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Phylogenetic Relationships Among Some Species Of The Genus *Euphorbia* L. (Euphorbiaceae) In Kurdistan Region – Iraq

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Abstract
A phylogenetic analysis of *Euphorbia* is presented using sequences from nine species of nuclear ribosomal DNA ITS and 28S rRNA. *Euphorbia* is among the largest genera and most diverse of four recently recognized subgenera within *Euphorbia*, with about 2000 species that are renowned for their remarkably diverse growth forms. Relationships within this group have been difficult to discern due mainly to homoplasious morphological characters and inadequate taxon sampling in previous phylogenetic studies. To clarify phylogenetic relationships in the genus, we used Maximum Parsimony (MP) and Bayesian Inference (BI) analyses. The combined analysis of two molecular markers resulted in only one most parsimonious tree and also generated new supported clades, in general, the backbone of the ITS tree from the concatenated matrix is better supported than 28S rRNA tree, although the order of divergence among the constituent clades is different and the topology is better supported and it resolves most of the same subclades that is common between them. It's concluded that the first comprehensive investigation on the genetic variation in *Euphorbia* and it provides resources for phylogenetic research in the genus, facilitating further studies on its taxonomy, evolution, and conservation.

Keywords: Maximum Parsimony, Bayesian Inference, ITS, 28S rRNA, *Euphorbia*.

Introduction

The Spurge family (Euphorbiaceae) is one of the most complex, largest and most diverse families of angiosperms and occurs in arid and semiarid environments It comprises approximately 300 genera and 8000 species, and it is established as an important source of medicines and toxins (Narendra et al., 2015). In Iraq, this family contained about 7 genus and 51 species (Pandey, 2009). In addition, *Euphorbia* L. is the largest genus in the spurge family with about 2100 cosmopolitan species (Bolaji et al., 2014). Unlike most other large angiosperm clades recognized as genera, species richness in *Euphorbia* is coupled with remarkable structural variability.

The combination of these attributes distinguishes the genus as a promising group in which to investigate fundamental evolutionary questions about the origin of morphological novelty and diversification (Horn et al., 2012). Molecular phylogenetic studies have improved the delimitation of the *Euphorbia* as well as its infrageneric classification. Here, only *Euphorbia* has been consistently placed in the subtribe *Euphorbiinae*, for several traditionally segregated genera including *Chamaesyce*, *Cubanthus*, *Elaeophorbia*, *Endadenium*, *Monadenium*, *Pedilanthus*, *Poinsettia*, and *Synadenium* are now understood to be deeply nested within *Euphorbia* (Steinmann and Porter, 2002, Bruyns et al., 2006, Steinmann et al., 2007, Zimmermann et al., 2010, Bruyns et al., 2011, Horn et al., 2012).

It has recently been established that the genus is composed of four subgenera, subg. *Esula*, *Athymalus*, *Chamaesyce*, and *Euphorbia* have been gradually recovered in *Euphorbia*, although the phylogenetic relationship among them has been in flux (Steinmann and Porter, 2002, Bruyns et al., 2006, Bruyns et al., 2011, Horn et al., 2012). Of these four, subg. *Euphorbia* is the largest and most morphologically diverse, and this makes it a particularly good group in which to investigate the causes of morphological evolution. Previous studies had established the monophyly of several clades belonging to subg. *Euphorbia*, but in each case the focus on the entire genus or was limited in geographic scope (Horn et al., 2012). Over the past decade, molecular phylogenetic studies have made much progress in understanding the broad scale relationships within *Euphorbia* (Steinmann and Porter, 2002, Bruyns et al., 2006, Park and Jansen, 2007, Bruyns et al., 2011, Horn et al., 2012, Yang et al., 2012).

Steinmann and Porter (2002)circumscribed *Euphorbia* as the clade including all species with cyathia and furthermore established the presence of four major clades within the genus. Bruyns et al. (2006) formally recognized these four clades as subgenera: *E.* subg. *Esula* Pers., E. subg. *Rhizanthium* (Boiss.) Wheeler, *E.* subg. *Chamaesyce* Raf., and *E.* subg. *Euphorbia*. Horn et al. (2012)analyzed ten gene regions from all three plant genomes to firmly establish that subg. *Esula* is the first clade to diverge, followed by subg. *Rhizanthium*, which is sister in turn to the clade of subg. *Chamaesyce* and subg. *Euphorbia*. With the relatively sparse taxon sampling in all previous phylogenetic studies, many

species in *Euphorbia* had not been placed to their corresponding subgenus, and relationships within the subgenera are still incompletely resolved. From an evolutionary and taxonomic standpoint, there is a need to develop a comprehensive sectional classification for each of the subgenera. An understanding of the evolution of reproductive and vegetative traits that encompass the most striking structural variants for which *Euphorbia* is renowned is necessary to achieve this goal. Hence, our primary objective in presenting a new phylogenetic hypothesis of *Euphorbia* is to use it as framework for a series of evolutionary analyses that illuminate the origin and evolution of the kaleidoscopic structural diversity within the entire clade. We discuss the biological significance of these results, and comment on their possible significance to the diversification of *Euphorbia*.

