic and phylogenetic studies is not very clear, there is no doubt that these markers can be used for diagnostic purposes. RAPD markers unique to individuals from 1 species within a genus will be species-specific (inter-specific). Similarly, genus-specific markers can be generated if the fragment is a unique polymorphism to individuals belonging to a certain genus (Bardakci, 2001; Nafees *et al*., 2015).

In this work, chromosome numbers and detailed karyotype features of 13 species of *Caralluma s. l.* collected from different locations in Saudi Arabia were studied. Also, SDS-PAGE (of seed storage protein) and Random DNA (RAPD-PCR) (of seedlings) techniques were also used in this study in an attempt to distinguish these species. Data were analyzed by a clustering method and similarity coefficients using NTSYSpc version 2.02i. Different phenograms were produced for the studied *Caralluma* species based on the analysis of the karyological data, protein banding patterns, DNA banding patterns. Data from the protein and DNA banding patterns were combined and analyzed to produce another phenogram based on these particular molecular attributes. Finally, the data produced from all attributes were combined and the relationships between the species were discussed.

Materials and Methods

Plant materials: Materials of the 13 species were collected from various habitats in Saudi Arabia during the period 2009-2011 as shown in Table 1.

Cytological studies: Cytological preparations were carried out on root tips obtained from seeds germinated on sterile moist filter paper in Petri dishes at 25°C. Root tips were pretreated with 0.05% colchicine solution for 2-3 hrs. and fixed in Carnoy for 24 hr. and then stored in 70 % ethanol at 4°C. Cytological preparations were made using the Feulgen squash method. The well-spread c-metaphase chromosomes were photographed from temporary preparations at magnifications of 2500 *x*.

A karyogram for each taxon was constructed by arranging the chromosomes in homologous pairs by order of their length and arm ratio as measured from the photographic prints. The number of chromosome types was determined as described by Levan *et al*. (1965). Measurements of chromosome length were taken on the same photographs of the karyogram.

The variation in chromosome length (MCL) and chromosome arm ratio (MAR) within the karyotype has been estimated by calculating the standard error (SE) of these parameters. Karyotype asymmetry deduced from the ratio between the short arms of the chromosomes and their total length was expressed as total form percent (TF%) as proposed by Huziwara (1962). Karyotype asymmetry expressed by the ratio between chromosome arms has been also estimated as the intrachromosomal asymmetry index (A_1) as suggested by Romero-Zarco (1986). If all chromosomes complement is metacentric, the value of A_1 is found to be close to zero, on the other hand, it will be near to one if all chromosomes are telocentric.

Karyotype asymmetry due to the ratio between lengths of different chromosomes has been also estimated as the interchromosomal asymmetry index (A_2) using Pearson's dispersion coefficient that is the ratio between the standard deviation and the mean chromosome length (Romero-Zarco, 1986).

The existence of previous chromosome counts for the studied taxa has been verified in the indexes of plant chromosome numbers by IPCN Chromosome Reports (http://www.tropicos.org/NameSearch.aspx).

Table 1. A list of the examined taxa of *Caralluma* and their sources & localities.

No.*	Taxa	Sources & Localites
1.	*C. commutata* A.Berger.	Hail - Al Madinah road, Saudi Arabia
2.	*C. deflersiana* Lavranos.	Jeddah – Jizan road, Saudi Arabia
3.	*C. edulis* (Edgew.) Benth. ex Hook.f.	Jabal Mershid, 50 km east of Qunfudhah, Saudi Arabia
4.	*C. eremastrum* O.Schwartz.	Jabal Mershid, 50 km east of Qunfudhah, Saudi Arabia
5.	*C. penicillata* White et Sloane.	Al Madinah - Makkah road, Saudi Arabia
6.	*C. petraea* Lavranos.	In rocky ground, Abha, Saudi Arabia
7.	*C. plicatiloba* Lavranos.	Jeddah – Taif road, Saudi Arabia
8.	*C. quadrangula* (Forssk.) N.E.Br.	Jeddah – Taif road, Saudi Arabia
9.	*C. retrospiciens* (Ehrenb.) N.E.Br.	Hail - Al Madinah road, Saudi Arabia
10.	*C. russeliana* (Brongn.) Cufod. = *C. acutangula* (Decne.) N.E.Br.	Jeddah – Taif road, Saudi Arabia
11.	*C. shadhbana* Lavranos.	Abha – Najran road, Saudi Arabia
12.	*C. sinaica* (Decne.) A.Berger.	Hail - Al Madinah road, Saudi Arabia
13.	*C. subulata* (Forssk.) Decne.	Jeddah – Jizan road, Saudi Arabia

No.* = Numbers of taxa as listed in Figs. 1-4.

SDS-PAGE protein electrophoresis: Seeds of the studied species of *Caralluma* were collected and subjected to SDS-PAGE as described by Laemmli (1970) and modified by Studier (1973). Three replicates of 0.1 gm of seeds were mixed, each with an equal weight of pure, clean, sterile fine sand and powdered using mortar and pestle. Extraction of proteins was carried out using Tris-HCl (8.8). The powder was homogenized with 1 ml of extraction buffer for 2 hr. at 20°C. SDS-PAGE was carried out in 12.5 % acrylamide gels in Tris-Glycine running buffer (pH 8.3) at 150 V for 3 hr. using a low molecular weight protein of Sigma as a marker in each run. Gels were then stained in Comassie brilliant blue R-250 for 30 min., distained, photographed, scanned and analysed using the Gel Doc 2000 Bio-Rad system. The molecular weight standard used for the gel analysis was the Bio-Rad protein marker Mid-Low range (94.0 -14.4 kDa).

RAPD-PCR DNA electrophoresis: Ten seeds were germinated separately for each species of *Caralluma* in clay pots for two weeks. Ten young seedlings from ten different seedlings for each sample were harvested and mixed together. Then about 0.5 g of these seedlings was used for DNA extraction following the Dellaporta method (Dellaporta *et al.*, 1983). A total of fifteen 10-mer random DNA oligonucleotide primers (UBC) were independently used in the PCR reactions according to Williams *et al*. (1990). The primers synthesized by the University of British Colombia. Only six primers were generated reproducible polymorphism in the DNA profiles. Each experiment was repeated two times and only stable products were scored. The code and sequences of these primers were listed in Table 2.

Table 2. List of primers and their nucleotide sequences.

Primer code	Sequences
OPB01	5'-TGCTCTGCCC-'3
OPB02	5'-GGTGACGCAG-'3
OPB03	5'-GTCCACACGG-'3
OPB04	5'-TGGGGGACTC-'3
OPB05	5'-CTGCTGGGAC-'3
OPB06	5'-GTAGACCCGT-'3

The optimal conditions for PCR amplification was as follows: one cycle at 94°C for 2 min followed by 40 cycles at 94°C for 30 sec, 37°C for 60 sec and 72°C for 2 min. The reaction was finally incubated at 72°C for 10 min. The RAPD products were electrophoresed in 1.4 % agarose gel in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8). The run was performed at 100V for one hour. The gels were stained in 0.2 µg/ml ethiditim bromide and photographed by gel documentation system (GelDoc BioRad 2000) under UV transilluminator. Each PCR reaction was repeated twice in order to ensure that RAPD banding patterns were consistent and reproducible and only stable products were scored. A mix of a 1018 bp fragment and its multimers and pBR328 DNA fragments with lengths of 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517/506, 396, 344, 298, 220/201 and 154/134 bp was used as marker (3000, 2000, 1500, 1200, 1000, 800, 600, 500, 400, 300 and 200 bp markers).

Numerical analysis: The SDS-PAGE and RAPD-PCR data obtained for each species were pooled together and coded to create the data matrix used for computation, where the absence of a band was scored as "0" and the presence of a band was scored as "1" for each species (Tables 4 & 5). The relationships between the studied species, expressed by a similarity coefficient, have been represented using a phenogram based on the analysis of the recorded characters using NTSYSpc version 2.02i (Rohlf, 2001). The similarity index was estimated using the Dice coefficient of similarity (Nei & Li, 1979). The average of the similarity matrices was used to generate a tree by the Unweighted Pair-Group Method Arithmetic Average (UPGMA). The similarity matrix was developed by the SPSS computer package system ver.16.

