



Journal Homepage: - www.journalijar.com
**INTERNATIONAL JOURNAL OF
 ADVANCED RESEARCH (IJAR)**

Article DOI: 10.21474/IJAR01/5537
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/5537>



RESEARCH ARTICLE

ANATOMICAL AND MOLECULAR CHARACTERIZATION OF SELECTED SPECIES OF *EUPHORBIA* L. (EUPHORBIACEAE) FROM KERALA, INDIA.

Lekshmi S., Dinesh Raj R., Asha Ramachandran and Sivaprasad A.

Dept. of Botany and Biotechnology, Bishop Moore College, Mavelikkara, Alappuzha (Dt), Kerala, India - 690 110.

Manuscript Info

Manuscript History

Received: 04 August 2017
 Final Accepted: 06 September 2017
 Published: October 2017

Key words:-

Anatomy, Morphological variation,
 Genetic diversity, *Euphorbia*, UPGMA.

Abstract

The study has documented the anatomical and molecular variability of the genus, *Euphorbia* from the southern districts of Kerala. Expeditions across the study area identified six species, namely *E. hirta*, *E. microphylla*, *E. heterophylla*, *E. milii*, *E. tirucalli* and *E. pulcherrima*. Anatomy of stem, root and leaves revealed much variability among species. Among the anatomical characters, leaf characters such as nature and type of stomata, nature of trichomes and stem characters like nature of hypodermis and cortex etc. are of high taxonomic significance. An artificial key for the identification based on anatomical characters was prepared. Molecular characterization using chloroplast loci, *matK* and nuclear loci, *ITS* revealed the phylogenetic interrelationships of the species. Nuclear diversity parameters showed an elevated level of diversity for the nuclear loci, *ITS* when compared to the chloroplast loci. Clustering pattern in the UPGMA dendrogram was mostly in accordance with the subgeneric identity of the species. This work revealed the blue print of anatomical and molecular variations exhibited by *Euphorbia* in the studied locality.

Copy Right, IJAR, 2017, All rights reserved.

Introduction:-

Euphorbia (Euphorbiaceae) is the second-largest genus of flowering plants with over 2150 species (Frajman and Schonswetter, 2011). In India, *Euphorbia* is represented by more than 195 species (Aditya, 2010) and widely distributed in varied habitats. The morphological diversity in this genus includes geophytes, herbs, shrubs, understory and canopy trees, and an array of succulent and xerophytic forms. Despite this vast vegetative variation, the entire genus is united by a distinctive morphological synapomorphy, the cyathium – a pseudanthial inflorescence that looks superficially like a typical dicot flower (Horn et al., 2012). This structure is intermediate between a flower and an inflorescence in developmental terms (Prenner and Rudall, 2007) and is comprised of a cup-like involucre that surrounds multiple male flowers (reduced to single stamens) and a single female flower (reduced to a single pistil). From this basic structure various elaborations have evolved, including colorful subtending bracts, cyathial nectary glands with petaloid appendages, and fusion or addition of cyathial glands. Some of these cyathial traits represent synapomorphies for particular clades within the genus (Dorsey, 2013).

It has been well established that the genus is composed of four subgenera, *Rhizanthium*, *Esula*, *Euphorbia*, *Chamaesyce*, (Steinmann and Porter, 2002; Bruyns et al., 2006; Bruyns et al., 2011; Horn et al., 2012). Several phylogenetic studies have been reported earlier and have made much progress in our understanding about the broad scale relationships within *Euphorbia*. (Steinmann and Porter, 2002; Bruyns et al., 2006, 2011; Park and Jansen,

Corresponding Author:- Dinesh Raj R.

Address:- Dept. of Botany and Biotechnology, Bishop Moore College, Mavelikkara, Alappuzha (Dt), Kerala, India - 690 110.

2007; Horn et al., 2012; Yang et al., 2012; Dorsey, 2013). Even so, the deeper relationships within the genus remain unclear. Importantly, several hypotheses exist as to the relationships among the four subgeneric clades, and the relationships of major subclades within each of the subgenera are even more ambiguous (Horn et al., 2012). Several floristic studies of the family Euphorbiaceae have been reported from India, considering the economic importance of the family (Balakrishnan and Chakrabarty, 2007; Aditya, 2010 and references therein; Ramarajan et al., 2015 and references therein; Beg, 2015). Whereas, a systematic approach to characterize and apportion the morphological and genetic variability of the species is scanty. Furthermore, no accessions from India have been used in any of the phylogenetic studies reported (Frajman and Schonswetter, 2011; Horn et al., 2012, 2014).

