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TAXONOMIC AND GENETIC RELATIONSHIPS OF SOME EUPHORBIACEAE SPECIES FROM TAIF DERIVED FROM MOLECULAR ANALYSES

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Abstract

For genetic documentation and studying the taxonomic relationships, 9 species of family Euphorbiaceae; *Euphorbia peplus, E. indica, E. prostrata, E. schimperi, E. tirucalli, E. granulata, Clutia myricoides, Ricinus communis, Chrozophora oblongifolia*, were investigated depending on six isozymes and different molecular markers (RAPD, SSR, ITS, ITS2, matK and rbcL). Little data was obtained through the utility of RAPD and SSR primers. The four barcoding loci did not reveal discriminatory bands for all the examined species. The three loci; ITS, matK and rbcL, discriminated *E. peplus* and *E. tirucalli* from other species within genus *Euphorbia.* Banding patterns of the six isozyme systems showed great variability. The UPGMA dendrogram obtained depending on the 40 isozyme bands showed different findings from that of DNA. The DNA data was more accurate than isozymes data as it grouped the three species belonging to subg. *Chamaesyce* (*E. indica*, *E. prostrata* and *E. granulata*), but isozymes was more effective in separating the subfamily Acalyphoideae.

Key words: *Euphorbiaceae*; Genetic relationships; Isozyme; DNA markers.

Introduction

Euphorbiaceae is one of the major flowering plant families: with 5000 species belonging to 334 genera grouped in 52 tribes and 5 subfamilies, is considered as the sixth largest family of Angiospermae (Webster, 1975).Due to their wide distribution in tropical, subtropical and temperate regions and possessing variable vegetative forms varying from trees to herbs and from non-succulent, succulent, *Euphorbia* has 2000 species and is believed to be the second or the third largest genera worldwide (Govaerts *et al.*, 2000; Frodin, 2004; Bruyns *et al*., 2006; Riina *et al*., 2013). *Euphorbia* has four subgenera, *Rhizanthium, Esula, Euphorbia*, and *Chamaesyce* (Steinmann & Porter, 2002; Bruyns *et al*., 2006; Park & Jansen, 2007; Zimmermann *et al*., 2010). The previous phylogenetic researches revealed that the taxonomy of Euphorbiaceae species, especially *Euphorbia* species, was complicated and showed much taxonomic alterations comparing with the traditional taxonomy that depended much on the morphological markers (Webster, 1994; Radcliffe-Smith, 2001). Recently, the molecular markers especially DNA barcoding genes, become powerful tools and can be used to solve these complications in Euphorbiaceae taxonomy. The universal barcoding genes such as the two plant DNA regions matK and rbcL in addition to the two internal transcribed spacers ITS or ITS2, are short DNA sequences that can be used for the identification, differentiating and taxonomic assignment at species, genera and familial level (Fazekas *et al*., 2012; Staats *et al*., 2016). The simplicity of SSR and RAPD approaches also makes them valuable tools for the investigation of DNA fingerprinting. On the other side, isozymes are still powerful biochemical markers for genetic, taxonomic and evolutionary studies. Although their selective neutrality has been before discussed, yet they have several advantages such as frequent polymorphism, codominance, rapidity, simplicity, using small amount of plant material and relatively inexpensive assay.

In Saudi Arabia, Euphorbiaceae is represented by 15 genera, the largest genus, *Euphorbia,* is represented by 38 species (Collenette, 1999; Chaudhary, 2001). Saudi Arabia has a contrast climate that encourages the environmental changes that make the vegetation structure more variable and complex (Thomas *et al*., 2014). Despite the above, there is no detailed biochemical and molecular knowledge concerning most species of family Euphorbiaceae found in Taif region, therefore, our study aimed to examine the genetic relatedness among nine species of family Euphorbiaceae for establishing excess information for better documentation, differentiation and phylogenetic analysis.

Materials and Methods

Plant materials: Leaves of 9 species belonging to family Euphorbiaceae were collected and identified according to Collenette (1999) and Chaudhary (2001) from highlands of Taif in Saudi Arabia (Table 1).