Five different phenograms were produced for the studied *Caralluma* species based on the analysis of the karyological data, protein banding patterns, DNA banding patterns. Data from the protein and DNA banding patterns were combined and analyzed to produce another phenogram based on these particular molecular attributes and finally, the data produced from all attributes were used to produce another phenogram.

Results and Discussion

Cytological observations: The karyological features of the studied species in this study (13 taxa) are presented in Table 3 and Figs 1, 2, 3 & 4. All taxa were found to have a basic chromosome number of $x = 11$, on the other hand, somatic chromosome numbers were varied between $2n = 22$ in 8 taxa (1, 2, 3, 4, 5, 6, 7 & 12) to $2n = 44$ in 5 taxa (8, 9, 10, 11 & 13).

Mean chromosome length (MCL) values vary between the taxa studied of *Caralluma* (Table 3). The highest value (9.40±0.44 µm) is recorded in *C. commutata*, while the lowest value (3.22±0.20 µm) is found in *C. subulata*. The most variable chromosomes in length are found in *C. quadrangula*. (SE of MCL ±0.61 µm), whereas the most similar chromosomes are scored in *C. penicillata* (SE of MCL ±0.19 µm).

The highest MAR value (2.30±0.31) was recorded in *C. plicatiloba*, whereas the lowest (1.26±0.07) was found in *C. subulata*. Also, the highest TF% (46.88%) was recorded in *C. subulata*, whereas, the lowest TF% (31.82%) was recorded in *C. plicatiloba.* Most of taxa studied have karyotypes of metacentric to submetacentric chromosomes. The degree of karyotype asymmetry as indicated by TF% values are correlated with A_1 and A_2 values show the high degree of karyotype symmetry in the majority of the taxa studied (Table 3).

The karyotype features of species help us as a taxonomic value in providing a logical basis for the redistribution of genera in tribes. In karyotype, symmetrical karyotypes are more primitive than asymmetrical ones, longer chromosomes are more primitive than shorter ones, metacentric chromosomes are more primitive than sub-metacentric to acrocentric chromosomes, low basic numbers are primitive than high ones (Sharma, 1990). In several instances, studies of karyotype morphology have led the way to a new and better understanding of the systematic relationships within a major group of plants and to a complete reorganization of the taxonomic system of the group (Stebbins, 1956).

Table 3. Somatic chromosome features of the studied taxa of the genus *Caralluma*.

No.	Taxa	*x*	2*n*	MCL ± SE (µm)	MAR ± SE (*r*- value)	TF %	A_1	A_2	SAT	Chr. Type					Previous chr. count
										M	m	sm	st	t	
1.	*C. commutata* A.Berger.	11	22*	9.40 ± 0.44	1.44 ± 0.08	41.36	0.28	0.15	--	--	7	4	--	--	--
2.	*C. deflersiana* Lavranos.	11	22	6.31 ± 0.36	1.91 ± 0.20	35.64	0.43	0.19	--	--	5	4	2	--	22
3.	*C. edulis* (Edgew.) Benth. ex Hook.f.	11	22*	8.86 ± 0.42	1.54 ± 0.11	40.40	0.32	0.16	--	--	7	4	--	--	--
4.	*C. eremastrum* O.Schwartz.	11	22*	5.66 ± 0.36	2.02 ± 0.25	34.65	0.42	0.21	--	--	5	4	2	--	22
5.	*C. penicillata* White et Sloane.	11	22	4.98 ± 0.19	1.82 ± 0.16	36.24	0.42	0.12	--	--	4	6	1	--	22
6.	*C. petraea* Lavranos.	11	22*	7.75 ± 0.26	1.97 ± 0.20	35.31	0.44	0.11	--	--	4	6	1	--	--
7.	*C. plicatiloba* Lavranos.	11	22	5.03 ± 0.35	2.30 ± 0.31	31.82	0.48	0.23	--	--	6	2	3	--	22
8.	*C. quadrangular* (Forssk.) N.E.Br.	11	44*	7.13 ± 0.61	1.74 ± 0.12	36.89	0.40	0.28	--	--	8	3	--	--	--
9.	*C. retrospiciens* (Ehrenb.) N.E.Br.	11	44*	5.51 ± 0.54	1.66 ± 0.10	37.57	0.37	0.32	--	--	6	5	--	--	--
10.	*C. russeliana* (Brongn.) Cufod.	11	44*	7.02 ± 0.56	1.53 ± 0.10	40.02	0.32	0.26	--	--	7	4	--	--	--
11.	*C. shadhbana* Lavranos.	11	44*	5.71 ± 0.23	1.81 ± 0.14	36.69	0.42	0.14	--	--	5	6	--	--	22
12.	*C. sinaica* (Decne) A.Berger.	11	22	4.89 ± 0.53	1.65 ± 0.12	37.95	0.35	0.36	--	2	3	6	--	--	22
13.	*C. subulata* (Forssk.) Decne.	11	44*	3.22 ± 0.20	1.26 ± 0.07	46.88	0.19	0.20	--	--	10	1		--	22

MCL = Mean chromosome length, MAR = Mean arm ratio, SE = Standard error, TF% = Total form percent, A_1 = Intrachromosomal asymmetry index, A_2 = Interchromosomal asymmetry index, SAT = A satellite, Chr. = Chromosome, M = Metacentric chromosome, m = Metacentric region chromosome, sm = Submetacentric chromosome, st = Subtelocentric chromosome, t = Acrocentric chromosome, * = New chromosome record

Polyploidy numbers of chromosomes were recorded within the studied taxa of *Caralluma* as a tetraploid of 2*n* = 44 in five taxa (*C. quadrangula*, *C. retrospiciens*, *C. russeliana*, *C. shadhbana* and *C. subulata*).

The most obviously karyological features is polyploidy numbers of chromosomes which was recorded within the studied taxa of *Caralluma* as a tetraploid of 2*n* = 44 in five taxa (*C. quadrangula*, *C. retrospiciens*, *C. russeliana*, *C. shadhbana* and *C. subulata*). These chromosome count (2*n* = 44) were scored for *C. quadrangula*, *C. retrospiciens* and *C. russeliana* for the first time, on the other hand, previous chromosome count reported for the remaining taxa (*C. shadhbana* and *C. subulata*) was 2*n* = 22.

Longest chromosome (MCL > 5 µm) were observed in ten taxa (*C. commutate*, *C. deflersiana*, *C. edulis*, *C. eremastrum*, *C. petraea*, *C. plicatiloba*, *C. quadrangula*, *C. retrospiciens*, *C. russeliana* and *C. shadhbana*). The remaining three taxa (*C. penicillata*, *C. sinaica* and *C. subulata*) are characterized by small chromosomes (MCL < 5 µm).

The degree of karyotype asymmetry as indicated by MAR, TF%, A_1 and A_2 values are divided the taxa studied into three groups, the first one comprises four taxa (*C. commutata*, *C. edulis*, *C. russeliana* and *C. subulata*) are characterized by highly symmetrical karyotypes. The second group comprises seven taxa (*C. deflersiana*, *C. penicillata*, *C. petraea*, *C. quadrangula*, *C. retrospiciens*, *C. shadhbana* and *C. sinaica*) are characterized by less symmetrical karyotypes or chromosomes. The last group consists of only two taxa; *C. eremastrum* and *C. plicatiloba* which is characterized by asymmetrical karyotypes.

Caralluma sinaica is the only species that have chromosomes of median point centromere (M type chromosomes) On the other hand, *C. deflersiana*, *C. eremastrum*, *C. penicillata*, *C. petraea* and *C. plicatiloba* are characterized by the presence of subtelocentric chromosomes type (st or subterminal region centromere).

SDS-PAGE protein banding pattern analysis: In this study, SDS-PAGE was performed to examine the seed protein banding patterns of the 13 taxa of the genus *Caralluma*. The electrophoretic protein patterns of those taxa revealed the presence of 18 different bands. The electropherograms of those isolates are shown in Fig. 5 and the distribution of the bands is described in Table 4.