The anatomical structures exhibit a wide range of variation in correlation with the diversity of habit in *Euphorbia* (Metcalf and Chalk, 1950). Anatomical characterization of a few species of *Euphorbia* and its systematic potential is reported elsewhere (Kakkar and Paliwal, 1974; Lukovic et al., 2009; Essiett et al., 2012; Aldhebiani and Jury, 2013; Zahra et al., 2014). All these studies exposed the epidermal and leaf anatomy, leaving anatomy of stem, root and the secondary structure largely unattempted, except a few (Zhang, 2008).

The present study is aimed to fill this gap, by studying the anatomical and molecular variability of the species *Euphorbia* from the southern districts of Kerala, a region known for its rich biodiversity. Being a part of Western Ghats - Sri Lanka biodiversity hotspot, this region exhibits much diversity and endemism of species (Gunawardene et al., 2007). High rate of endemism for Euphorbiaceae family in this region is reported (Volga et al., 2013). Analyzing the genetic and structural diversity of species in this region, and combining it with global data, will offer a very deep understanding in the variability and distribution of the genus, *Euphorbia*.

Materials and methods:-

Plant materials: Several collection expeditions were conducted throughout the southern districts of Kerala, i.e., Thiruvananthapuram, Kollam and Alappuzha. Ornamental species were also included in the study. Both vegetative and floral parts were collected after taking photographs of the entire plant using a digital camera (Olympus). The field observations like habit, habitat, inflorescence, flower colour, nature of fruits etc. were noted in a field diary. The specimens of appropriate size with relevant parts were collected for herbaria. The herbaria were prepared as per the standard protocol seen elsewhere (Sambamurthy, 2005). The floral and vegetative parts were closely examined and identified using available floras (Gamble, 1921, Manilal and Sivarajan, 1982, Sasidharan, 2006). The identity of the taxon was confirmed by referring Jawaharlal Nehru Tropical Botanic Garden and Research institute (JNTBGRI) herbarium, TBGT.

Anatomical study: Stem and root from 5 cm away from the apex and adult leaves were collected and fixed in FAA 70 (Johansen, 1940), which was replaced by 70% ethanol (Berlyn and Miksche, 1976). Fresh/preserved material was sectioned by hand and stained with Saffranin.

Molecular analysis: List of species used for molecular analysis is given Table 1. Molecular analysis were outsourced at Regional Facility for DNA Fingerprinting (RFDF) of Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. Genomic DNA was isolated using NucleoSpin[®] Plant II Kit (Macherey-Nagel) kit as per the manufacturer's instructions. The quality of the DNA isolated was checked using agarose gel electrophoresis. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). One chloroplast loci, *matK* (f- CGATCTATTCATTCAATATTTTC, r- TCTAGCACACGAAAGTCGAAGT) and one nuclear loci, ITS (f- GGAAGTAAAAGTCGTAACAAGG and r TCCTCCGCTTATTGATATGC) were sequenced. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM MgCl₂), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hot start II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml Ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the Bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes. Sequencing reaction was done in a PCR thermal cycler (GeneAmp

PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufacturer's protocol. The sequencing PCR temperature profile consisted of a 1 cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes for all the primers. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

Table 1:- List of *Euphorbia* species used for molecular analysis.

Sl.No.	Species	Geographic origin	Status	Subgenus
1	<i>E. hirta</i>	Thiruvananthapuram	wild	<i>Chamaesyce</i>
2	<i>E. microphylla</i>	Mavelikara	wild	<i>Chamaesyce</i>
3	<i>E. heterophylla</i>	Thiruvananthapuram	wild	<i>Chamaesyce</i>
4	<i>E. milii</i>	Thiruvananthapuram	ornamental	<i>Euphorbia</i>
5	<i>E. tirucalli</i>	Thiruvananthapuram	ornamental	<i>Euphorbia</i>
6	<i>E. pulcherrima</i>	Thiruvananthapuram	ornamental	<i>Chamaesyce</i>

Sequence analysis:-

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010). The sequences were subjected to homology searches at National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search (BLAST) algorithm in order to assess the authenticity of the sequence obtained. Nucleotide diversity parameters like Number of polymorphic sites (S), Total number of mutations (Eta), Average number of nucleotide differences (k), Nucleotide diversity, Pi (π) (Watterson, 1975). Theta (θ) (per sequence) from Eta.; Theta (θ) (per site) from Eta ; Tajima's D (Tajima, 1989) were computed the software DnaSP, V5 (Librado and Rozas, 2009). For Tajima's D, negative values indicate an excess of low frequency polymorphisms, whereas positive values indicate an excess of intermediate variants. Unweighted pair group method with arithmetic average (UPGMA) based clustering analysis of sequences was performed with the help of MEGA Version 5 (Tamura et al., 2011).