Isozyme electrophoresis: Aldehyde oxidase (AO), alcohol dehydrogenase (ADH), α-and β-esterases (EST), malate dehydrogenase (MDH) and peroxidase (PRX) were detected utilizing 10% Native-polyacrylamide gel electrophoresis procedure (Stegemann *et al.*, 1985). AO, ADH, MDH, α-& β-EST and PRX gels were stained according to protocols of Wendel & Weeden (1989), Weeden & Wendel (1990), Jonathan & Wendell (1990), Scandalios (1964) and Heldt (1997), respectively.

DNA isolation and amplification: CTAB method was used to isolate the DNA of the nine Euphorbiaceae species (Doyle & Doyle, 1987). Different primers for RAPD, SSR and barcoding genes were investigated (Table 3). These primers were supplied by Macrogen Inc. (Seoul, Korea). For PCR amplification, each reaction/sample included 1 µL DNA, 10 µL Taq Master Mix, 1 µL each, forward and reverse primers and 13 µL dis. H_2o. The thermal cycling was performed depending on the following program: initial denaturation of 94°C for 4 min, followed by DNA denaturation stage for 1 min at 94°C, annealing stage for 30s at different temperatures as mentioned in Table 3 and exninesion stage for 1 min at 72°C, these stages were repeated 35 cycle and a final exninesion at 72°C for 7 min.

Table 1. Names and sources of the nine species of Euphorbiaceae under study.

No.	Species	Origin	Location	Co-ordinates	
				Latitude (N)	Longitude (E)
1.	*Euphorbia peplus* L.	Wild	Taif Univ. camp	21°25'59.5"	40°29'29"
2.	*Euphorbia indica* Lam.	Wild	Taif Univ. camp	21°25'59.5"	40°29'29"
3.	*Euphorbiaprostrata* Aiton.	wild	Taif Univ. camp	21°25'59.5"	40°29'29"
4.	*Euphorbiaschimperi* C. Presl	wild	WadiSeesed	21°17' 37.0"	40°29' 30"
5.	*Euphorbia tirucalli* L.	cultivated	Taif Univ. camp	21°25'59.5"	40°29'29"
6.	*Euphorbia granulate* Forssk.	wild	Al-Rodaf	21° 13'49"	40° 25'19"
7.	*Clutiamyricoides* L.	wild	Al-Shafa	21°8'10.83"	40°22'4.83"
8.	*Ricinuscommunis* L.	wild	Taif – Hawia road	21°18'5.5"	40°27'12.8"
9.	*Chrozophoraoblongifolia* L.	wild	Taif – Hawia road	21°18'5.5"	40°27'12.8"

Table 2. Types of bands and their polymorphism (%) detected in the six *Euphorbia* species (*E.*) and the nine Euphorbiaceae species (All) by different DNA markers.

Marker	Sequence (5'-3')	Anneal. temp.	Unique bands		Polymorphic bands		Total bands		Polymorphi-sm (%)	
			E.	All	*E.*	All	*E.*	All	*E.*	All
RAPD	TGCCGAGCTG	36˚C	1	3	0	0	1	3	100	100
SSR 1	F TGTGGGCTGGTGATAGATGT R GCTTCATCCCACGGACTA	50˚C	2	6	0	2	2	8	100	100
SSR 2	F AGACGTTATTTGGAGCAGCA R TCTCGGATCAACATGAGCTG	50˚C	0	0	1	1	1	1	100	100
matK	F CGTACAGTACTTTTGTGTTTACGAG R ACCCAGTCCATCTGGAAATCTTGGTTC	52˚C	0	0	1	1	1	1	100	100
ITS	F ACGAATTCATGGTCCGGTGAAGTGTTCG R TAGAATTCCCCGGTTCGCTCGCCGTTAC	52˚C	0	0	1	1	1	1	100	100
ITS2	F ATGCGATACTTGGTGTGAAT R TCCTCCGCTTATTGATATGC	52˚C	0	0	1	1	1	1	100	100
rbcL	F ATGTCACCACAAACAGAGACTAAAGC R GTAAAATCAAGTCCACCRCG	52˚C	0	0	1	1	1	1	100	100
Total			**3**	**9**	**5**	**7**	**8**	**16**	**100**	**100**

Table 3. Isozyme polymorphism detected in the seven *Euphorbia* species (*E.*) and the nine Euphorbiaceae species (All).