The highest number of bands recorded in two taxa was 11 bands in C. *quadrangula* and *C. subulata*, while the lowest number of bands found was seven bands and was recorded in seven taxa (*C. commutate*, *C. eremastrum*, *C. penicillata*, *C. plicatiloba*. *C. retrospiciens*, *C. russeliana* and *C. sinaica*).

The molecular weight of the products ranged from 104.60 to 11. 60 kDa. The highest molecular weight band recorded was 104.60 kDa. and this band was found in all taxa studied, while the lowest molecular weight band recorded was 11.60 kDa. This band was recorded in only three taxa (*C. deflersiana*, *C. sinaica* and *C. subulata*) (Table 4).

A total of 15 polymorphic bands were observed in the protein profiles of the studied taxa (Table 4). No unique band was observed of the polymorphic bands for all the studied taxa. With respect to the monomorphic bands, three bands were detected (at approximately 104.60, 69.70 and 15.30 kDa.). These bands allow better discrimination between the studied *Caralluma* species.

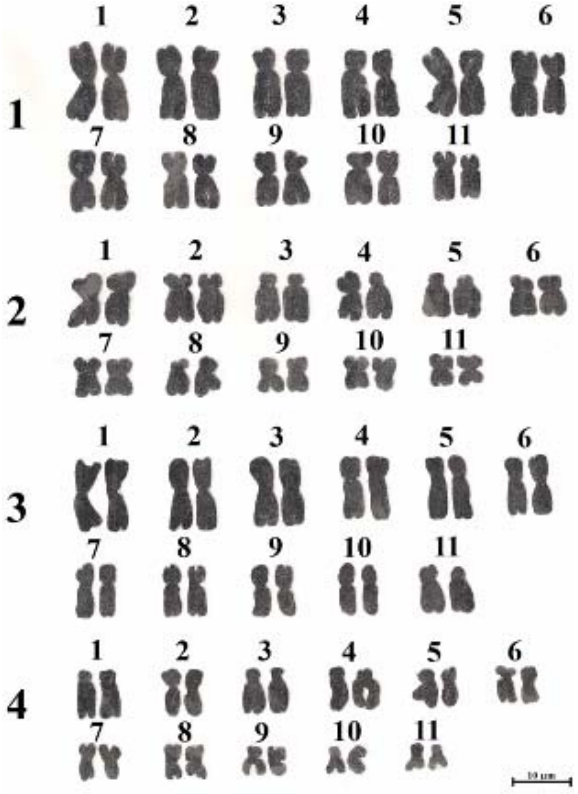

Fig. 1. Karyotypes of studied taxa of *Caralluma*: (1) *C. commutata* A.Berger., (2) *C. deflersiana* Lavranos, (3) C. *edulis* (Edgew.) Benth. Ex Hook. F. and (4) C. *erematrum* O. Schwartz.

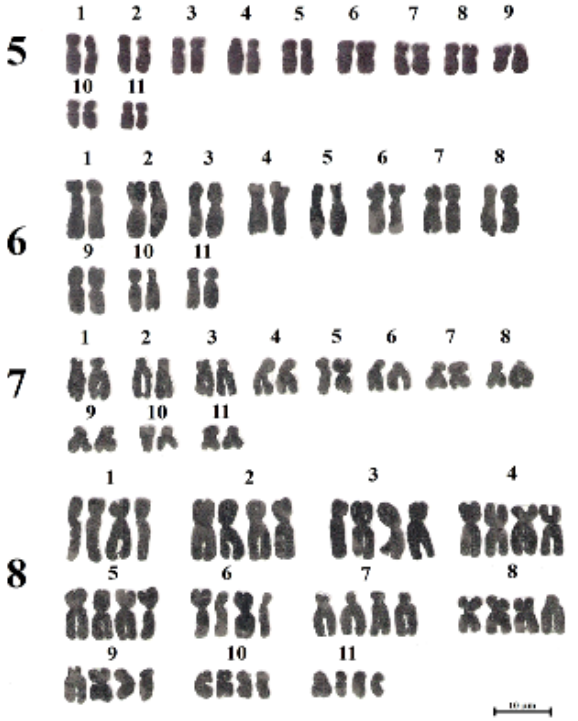

Fig. 2. Karyotypes of studied taxa of *Caralluma*: (5) *C. Penicillata* White et Sloane., (6) *C. petraea* Lavranos, (7) C. *plicatiloba* Lavranos and (8) C. *quadrangular* (Forssk.) N.E.Br.

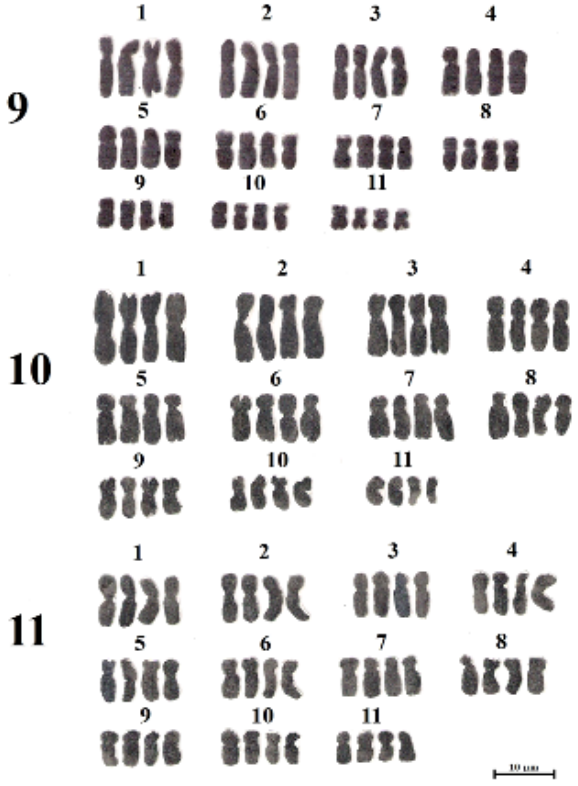

Fig. 3. Karyotypes of studied taxa of *Caralluma*: (9) *C. retrospiciens* (Ehrenb.) N.E.Br., (10) *C. russeliana*, (Brongn.) Cufod. and (11) C. *shadhbana* Lavranos.

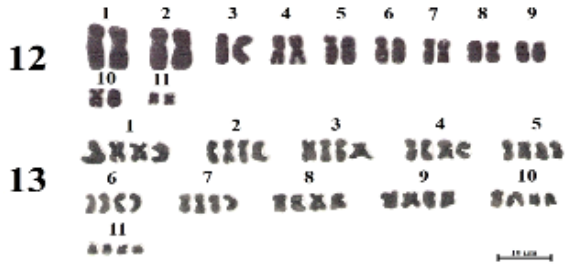

Fig. 4. Karyotypes of studied taxa of *Caralluma*: (12) *C. sinaica* (Decne.) A.Berge. and (13) *C. subulata* (Forssk.) Decne.

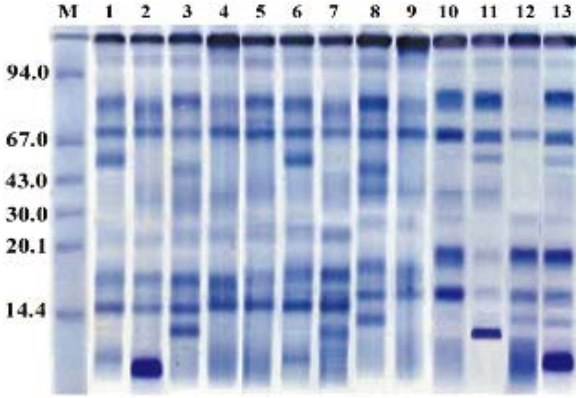

Fig. 5. Electrophoretic banding profiles of seed proteins extracted in Tris-HCl buffer of the studied taxa of *Caralluma.*

Table 4. Survey of polymorphic and monomorphic SDS-PAGE bands of total seed protein from the studied taxa of *Caralluma*. [1 = Present and 0 = Absent].