Results and Discussion:-

Anatomical Characterization:-

Euphorbia heterophylla:-

Stem: Transverse section (TS) of stem circular in outline and fistular in nature. Epidermis single layered and consists of sub-rectangular cells with straight anticlinal walls. The epidermal cells heavily cutinised and cuticularised; Young stem shows epidermal hairs but lost during growth. Epidermis followed by 2-3 layered collenchymatous hypodermis. Cortex parenchymatous, 2-3 layers of outer cortex with dense chloroplasts, cells smaller and compactly placed; chlorenchyma reaching epidermis interrupting collenchymatous hypodermis at intervals. Inner cortex 4-5 layered, cells comparatively smaller, compactly placed, with few chloroplasts. Latex cells found in the cortex intermittently. Starch granules found in inner cortical cells. Endodermis and pericycle not distinct. Vascular bundles are conjoint, collateral and open. Secondary thickening normal and resulted in continuous vascular cylinder. Phloem is slightly developed and appears almost identical to cambium. Secondary xylem well developed and consists of vessels, tracheids, fibres and parenchyma. Vessels are clustered in the primary bundle region and mostly in uniseriate rows. Annual rings absent. The central region is hollow pith is lined with broken cell walls.

Leaf: T.S. of leaf shows mount towards the abaxial surface. Both upper and lower epidermis uniseriate and consists of cutinised and cuticularised cells. Stomata anomocytic and restricted to abaxial epidermis. Long, multi-cellular, non glandular trichomes present in adaxial epidermis. Four-five layers of collenchymatous hypodermis present in the midrib region. Mesophyll heterogenous and differentiated into palisade and spongy parenchyma; both densely filled with chloroplasts. Palisade single layered; spongy parenchyma 3-4 layered; cells irregular. Palisade cells shorter towards margin; ground tissue parenchymatous. Vasculature in the form of central shallow 'C' shaped arc and bundle sheath parenchymatous. Vessels in patches are separated by parenchymatous cells; patches of internal phloem lie scattered towards metaxylem.

Root: Primary structure is typical with triarch xylem. Secondary thickening normal and resulted in extensive xylem cylinder and narrow phloem cylinder. Secondary cortex narrow. Pith absent. Growth rings absent. Vessels large, uniseriate in multiples or solitary. Fibres arranged in radial rows. Phloem with scattered patches of stone cells.

Cortical cells horizontally stretched and irregular. Extrastelar secondary growth is due to single layered phellogen arise from the outer cortex. Periderm narrow.

Euphorbia hirta

Stem: T.S. of stem circular in shape. The epidermis uniseriate, undulated with thin cuticle. Multiseriate and uniseriate epidermal hairs present in high frequency. Epidermis followed by 6-7 layers of rounded parenchymatous cortex. Collenchymatous hypodermis absent. Endodermis and pericycle indistinct. Vascular cambium cuts off phloem outwardly and xylem inwardly. Vasculature continuous. Pith extensive and parenchymatous.

Leaf: T.S. through midrib appears crescent shape in outline, with mount towards abaxial side. The epidermal cells at the midrib region of both surfaces polygonal in surface view, with slightly wavy anticlinal walls. Anomocytic stomata observed on abaxial epidermis. Mesophyll heterogeneous with two layers of palisade and 3-4 layers of spongy tissue of irregular cells. The palisade layer interrupted in the midrib region by a few rows of angular chlorenchyma. The midrib shows a central arc of vascular tissue consisting of a radiating xylem and lower soft phloem. Bundle sheaths wreath shaped and showed Kranz syndrome. Vascular elements scanty. Abaxial epidermis possess glandular trichomes.

Root: Secondary thickening normal and vascular cylinder continuous. Cortex and phloem narrow, xylem extensive. Pith absent. Distinct growth rings absent. Vessels scattered, mostly solitary or in doubles. Phloem with scattered patches of stone cells. Outer cortical cells horizontally stretched. Cork thick, 2-3 layered. Cork cambium superficial.

Euphorbia microphylla:-

Stem: Young stem roughly circular in outline. Epidermis single layered, cutinised and cuticularised. Epidermis followed by four to six layers of parenchymatous cortex. Collenchymatous hypodermis and chlorenchymatous layers absent. Endodermis and pericycle not distinct. Vasculature continuous and broad. Vessels either solitary or in short uniseriate rows. Fibres in radial rows. Pith large and parenchymatous.