Material and Methods

Taxon sampling

The plant taxa used in the present study were collected from the different districts of Kurdistan region-Iraq that preserved in the Herbarium of College of Education/Salahaddin University-Erbil (Table 1). Ten distinct taxa consist of nine in group taxa and one out group *Andrachne aspera* were used in the analysis.

DNA extraction

Total DNA was extracted from the collected specimens. The extraction method was based on the CTAB protocol of Doyle and Doyle (1990) with some modification (1× CTAB: 10 mL of 1.0 M Tris-HCl, PH 8; 4.0 mL of 0.5 M EDTA, PH 8; 28.0 mL of 5 M NaCl; 2% CTAB; 2.0 g PVP; and 158 mL ddH_2O), the washing process of the DNA pellet has been conducted twice with 0.5 mL of 80% ethanol, then DNA was dissolved in 25 µL TE-buffer.

PCR and DNA sequencing

The two noncoding regions of nrDNA ITS and 28S rRNA were amplified by using the primers as shown in (Table 2). The primers were ordered from Macrogen Company, Seoul, Korea. The total volume of amplification reactions was 25.0 µL and Master Mix made up of 12.5 µL, 3.0 µL genomic DNA extract, 2.0 µL of each primer, 5.5 µL free nuclease water. The PCR-Thermal cycler for 28S rRNA gene started with 5 min for initial denaturation at 94°C followed by 35 cycles: denaturation at 94°C for 30 sec.; annealing at 54°C for 60 sec.; extension at 72°C for 60 sec. and the final extension at 72°C for 5 min. While, the PCR program for ITS gene started with 5 min for initial denaturation at 94°C followed by 35 cycles: denaturation at 94°C for 30 sec.; annealing at 56°C for 20 sec.; extension at 72°C for 20 sec. and the final extension at 72°C for 5 min. The resultant PCR products were checked on 1.5% agarose gel run in TAE buffer. The gel was stained with Safe red dye and photographed under UV transilluminator. PCR products were purified by using Kits (Promega Company-Madison-USA). The purified PCR products were sent to the National Science and Technology Development Agency (NSTDA) in Thailand for sequencing.

Sequence alignment and phylogenetic analysis

All the DNA sequences were edited and aligned with ClastalW option available in BioEdit, Version 7.0.4.1 (Hall, 2001) and manual adjustment, there are 10 accessions for each ITS, 28S rRNA, including the out group species. Bayesian inference (BI) and Maximum parsimony (MP) analyses were conducted for each dataset separately built from the two markers that included 10 terminal taxa with all sequences available. For MP, PAUP- 4.0a164 (Swofford, 2000) was also used. Using heuristic search with 100 replicates of random taxon additions, Tree-Bisection-Reconnection (TBR) branch swapping, MulTrees on, and steepest decent off was performed. The maximum numbers of saved trees were 100 for each replicate. The bootstrap values were calculated from 100 replicates, the consistency index (CI), retention index (RI), rescaled consistency (RC), and homoplasy index (HI) were measured (Felsenstein, 1985).

Before running BI, the optimal substitution models were estimated using the Akaike information criterion (AIC) in MrModeltest2 version 2.3 (Nylander et al., 2004). The general time reversible model of nucleotide substitution with gamma-shaped rate variation and a proportion of invariable sites (GTR+I+G) was the estimated best-fit model for ITS region and (HKY+G) was the estimated best-fit model for 28S rRNA. For BI analyses we used MrBayes v.3.2 (Ronquist and Huelsenbeck, 2003). The priors on state frequencies and rates and variation across sites were estimated automatically by the program. Four Markov chains starting with a random tree were run simultaneously, two independent analyses were run with 2 million generations set for ITS and 5 million generations for 28S rRNA datasets with four chains (one cold and three heated) for each generation and the temperature parameter set to 0.1. Trees were sampled every 100th generations. After that (25% of initial tree sampled) were removed by burn-in period samples, a tree with maximum 50% (majority rule consensus tree) was plotted. The value of posterior probability (PP) was calculated and the final tree was plotted by using FigTree software version 1.4.3 (Rambaut, 2016).