Molecular weights kDa.	*Caralluma* species												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
104.60	1	1	1	1	1	1	1	1	1	1	1	1	1
086.20	1	0	1	0	1	1	0	1	0	1	1	0	1
073.40	0	1	0	1	0	0	1	0	1	0	0	0	0
071.50	0	0	0	0	1	0	0	1	0	0	0	0	0
069.70	1	1	1	1	1	1	1	1	1	1	1	1	1
050.80	1	0	0	0	0	1	0	1	0	0	1	0	1
044.90	0	0	1	0	0	0	0	1	0	0	0	0	0
040.50	0	1	1	1	0	1	0	1	1	1	1	0	1
032.30	0	0	0	0	0	0	0	1	1	1	0	1	1
028.70	1	1	1	1	1	1	1	0	0	0	0	0	0
019.30	0	0	0	0	0	0	0	0	0	1	1	1	1
017.90	0	0	0	0	0	0	0	1	1	0	0	0	0
016.80	1	1	1	1	1	1	0	0	0	0	0	0	0
015.30	1	1	1	1	1	1	1	1	1	1	1	1	1
014.90	0	0	0	0	0	0	0	1	0	0	0	1	1
012.70	0	0	1	0	0	0	1	0	0	0	1	0	0
012.00	0	0	0	0	0	1	1	0	0	0	0	0	1
011.60	0	1	0	0	0	0	0	0	0	0	0	1	1
Total number of bands	**7**	**8**	**9**	**7**	**7**	**9**	**7**	**11**	**7**	**7**	**8**	**7**	**11**

RAPD-PCR DNA banding pattern analysis: In this study, randomly amplified polymorphic DNA (RAPD) based on polymerase chain reaction (PCR) analysis was conducted to fingerprint and elucidate the similarity indices between 13 species of *Caralluma*.

Fifteen random primers were initially tested, but six-revealed distinct inter and intra specific polymorphisms between the 13 *Caralluma* species. Only six primers generated reproducible polymorphisms in the DNA profiles. The code and sequences of these primers are listed in Table 2. These primers are named OPB01, OPB02, OPB03, OPB04, OPB05 and OPB06. Fig. 6 illustrate the RAPD profiles generated by these primers respectively. The presence of bands is expressed as (1) and absence as (0) in Table 5.

The six primers revealed considerable variations between the studied species. A total of 71 DNA bands were detected (Table 5). The highest number of bands recorded within the studied species was 69 bands observed in *Caralluma commutata* A. Berger. (1), on the other hand, the lowest number (59 bands) was observed in two species (*C. deflersiana* Lavranos. - 2 and *C. quadrangular* (Forssk.) N. E. Br. - 8). The size of the DNA bands ranged from 4122 bp (OPB02) to 122 bp (OPB04). The profiles of the DNA bands varied with the primer used (Fig. 6).

Similarity matrix was developed by SPSS computer package system for the studied species of *Caralluma* based on RAPD-PCR analysis (Table 6). The highest similarity value was 0.969 % between *C. retrospiciens* (Ehrenb.) N.E.Br. (9) and *C. subulata* (Forssk.) Decne. (13) and the lowest similarity value was 0.779 % between the species *C. deflersiana* Lavranos. (2) and *C. plicatiloba* Lavranos. (7). Increasing in the similarity value reflect the increasing in the closed phylogenetic relationship among the studied species.

Numerical analysis and relationships between the studies taxa

Relationships based on attributes of karyotype analysis: A UPGMA tree expressing the relationships among the studied species of *Caralluma* based on karyological variation is shown in Fig. 7. In this tree, *C. subulata* (13) is clearly delimited from the other species at a distance of 1.80 on the UPGMA distance scale. The other 12 species are clearly divided into two groups at distance of 1.54, one comprising two species (*C. edulis* - 3 and *C. commutata* -1) and the other is comprised of ten species. In the former group, *C. edulis* is delimited from *C. commutata* at distance of 0.96 on the UPGMA distance scale. In the other group, the ten species are divided into two clusters; one comprising three species (*C. sinaica* - 12, *C. russeliana* - 10 and *C. retrospiciens* - 9) and the second is comprised of seven species (*C. shadhbana* - 11, *C.* quadrangular - 8, *C. petraea* - 6, *C. plicatiloba* - 7, *C. eremastrum* - 4, *C. penicillata* - 5 and *C. deflersiana* - 2). In the first cluster, *C. sinaica* is delimited from the other two species at a high distance of 1.21 on the UPGMA distance scale. It is also noted that the two species; *C. russeliana* and *C. retrospiciens* are delimited from each other at a high UPGMA distance of 0.98. In the second cluster, the seven species are divided into two subcluster at a distance of about 1.18 on UPGMA distance scale. In the first subcluster *C. shadhbana* is delimited from *C. quadriangular* at UPGMA of about 0.93. In the second subcluster the *C. petraea* is separated from the other four species at UPGMA distance of about 0.78. The latter four species are divided into two sections one comprising the two species *C. plicatiloba* and *C. eremastrum* and the other is comprised *C. penicillata* and *C. deflersiana*.

Table 5. The presence (1) and absence (0) of DNA in the generated RAPD profiles of the 13 *Caralluma* species.

Primer	Molecular weight in bp.	*Caralluma* species												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
OPB01	3198	1	1	1	1	1	1	1	0	1	1	1	1	1
	1543	1	1	1	1	1	1	1	0	1	1	1	1	1
	1453	1	1	1	1	1	1	1	1	1	1	1	1	1
	989	1	1	1	1	1	1	1	1	1	1	1	1	1
	845	1	1	1	1	1	1	1	1	1	1	1	1	1
	766	1	1	1	1	1	1	1	1	1	1	1	1	1
	608	1	1	1	1	1	1	1	1	1	1	1	1	1
	521	1	1	1	1	1	1	1	1	1	1	1	1	1
	472	1	1	1	1	1	1	1	1	1	1	1	1	1
	329	1	1	1	1	1	1	1	1	1	1	1	1	1
	284	1	1	1	1	1	1	1	1	1	1	1	1	1
	198	1	1	1	1	1	1	1	1	1	1	1	1	1
Total number of bands		**12**	**12**	**12**	**12**	**12**	**12**	**12**	**10**	**12**	**12**	**12**	**12**	**12**
OPB02	4122	1	0	1	1	0	1	1	1	1	1	1	1	1
	4033	1	1	1	1	1	0	1	1	1	1	1	1	1
	3476	1	1	1	1	1	1	1	1	1	1	1	1	1
	2866	1	0	1	1	1	0	1	1	1	0	1	1	1
	2019	1	1	1	1	1	1	1	1	1	1	1	1	1
	1983	1	1	1	1	1	0	1	1	1	1	0	1	1
	1756	1	1	1	1	1	1	1	0	1	1	1	1	1
	1604	1	0	1	1	1	1	1	0	1	1	1	0	1
	1388	1	1	0	1	1	1	1	1	1	1	1	1	1
	1165	1	1	1	1	1	1	1	1	1	1	1	1	1
	976	1	1	1	1	1	1	1	1	1	1	1	1	1
	806	1	1	1	1	1	1	1	1	1	0	1	1	1
	623	1	1	1	1	1	1	0	1	1	1	1	1	1
	587	1	1	1	1	1	1	1	1	1	1	1	1	1
	398	1	0	1	1	1	1	1	1	1	1	1	1	1
	389	1	1	1	1	1	1	1	1	1	1	1	1	1
	278	1	0	0	1	1	0	0	0	0	0	0	0	0
	176	1	1	1	1	1	1	1	1	1	1	1	1	1
Total number of bands		**18**	**13**	**16**	**18**	**17**	**14**	**16**	**15**	**17**	**15**	**16**	**16**	**17**
OPB03	3299	1	1	0	0	1	1	1	1	1	1	1	1	1
	2803	1	1	0	0	1	1	1	1	1	1	1	1	1
	1398	1	1	1	1	1	1	1	1	1	1	1	1	1
	966	1	1	1	1	1	1	1	1	1	1	1	1	1
	738	1	1	1	1	1	1	1	1	1	1	1	1	1
	685	1	1	1	1	1	1	1	1	1	1	1	1	1
	592	1	0	0	0	0	1	1	1	0	0	1	0	0
	518	1	1	1	1	1	1	1	1	1	1	1	1	1
	454	0	0	1	0	0	0	0	0	0	0	0	0	0
	403	1	1	1	1	1	1	1	1	1	1	1	1	1
Total number of bands		**9**	**8**	**7**	**6**	**8**	**9**	**9**	**9**	**8**	**8**	**9**	**8**	**8**

Table 5. (Cont'd.).