Isozyme	Monomorphic bands		Polymorphic bands				Total		Polymorphism (%)	
			Unique		Non-unique					
	E.	All	*E.*	All	*E.*	All	*E.*	All	*E.*	All
α-EST	0	0	11	9	4	6	15	15	100	100
β-EST	0	0	7	6	3	5	10	11	100	100
ADH	0	0	0	0	0	0	0	0	0	0
AO	1	1	0	0	0	0	1	1	0	0
MDH	0	0	0	0	0	1	0	1	0	100
PRX	0	0	5	4	4	8	9	12	100	100
Total	1	1	23	19	11	20	35	40	97.1	97.5

Statistical analysis: Isozyme and DNA patterns were analyzed by gel analyzer program III. The polymorphism percentage by dividing the polymorphic bands on the total recorded bands (Fig. 1). For UPGMA cluster analysis construction, each band of isozyme and DNA patterns was coded as 1or 0 for its presence or absence, respectively, using NTSYS-pc 2.2 program (Rohlf, 1998).

Results and Discussion

DNA analysis: DNA extracted from the nine Euphorbiaceae species was tested with one RAPD primer, two microsatellites (SSR) and four DNA genomic regions (ITS, ITS2, matK and rbcL). The utility of DNA barcoding genesfor resolving the phylogenetic relationships in Euphorbiaceae has been previously established (Loo *et al*., 1995; Cameron *et al*., 2001; Gustafsson *et al*., 2002; Wurdack *et al*., 2005; Pang *et al*., 2010; Cardinal-McTeague & Gillespie, 2016). The sequences of primers, types of bands and polymorphism percentages are shown in Table 2 and Fig. 2. Little data was obtained through the utility of RAPD and SSR primers, this may be due to some of the problems with them that were related to reproducibility, designing suitable primers and the PCR

amplification conditions. The 7 DNA markers generated 16 bands with polymorphism percentage of 100% indicating a high level of polymorphism. Nine unique bands were identified by RAPD and SSR-1. Three of them distinguished *E. schimperi* (subfamily *Euphorbioideae* subg. *Esula* sect. *Aphyllis*) and *E. tirucalli* (subg. *Euphorbia* sect. *Tirucalli*), whereas, the remaining bands characterized *Clutiamyricoides* and *Chrozophora oblongifolia* (subfam. *Acalyphoideae*, tribe *Clutieae* and *Chrozophoreae* respectively). However, the four barcoding loci did not reveal discriminatory bands for all the examined species. In some cases, DNA barcoding analyses fail to discriminate between species because of the considerable similarity between their DNA sequences in the amplified region (Arif *et al*., 2010). The PCR amplification percentage was higher in rbcL locus (90%) than those of ITS, ITS2 and matK (50%). ITS2 characterized *E. peplus* (subfam. *Euphorbioideae* subg. *Esulasect. Tithymalus*) only, the three loci; ITS, matK and rbcL, discriminated *E. peplus* and *E. tirucalli*f rom other species within genus *Euphorbia*. Moreover, ITS and matK discriminated *Clutia myricoides*, and *Ricinus communis* (subfam. *Acalyphoideae*, tribe *Acalypheae*) from *Chrozophora oblongifolia* (Fig. 2). The UPGMA dendrogram resulted depending on the 16 DNA bands showed little variability within species than that of isozyme data (Fig. 4). At coefficient 0.00, *E. indica*, *E. prostrata* and *E. granulata* (subfam. *Euphorbioideae* subg. *Chamaesyce*) were grouped together. *E. peplus* joined with *Ricinus communis* at 0.89, whereas, *E. schimperi*, *E. tirucalli*, *Clutia myricoides* and *Chrozophora oblongifolia* diverged into separate clades.

Fig. 1. Photos of the nine Euphorbiaceae species under study. (A) *E. peplus*, (B) *E. indica*, (C) *E. prostrata*, (D) *E. schimperi*, (E) *E. tirucalli*, (F) *E. granulata*, (G) *Clutiamyricoides*, (H) *Ricinuscommunis* and (I) *Chrozophoraoblongifolia*.