Table 1. Specimen numbers of *Euphorbia* species which their DNA have been studied, and their preserved locations in the Herbarium of College of Education, Salahaddin University-Erbil with collection date.

Species	Specimen number & Herbarium symbol		Specimen location	Date of collection
E. aleppica	8023	ESUH	Shaqlawa	22.7.2019
E. falcata	8031	ESUH	Mergasur	12.9.2018
E. macroclada	8034	ESUH	Amadiya	8.6.2017
E. cuspidata	8039	ESUH	kalak	13.5.2019
E. helioscopia	8046	ESUH	Jundian	15.4.2019
E. altissima	8053	ESUH	Sarsang	18.7.2018
E. petiolata	8059	ESUH	Rowanduz	26.8.2018
E. chamaesyce	8063	ESUH	Aqra	22.6.2016
E. granulata	8068	ESUH	Kirkuk	7.3.2014

Table 2. List of primers and their sequences that have been used in the study.

Primer name	Product size	Primer Sequences		References
		Foreword	Foreword	
28S rRNA	700 bp	TCT GAC ATG TGT GCG AGT CA	GAT TCG GCA GGT GAG TTG TT	(Chen et al., 2010)
ITS	400 bp	ATG CGA TAC TTG GTG TGA AT	TCC TCC GCT TAT TGA TAT GC	(Taberlet et al., 1991)

Results and discussion

Summary statistics for the DNA matrices are given in (Table 3). In general, the backbone of the ITS tree from the concatenated matrix (Figure 1) is better supported than 28S rRNA tree, although the order of divergence among the constituent clades is different and the topology is better supported and it resolves most of the same subclades that is common to (Figure 2). The analyses were carried out for separate regions, consisted of nine in groups and one outgroup taxa. The tree topology of the maximum parsimony (MP) showed same results with Bayesian (BI) analysis.

The tree of nrDNA ITS consist of five clades as follow: clade A consists of *E. aleppica* and *E. falcata* are well supported with bootstrap support (96 – 1.00); clade B consists of *E. macroclada* and *E. cuspidata* with bootstrap support (92 – 1.00) both are perennial herb; clade C consists of *E. helioscopia* and *E. altissima* were highly supported with bootstrap support (100 – 1.00); clade D consists of *E. petiolata* and *E. chamaesyce* were highly supported with bootstrap support (100 – 1.00); and finally clade E consist of only one species which is *E. granulata* poorly supported with bootstrap support (63 – 0.62).

The tree of 28S rRNA consists of three clades as follow: Clade A consists of *E. petiolata*; *E. altissima; E. cuspidata; E. macroclada* and *E. falcata* well supported with bootstrap support (60 – 0.67); while clade B consists of two sister species *E. chamaesyce* and *E. aleppica* well supported with bootstrap support (82 – 1.00) and finally clade C consists of two paraphyletic species which are *E. granulata* and *E. helioscopia* with bootstrap support (100 – 0.85).

Individual analyses of the ITS and 28S rRNA data sets produced estimates of phylogenetic relationships (MCC trees) that resolved most of the same monophyletic groups, although the relationships among these clades are not consistent between the two trees (Figure 1 and 2).

Table 3. A summary of alignment and tree statistics of 28S rRNA, ITS and combined analyses**.**

Parameters/Regions	ITS	28S rRNA
Aligned length	392	645
Number of parsimony informative characters	107	47
Number of variable parsimony uninformative characters	106	330
Number of constant characters	179	268
Tree length (steps)	409	454
CI (Consistency Index)	0.770	0.923
RI (Retention Index)	0.525	0.533
RC (Rescaled Index)	0.405	0.492
HI (Homoplasy index)	0.230	0.077
Model	GTR+I+G	HKY+G