Primer	Molecular weight in bp.	*Caralluma* species												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
OPB04	3122	1	0	1	1	0	1	1	1	1	1	1	1	1
	3033	1	1	1	1	1	0	1	1	1	1	1	1	1
	2476	1	1	1	1	1	1	1	1	1	1	1	1	1
	1866	1	0	1	1	1	0	1	1	1	0	1	1	1
	1019	1	1	1	1	1	1	1	1	1	1	1	1	1
	983	1	1	1	1	1	0	1	1	1	1	0	1	1
	756	1	1	1	1	1	1	1	0	1	1	1	1	1
	604	1	0	1	1	1	1	1	0	1	1	1	0	1
	623	1	1	1	1	1	1	0	1	1	1	1	1	1
	587	1	1	1	1	1	1	1	1	1	1	1	1	1
	398	1	0	1	1	1	1	1	1	1	1	1	1	1
	389	1	1	1	1	1	1	1	1	1	1	1	1	1
	278	1	0	0	1	1	0	0	0	0	0	0	0	0
	176	1	1	1	1	1	1	1	1	1	1	1	1	1
	169	1	1	1	1	1	1	1	1	1	1	1	1	1
	122	1	1	1	1	1	1	1	1	1	1	1	1	1
Total number of bands		**16**	**11**	**15**	**16**	**15**	**12**	**14**	**13**	**15**	**14**	**14**	**14**	**15**
OPB05	1387	0	1	0	0	0	0	0	0	0	0	0	0	0
	1340	1	1	1	1	1	1	1	1	1	1	1	1	1
	1226	1	1	1	1	1	1	1	1	1	1	1	1	1
	977	1	1	1	0	1	1	0	1	0	1	1	0	1
	719	1	1	1	1	1	1	1	1	1	1	1	1	1
Total number of bands		**4**	**5**	**4**	**3**	**4**	**4**	**3**	**4**	**3**	**4**	**4**	**3**	**4**
OPB06	1634	1	1	1	1	1	1	1	1	1	1	1	1	1
	1365	1	1	1	1	1	1	0	0	0	0	0	1	1
	1265	1	1	1	1	1	1	1	1	1	1	1	1	1
	1054	1	1	1	1	1	1	1	1	1	1	1	1	1
	912	1	1	1	1	1	1	1	1	1	1	1	1	1
	834	1	1	1	1	1	1	1	1	1	1	1	1	1
	670	1	1	1	1	1	1	1	1	1	1	1	1	1
	572	1	1	1	1	1	1	1	1	1	1	1	1	1
	481	1	1	1	1	1	1	1	1	1	1	1	1	1
	238	1	1	1	1	1	1	0	0	0	0	0	0	0
Total number of bands		**10**	**10**	**10**	**10**	**10**	**10**	**8**	**8**	**8**	**8**	**8**	**9**	**9**
Total number of bands by all primers		**69**	**59**	**64**	**65**	**66**	**61**	**62**	**59**	**63**	**61**	**63**	**62**	**65**

Table 6. Similarity matrix among the studied species of *Caralluma*.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1.	1.000	0.829	0.900	0.942	0.957	0.884	0.899	0.855	0.913	0.884	0.913	0.899	0.942
2.		1.000	0.809	0.797	0.866	0.818	0.779	0.788	0.821	0.846	0.794	0.862	0.851
3.			1.000	0.925	0.884	0.838	0.853	0.809	0.896	0.866	0.868	0.881	0.925
4.				1.000	0.926	0.826	0.868	0.797	0.910	0.853	0.855	0.896	0.912
5.					1.000	0.841	0.855	0.812	0.897	0.868	0.870	0.882	0.926
6.						1.000	0.836	0.791	0.851	0.877	0.908	0.836	0.881
7.							1.000	0.862	0.953	0.892	0.923	0.908	0.924
8.								1.000	0.877	0.846	0.877	0.891	0.879
9.									1.000	0.938	0.938	0.953	0.969
10.										1.000	0.908	0.892	0.938
11.											1.000	0.894	0.939
12.												1.000	0.954
13.													1.000

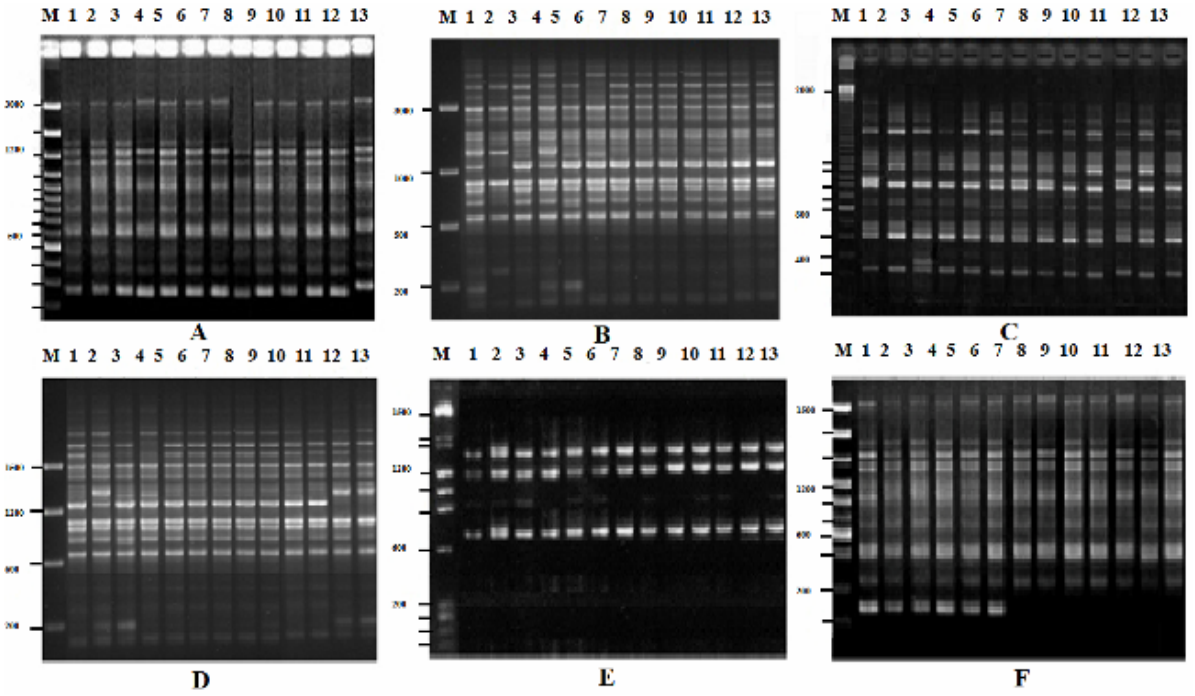

Fig. 6. RAPD fingerprints of the 13 of *Caralluma* species with the primers OPB (A-F).

Relationships based on attributes of seed protein electrophoresis: A UPGMA tree illustrating the relationship between the examined species of *Caralluma*, based on the polymorphism in the seed protein electrophoretic profile is shown in Fig. 8. In this tree, the *C. petraea* (6) is delimited from the other species of *Caralluma* at a UPGMA distance coefficient of about 1.70. The remaining species of *Caralluma* are separated in two clusters at a distance coefficient of about 1.48. The first cluster comprises seven species and the other including the other five species. In the former cluster the *C. plicatiloba* (7) and *C. shadhbana* (11) are delimited from the other species at a distance coefficient of about 1.31 and 1.25, respectively. The other remaining species are divided into two small subgroups; the first one includes two species (*C. sinaica* - 12 and *C. russeliana* - 10) and the second subgroup includes the other three species (*C. retrospiciens* - 9, *C. subulata* - 13 and *C. quadrangula* - 8).