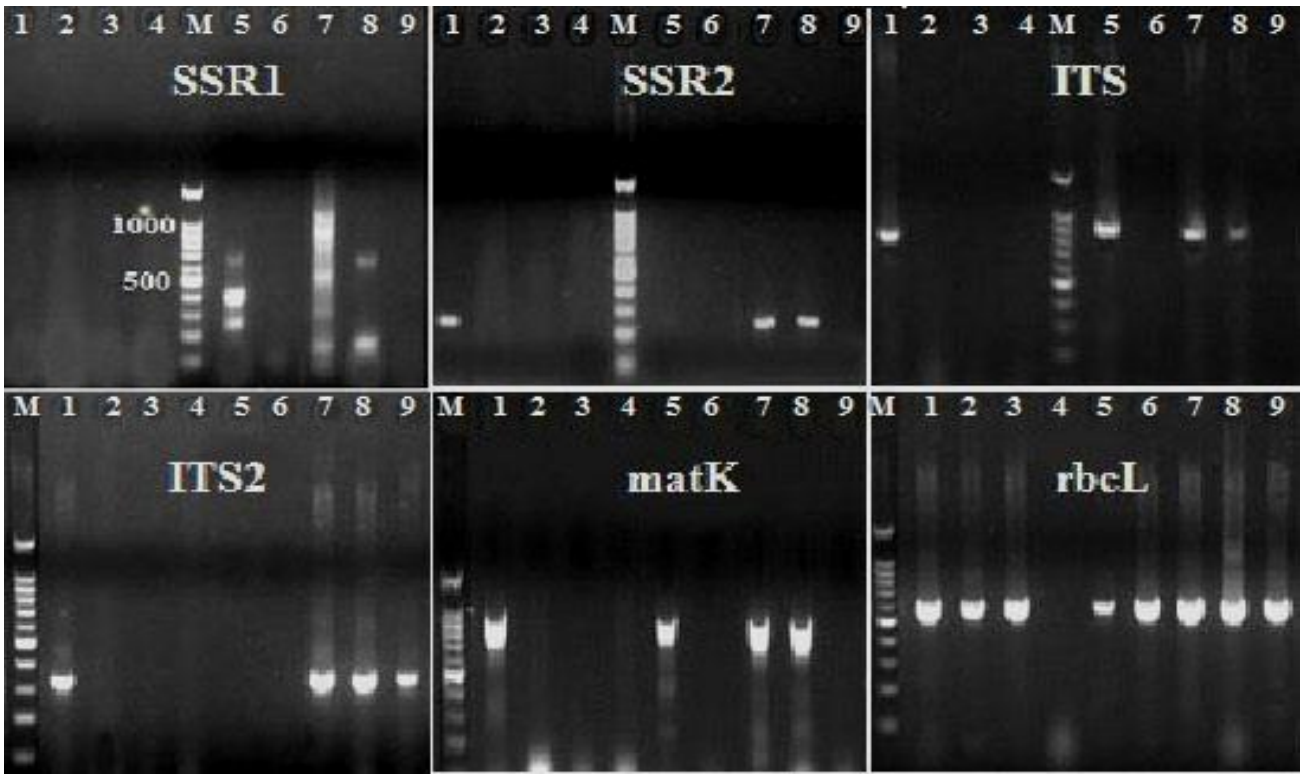

Fig. 2. Six DNA patterns of the nine Euphorbiaceae species; (1) *E. peplus,* (2) *E. indica,* (3) *E. prostrata,* (4) *E. schimperi,* (5) *E. tirucalli,* (6) *E. granulata,* (7) *Clutiamyricoides,* (8) *Ricinuscommunis,* (9) *Chrozophoraoblongifolia*. (M) marker.