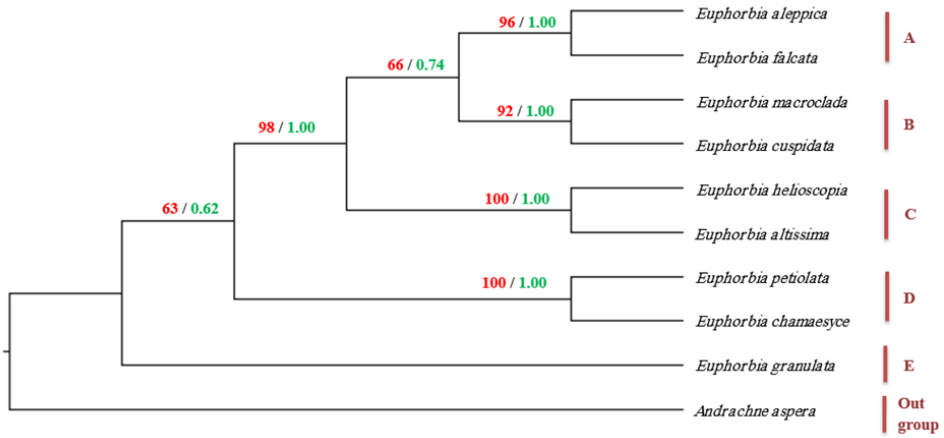

Figure 1. Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the combined sequences with heuristic search using maximum parsimony analysis. (Tree length of 392 steps, CI = 0.770, RI = 0.525, RC = 0.405 and HI =0.230). Numbers on the branches in red color indicate bootstrap support and numbers in green color are Bayesian posterior probability values and clades are identified by letters.

The ITS tree is composed of five main clades, whereas the 28S rRNA tree includes a basal grade subtending three major clades. Despite the fact that many of the same clades are inferred between the two analyses, there are several cases of significant incongruence. The main conflict between the trees lies in the placement of several clades that form a monophyletic group in the ITS tree. But that form a grade at the base of the 28S rRNA tree. Importantly, the diverging clade A in the basal grade of the 28S rRNA tree, whereas they are split into four separate clades in the ITS tree.

With our greatly increased sampling effort, we confirmed the results of several previous studies, which found that *Euphorbia* subg. *Euphorbia* is a strongly supported monophyletic group (Steinmann and Porter, 2002, Bruyns et al., 2006, Bernal et al., 2006, Zimmermann et al., 2010, Horn et al., 2012).

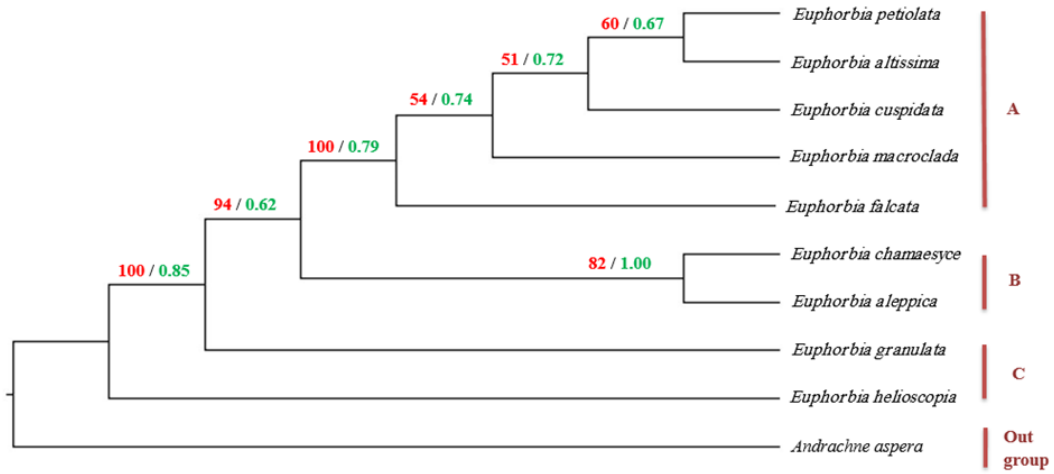

Figure 2. Strict consensus tree of most parsimonious tree resulting from phylogenetic analysis of the combined sequences with heuristic search using maximum parsimony analysis. (Tree length of 645 steps, CI = 0.932, RI = 0.533, RC = 0.492 and HI =0.077). Numbers on the branches in red color indicate bootstrap support and numbers in green color are Bayesian posterior probability values and clades are identified by letters.

Based on 28S rRNA tree *E. helioscopia* was seem to be ancestral form for all *Euphorbia* species (Barres et al., 2011, Horn et al., 2012, Dorsey et al., 2013, Tian et al., 2018, Wei et al., 2021), which were supporting our finding.

Conclusion

The analyses presented by this study resulted in only one most parsimonious tree and also generated new supported clades showing close relationships among the major clades and among the subclades within *Euphorbia* species. Based on our results and synthesis, it is clear that the evolution in the genus was critical to its overall diversification. Our findings point to the need for further phylogenetic explorations across plant lineages. To better perform phylogeny-based studies for *Euphorbia* in the future, we screened promising molecular markers both from nrDNA

IS and 28S rRNA regions. Lastly, the monophyly of *Euphorbia* and its species is supported, using a phylogenomic framework. These topological incongruences deserve further explorations to the underlying biologically relevant evolutionary history, using nuclear datasets.

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