In the latter cluster that comprises of five species (*C. penicillata* - 5, *C. eremastrum* - 4, *C. edulis* - 3, *C. delfersiana* - 2 and *C. commutata*- 1); at distance coefficient of about 1.43, the *C. penicillata* (5) is delimited from the other four species. The remaining species are divided into two subgroups at distance coefficient of about 1.30 of UPGMA scale. The first group includes *C. eremastrum* (4) and *C. edulis* (3) and the second includes *C. delfersiana* (2) and *C. commutate* (1).

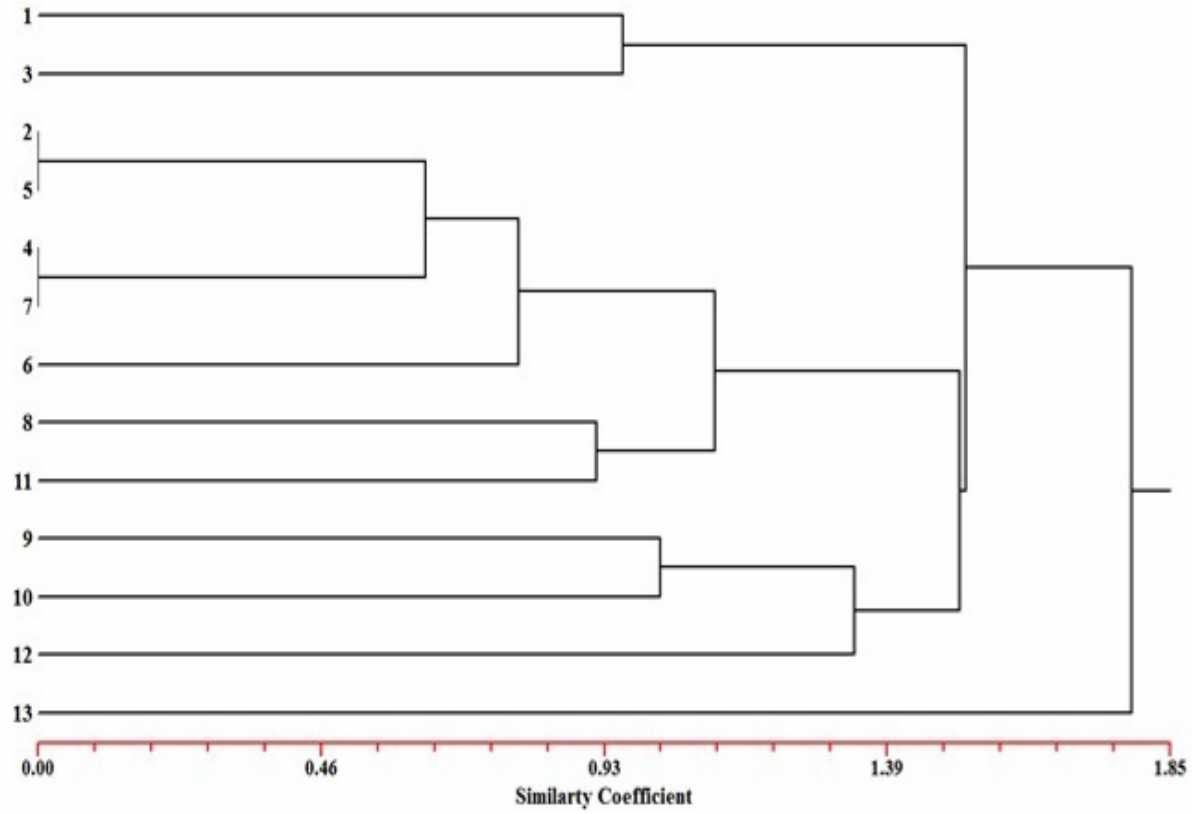

Fig. 7. UPGMA-phenogram constructed from 13 *Caralluma* taxa based on 25 attributes of karyotype analysis.

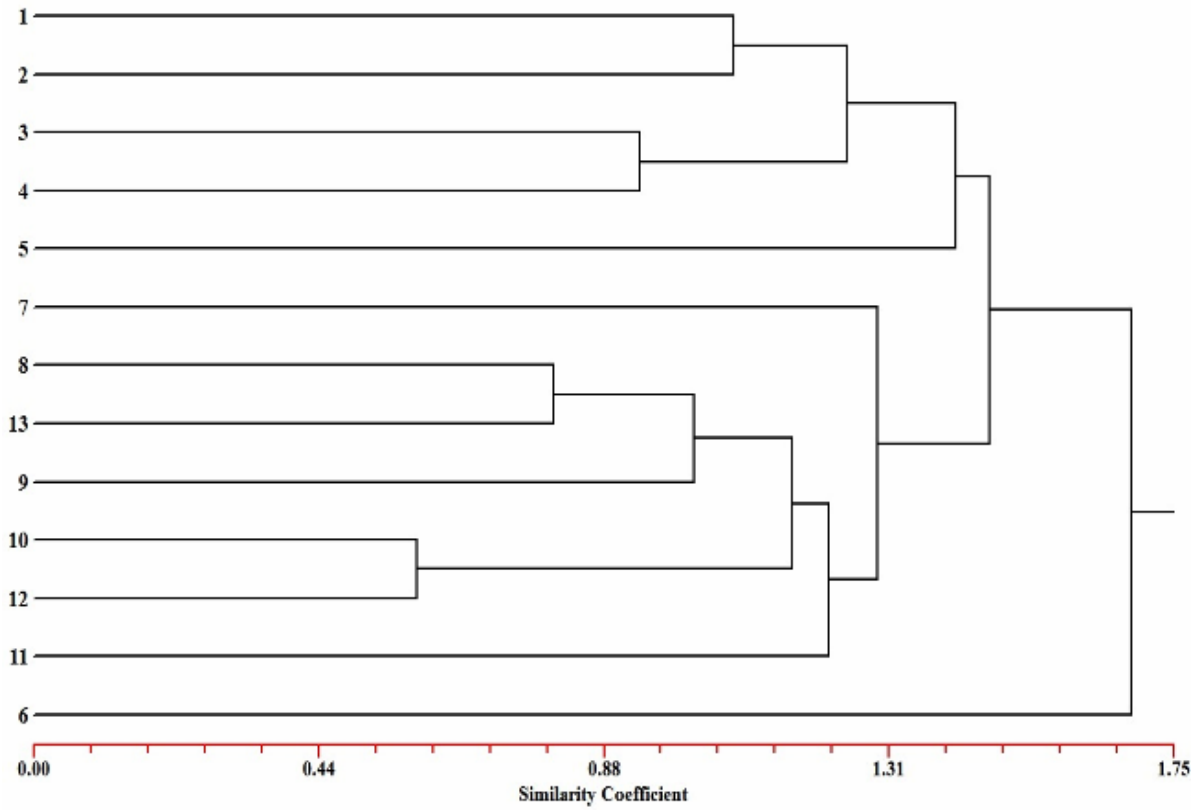

Fig. 8. UPGMA-phenogram constructed from 13 *Caralluma* taxa based on protein attributes after extraction with HCl buffer.