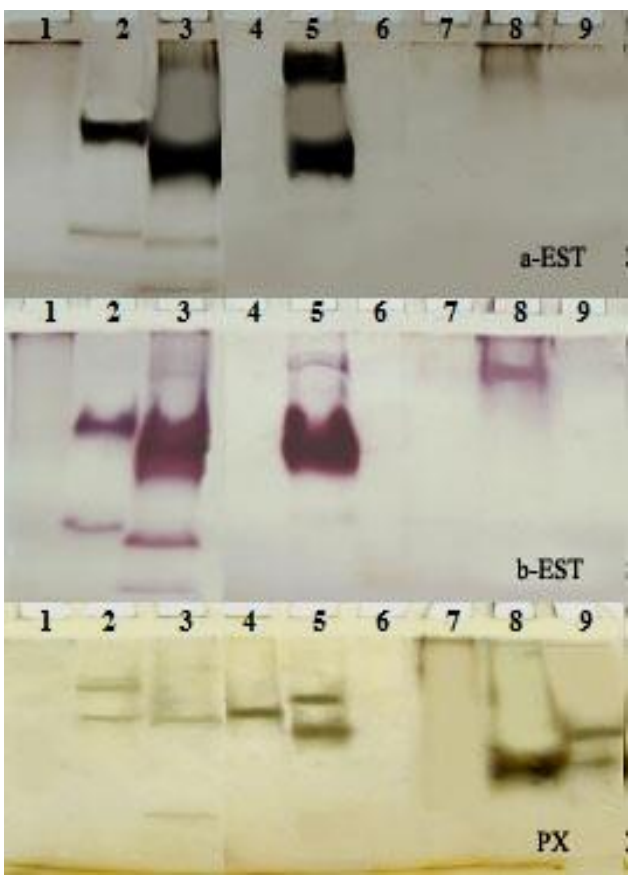

Fig. 3. Zymograms of three isozymes for the nine Euphorbiaceae species; (1) *E. peplus,* (2) *E. indica,* (3) *E. prostrata,* (4) *E. schimperi,* (5) *E. tirucalli,* (6) *E. granulata,* (7) *Clutia myricoides,* (8) *Ricinus communis,* (9) *Chrozophora oblongifolia*.

Isozyme analysis: Results in this research represented the first use of different isozyme markers to characterize the interspecific genetic variability and discriminating among 9 species of Euphorbiaceae in Saudi Arabian flora. Banding patterns of the six isozyme systems showed great variability (Fig. 3). Alcohol dehydrogenase (ADH) scored no band. Forty electrophoretic bands were detected in the other five zymograms. From which 39 were polymorphic with high polymorphism percentage (nearly 97 %) (Table 3). Aldehyde oxidase (AO) recorded the only monomorphic band. Twenty unique bands were produced by α-and β-esterases (EST) and peroxidase (PRX). These unique bands distinguished six species; *E. indica*, *E. prostrata*, *E. schimperi*, *E. granulata*, *E. tirucalli* and *Ricinus communis*. *E. prostrata* had the highest number of products (17 bands), whereas *E. tirucalli* recorded the least (4 bands). The UPGMA dendrogram obtained depending on the 40 isozyme bands showed different findings from that of DNA (Fig. 5). At coefficient 0.63, *Ricinus communis* and *Chrozophora oblongifolia* were grouped together. *E. peplus* joined with *Clutia myricoides* at 0.95. Except for *E. schimperi* and *E. tirucalli* that joined at coefficient 1.11, the other species of the genus *Euphorbia* formed separate clades offering higher genetic diversity. Wurdack *et al.*, (2005) revealed that two subfamilies; Acalyphoideae, Crotonoideae, and 10 genera (via. *Euphorbia*) of family Euphorbiaceae were found to be para- or polyphyletic reflecting poor relationships within them. The isolation of *Euphorbia* species was also confirmed by Aljibouri *et al*., (2013). These results showed that isozymes are useful when several genera and species are compared, as the assumption of variability is more expected than from some DNA markers.

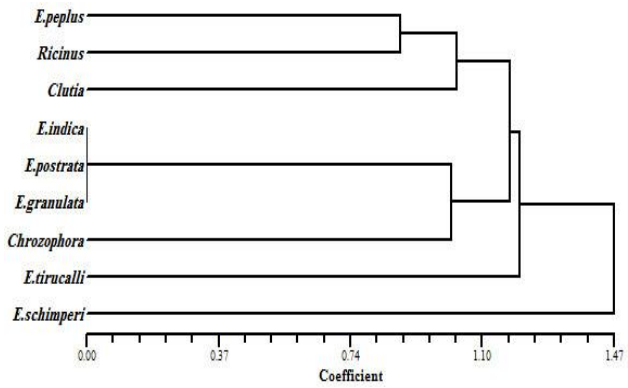

Fig. 4. UPGMA phenogram showing genetic relationships among the nine Euphorbiaceae species depending on DNA data.