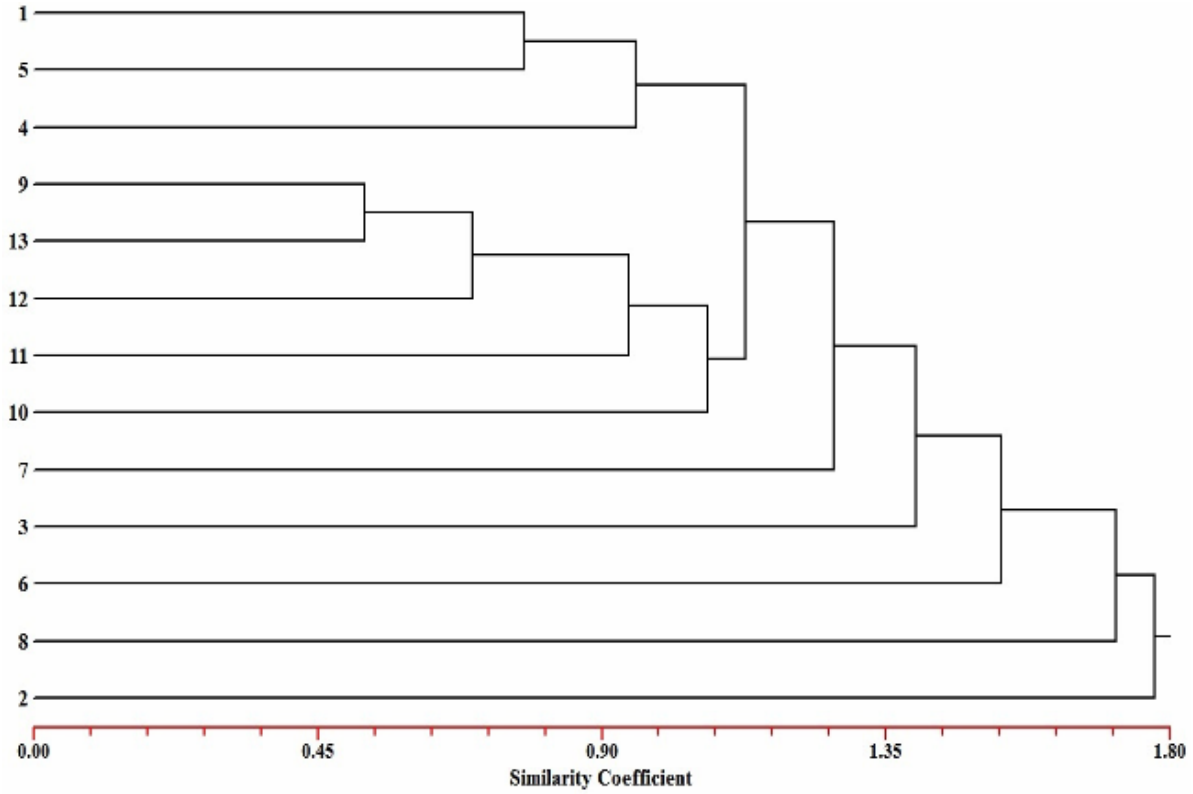

Fig. 9. UPGMA-phenogram constructed from 13 *Caralluma* taxa based on RAPD-PCR attrributes.

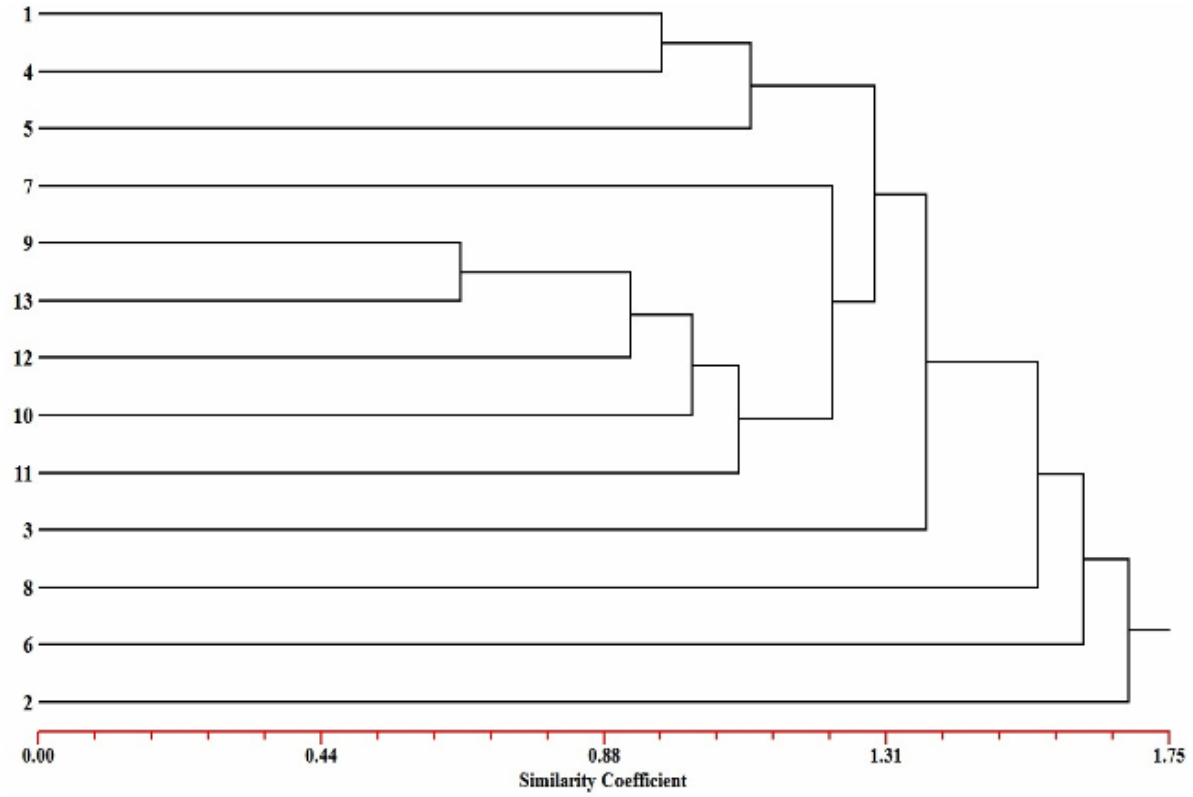

Fig. 10. UPGMA-phenogram constructed from 13 *Caralluma* taxa based on 89 attributes of both SDS-PAGE protein and RAPD-PCR analysis.

Relationships based on attributes of RAPD-PCR: A UPGMA tree expressing the relationship among the studied species of *Caralluma* based on RAPD-PCR attributes is shown in Fig. 9. In this tree, the five species (*C. deflersiana* - 2, *C. quadrangula* - 8, *C. petraea* - 6, *C. edulis* - 3 and *C. plicatiloba* - 7) are clearly delimited from the other taxa at a distance of 1.78, 1.70, 1.50, 1.40 and 1.30, respectively on the UPGMA distance scale. The other eight species are clearly divided into two groups at a distance of 1.15, one comprising five species (*C. russeliana* - 10, *C. shadhbana* - 11, *C. sinaica* - 12, *C. subulata* - 13 and *C. retrospiciens* - 9) and the other group comprises the other three species (*C. eremastrum* - 4, *C. penicillata* - 5 and *C. commutata* - 1). In the former group, the *C. russeliana* - 10 and *C. shadhbana* - 11 are delimited from the other three species at distance coefficient of about 1.10 and 0.90, respectively. At a relatively low distance coefficient of about 0.75 the *C. sinaica* (12) is separated from the other two species (*C. subulata* - 13 and *C. retrospiciens* - 9).

In the other group, the *C. eremastrum* (4) is delimited from the other two species (*C. penicillata* - 5 and *C. commutata* - 1) at UPGMA distance of about 0.90. The latter two species are distinguished from each other at a distance coefficient of about 0.85 on the UPGMA distance scale (Fig. 9).

Relationships based on attributes of seed protein electrophoresis and RAPD-PCR: A UPGMA tree illustrating the relationships between the examined species of *Caralluma*, based on both SDS-PAGE protein and RAPD-PCR analysis is shown in Fig. 10. The topology of this tree generally resembles that of the tree based on RAPD-PCR attributes (Fig. 10) in that the *C. deflersiana* - 4, *C. petraea* - 6, *C. quadrangula* - 8 and *C. edulis* - 3 are delimited from the other species at UPGMA distance coefficient of about 1.70, 165, 1.60 and 1.40, respectively. The remaining species are separated into two groups, one comprises six species (*C. shadhbana* - 11, *C. russeliana* - 10, *C. sinaica* - 12, *C. subulata* - 13, *C. retrospiciens* - 9 and *C. plicatiloba* - 7) and the second group comprises three species (*C. penicillata* - 5, *C. eremastrum* - 4 and *C. commutata* - 1). In the former group the *C. plicatiloba* (7) is delimited from other species at UPGMA distance coefficient of about 1.30.This finding may agree with Meve & Leide (2002) who placed *C. plicatiloba* in a separate genus *Apteranthes* Mikan, which is characterized by certain morphological traits as not pungent stems, podaria not grooved, leaf rudiments subsessile to petiolate or sometimes swollen), with stipular glands or stipular formings absent. The other three species (*C. shadhbana* - 11, *C. russeliana* - 10 and *C. sinaica* - 12) are separated from the other two species (*C. subulata* - 13 and *C. retrospiciens* - 9) at distance coefficient of about 1.15, 1.00 and 0.95, respectively (Fig. 10). Meve & Leide (2002) placed *C. russeliana* and *C. retrospiciens* in the genus *Desmidorchis* Ehrenb. thus suggesting a strong relation between them.

In the second group the *C. penicilata* (5) is delimited from *C. eremastrum* (4) and *C. commutata* (1) at UPGMA distance coefficient of 1.10. The latter two species are separated from each other at distance of about 0.95 of UPGMA scale. The delimitation of *C. deflersiana* (4), *C. petraea* (6) and *C. quadrangula* (8) from the other species at UPGMA distance coefficient of about 1.70, 165 & 1.60, respectively could be attributed to the fact that these species in particular is confined or endemic to Saudi Arabia. Moreover, *C. petraea* (6) was separated from the rest of the studied taxa in the trees based upon SDS-PAGE attributes at a UPGMA distance coefficient of about 1.70. However, *C. petraea* and *C. quadrangula* were shown to be not closely related by Meve & Leide (2002) and were placed by them in two different genera *Desmidorchis* Ehrenb. and *Monolluma* Plowes, *Caralluma edulis* (3) has a more widespread distribution as has been reported from Mauritania, Sudan, Eritrea, Somalia, Saudi Arabia, United Arab Emirates, Pakistan, India and possibly Iran and Afghanistan (Jansen, 2004). Thus, a certain link could be deduced between *C. edulis* (3) and the aforementioned three species.

Relationships based on attributes of karyotype, seed protein electrophoresis and RAPD-PCR: A UPGMA tree expressing the relationships among the studied species of *Caralluma* based on all attributes obtained from karyotype analysis, seed protein electrophoresis and RAPD-PCR analysis is shown in Fig. 11. In this tree, the three species *C. deflersiana* - 2, *C. petraea* - 6 and *C. quadrangula* - 8 are clearly separated from the other species at a distance of about 1.56, 1.50 and 1.48 on the UPGMA distance scale, respectively. More or less similar to the results obtained from the previous phenogram based on attributes from both RAPD -PCR and SDS-PAGE protein prophile attributes. The other species are divided into two groups at a high distance of about 1.40 on the UPGMA distance scale, one comprising the two species *C. edulis* - 3 and *C. commutata* - 1 and the other group is comprised of the other species (8 species). In the former group, the *C. edulis* is delimited from the other species (*C. commutata*) at a distance of 1.21 on the UPGMA distance scale. In the other group the species are clearly divided into two clusters at distance of 1.30 on the UPGMA distance scale. The first cluster is comprised of five species (*C. shadhbana* - 11, *C. subulate* - 13, *C. russeliana* - 10, *C. sinaica* - 12 and *C. retrospiciens* - 9), the first three species are delimited from the other two species at a UPGMA distance of about 1.23, 1.20 and 1.10, respectively, which were delimited from each other at a high UPGMA distance of 0.98. The second cluster is comprised of three species (*C. plicatiloba* - 7, *C. penicillata* - 5 and *C. eremastrum* - 4); *C. plicatiloba* is delimited from the other two species at a distance of about 1.28 on the UPGMA distance scale.

Gilbert (1990) placed *C. edulis* (3) and *C. sinaica* (12) in *C.* subg. *Caralluma*, Meve & Liede (2002) placed them in the genus *Caudanthera* Plowes. Moreover, the same authors stated that the close relationship between the two very distinct species had been suggested by Bruyns (1989) who showed that both species shared similar morphological traits as the slender stems with four rounded angles, large leaf rudiments that are accompanied by stipular glands and long-lived elongated-conical terminal portions of the stems. These

in turn bear one- to two-flowered inflorescences. In addition, both species are characterized karyologically by exceptionally large chromosomes (Albers & Meve, 2002). The same view was stated by (Bruyns *et al*., 2010) in their phylogenetic analysis of the genus using data analysis from four molecular markers: two plastid regions *psbAtrnH* and *trnT-F* and two nuclear regions *ITS* and *ncpGS*, placed them in Clade B Subclade II thus suggesting a close relationship between them. However, the present study does not give an indication of a strong relationship between the two taxa but may agree with the classification of Plowes (1995) who placed the two taxa in two different genera *Cryptolluma* Plowes and *Caudanthera*, respectively. More studies are still needed on the relationships between *C. edulis* and *C. sinaica*. A remarkable finding from this study was the presence of a certain degree of conflict between results obtained from the different attributes used. However, Bremer (1996) stated that separate analyses of data obtained by using different taxonomic tools might provide important information on the studied taxa. In her opinion, it is still the best way to reveal conflicts between different data sets. The knowledge of the conflicts Can promote further detailed studies on a particular group in order to improve our understanding of characters and phylogenetic hypotheses. Moreover, the present study re assured the conclusion of Mahmoud *et al*. (2010) who stated that two species of *Caralluma* viz., *Caralluma tuberculata* and *Caralluma edulis* studied by them and collected from different locations, were not clearly differentiated at the molecular level by using their studied RAPD primers. In their opinion, this may be due to the fact that many of *Caralluma* species are exhibiting more intermediate forms in their habitat due to their inter hybridiable potency.

Finally, the delimitation of *Caralluma s. l.* is still far from settled. It is an extremely variable genus as Cullen (2000) stated before. Moreover, the concept about what taxa would be placed in *Caralluma s. str*. widely varies among authorities. The present study is a preliminary one and more work is still needed on the *Caralluma* taxa of Saudi Arabia and their relationships between other taxa of the genus worldwide.

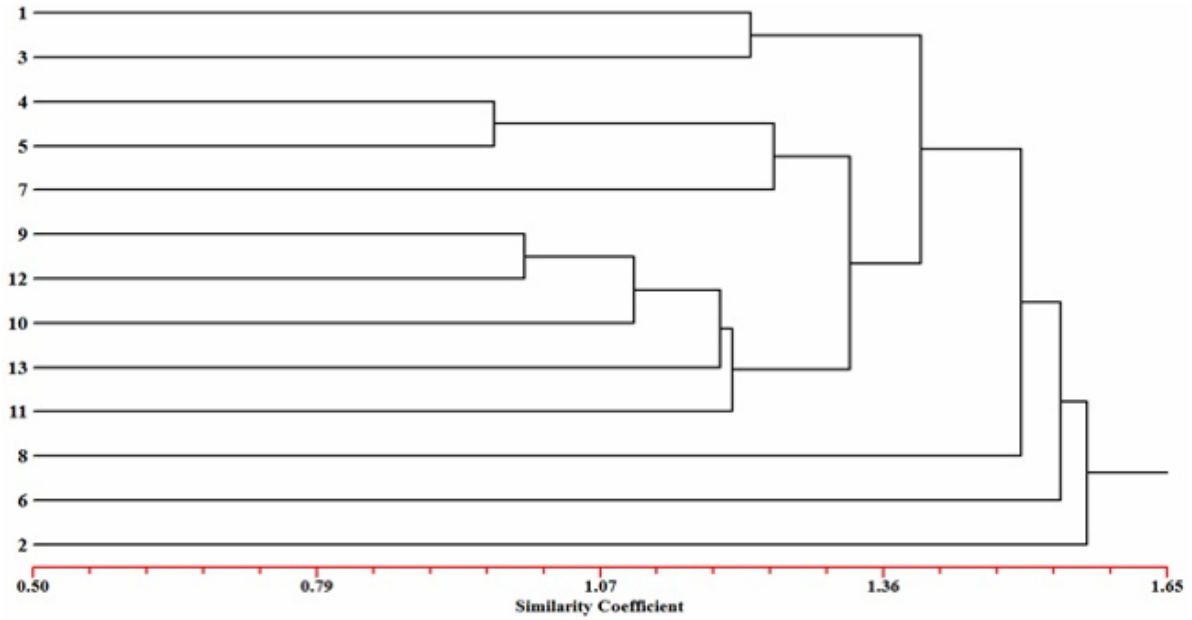

Fig. 11. UPGMA-phenogram constructed from 13 *Caralluma* taxa based on all attrributes.

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