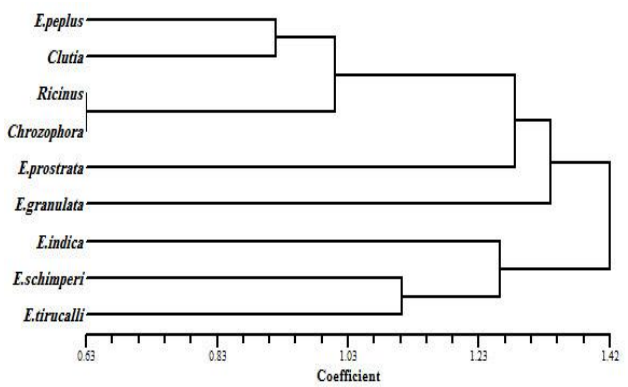

Fig. 5. UPGMA phenogram showing genetic relationships among the nine Euphorbiaceae species depending on isozyme data.

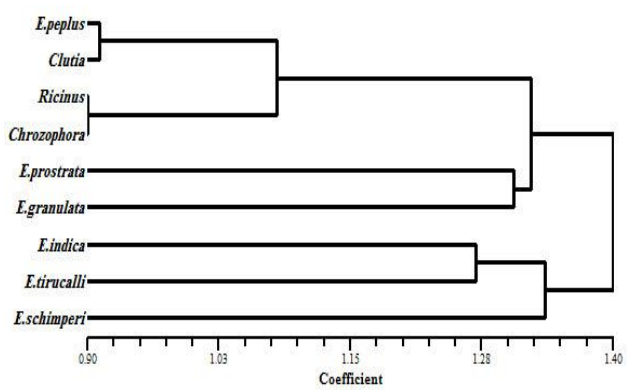

Fig. 6. UPGMA phenogram showing genetic relationships among the nine Euphorbiaceae species depending on combined isozyme and DNA data.

Combined molecular analysis: The two previous dendrograms (Figs 4&5) showed that there was no correlation between isozyme and DNA characters. Subsequently, data of isozyme and DNA patterns were combined together to form the UPGMA phenogram as shown in Figure 6. Four groups were formed at different coefficient values. The first included *E. peplus* and *Clutia myricoides* and the second consisted of *Ricinus communis* with *Chrozophora oblongifolia* as in isozymephenogram. The third that involved *E. prostrata* with *E. granulate* and the fourth that included *E. indica* and *E. tirucalli* were considered as new groups. *E. schimperi* formed a separate clade. Weak genetic relatedness between *E. peplus* and three species*; E. granulata*, *E. prostrata* and *Ricinus communis*was also reported by Aljibouri *et al*., (2013), Mohamed *et al*., (2016) and Moustafa *et al*. (2016) using RAPD and ISSR markers. The DNA and isozymesphenograms (Figs. 4&5) integrated together and distinguished the studied species into two subfamilies *Euphorbioideae* and *Acalyphoideae* with the exception of *Chrozohora* and *E. peplus.* Species belonging to subgenus *Esula* (*E. peplus* and *E. Schimperi*) were separated from other *Euphorbia* species according to the DNA data. Steinmann & Porter (2002) showed that some species of subg. *Esula*. sect. *Tithymalus*Boissier were polyphyletic and did not belong to subg. *Esula*. This might be due to some of the problems with them that are related to reproducibility, designing suitable primers and the PCR amplification conditions. The DNA data was more accurate than isozymes data as it grouped the 3 species belonging to subg. *Chamaesyce* (*E. indica*, *E. prostrata* and *E. granulata*), but isozymes was more effective in separating the subfamily *Acalyphoideae* despite the obvious morphological differences between them as *Ricinus* is a large shrub without latex, *Clutia* is small under shrub with latex and *Chrozophora* is a large herb without latex.

Despite the tireless efforts to characterize the germplasm collections of flora of Saudi Arabia, there are few molecular markers available. Curiously, the use of widely spread molecular approaches, as isozymes and DNA primers, in polymorphism and genetic diversity analyses is still insufficient and problematic. Thus, our results will be quite useful for the taxonomical studies by providing valuable information about genetic diversity of these species under study. Furthermore, our efforts in revealing novel molecular markers certainly should help the description of the genetic relatedness for 9 species of Euphorbiaceae. However, still there is a need for selection of new molecular approaches such as DNA sequencing process for more characterization and distinguishing among species of Euphorbiaceae.

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