Leaf: T. S. of lamina through the midrib region showed shallow, crescent shaped outline with less pronounced mount. Mesophyll heterogenous with one layer of palisade and 3-4 layers of spongy cells. Continuity of palisade tissue interrupted at the midrib region, by a few rows of angular chlorenchyma. Palisade and spongy tissue loaded with chloroplasts. The midrib region shows a central arc of vascular tissue consisting of radiating xylem and lower soft phloem. Bundle sheath parenchymatous. Anomocytic stomata observed in the abaxial epidermis. The epidermal cells at the midrib region of both the surfaces polygonal in surface view, with slightly wavy anticlinal walls. Vein bundles showed distinct Kranz anatomy, with wreath shaped bundle sheath cells.

Root: Secondary thickening normal and cambium formed from pericyclic region. Vascular cambium cuts off secondary xylem towards internally and secondary phloem towards externally. Distinct growth rings absent. Vessels solitary and scattered or in tangential rows. Phloem narrow with scattered patches of stone cells. Outer cortical cells horizontally stretched.

Euphorbia milii

Stem: The stem angular in shape in T.S. The epidermis uniseriate with smooth arched epidermal cells and thin cuticle. Below the epidermis, cortex massive with 20-25 layers of parenchymatous cells. The laticiferous ducts present in the cortex. Large sclereids of mechanical function is scattered in the inner cortex, outside the phloem. Xylem and phloem tissues limited. The pith region consists of relatively large parenchymatous cells. Secondary thickening not pronounced and secondary tissues are scanty.

Leaf: Epidermis single layered, cutinised and cuticularised. Mesophyll is differentiated into palisade and spongy parenchyma; both densely filled with chloroplasts. Palisade uniformly double layered; spongy parenchyma 3-4 layered; cells irregular loosely packed. Vasculature in the form of central shallow 'C' shaped arc. Stomata of anisocytic and paracytic are seen in a single leaf.

Root: T.S. of root circular in outline. Periderm consists of irregular narrow, brownish cork surrounding a narrow parenchymatous phelloderm. The vascular system is formed of a continuous ring of phloem and xylem, separated by a narrow cambium and traversed longitudinally by medullary rays. The phloem consists of a narrow zone consists of

sieve tubes, companion cells and phloem parenchyma. The xylem cylinder comparatively wide with less lignified elements. Vessels scattered, fibres less and not in radial rows.

Euphorbia tirucalli:-

Stem: T.S. of stem nearly circular in outline. Epidermis single layered, with uniseriate epidermal hairs. Cortex massive and consist of a few layers of chlorenchymatous hypodermis followed by 15-20 layers of parenchyma. Laticifers are distributed through the cortex. Endodermis not distinct. A few groups of fibres present in the pericycle region. Central vascular cylinder consists of phloem and xylem encircling a parenchymatous pith. Vascular bundles distinct, conjoint, collateral and open. Bundles vary in the size and shape. The phloem formed of sieve tubes, companion cells and phloem parenchyma. The xylem consists of a lignified tracheid and parenchyma. Secondary thickening is limited and vascular cylinder not formed.

Leaf: The leaf is very small in this species. T.S. shows epidermis, mesophyll and veins. In mesophyll, the spongy tissue abundant and palisade single layered. In spongy tissue, cells are packed with small inter-cellular space between cells. Very few chloroplasts exist in spongy tissue cells. Palisade tissue is underdeveloped in mesophyll. Leaf amphistomatic i.e., stomata present in both adaxial and abaxial epidermis. Stomatal types include anisocytic, paracytic and twin stomata. Midrib bundles large with less vascular elements. Bundle sheath parenchymatous.

Root: T.S. of root nearly circular in outline. It shows an irregular narrow brownish cork surrounding a narrow parenchymatous phelloderm. The vascular system is formed of a continuous cylinder of phloem and xylem, separated by a narrow cambium and traversed longitudinally by medullary rays. The phloem consists of sieve tubes, companion cells and phloem parenchyma. The xylem consists of a few vessels, fibres and parenchyma, but the fibres were very less.

Euphorbia pulcherrima:-

Stem: T.S. of stem nearly circular in outline and fistular in nature. Epidermis single layered and covered with thick cuticle; Young stem shows epidermal hairs. The cortex consists of 2-3 layers of collenchymatous cells, followed by a parenchymatous region of 6-7 layers. Endodermis and pericycle not distinct. Vascular bundles conjoint, collateral and open. Xylem consists of vessels, fibres and parenchyma. The central region is hollow pith and is lined with broken cell walls. Laticifers distributed throughout in the cortex and pith. Secondary thickening scanty.

Leaf: T.S. of leaf through midrib shows a prominent mount towards the abaxial epidermis. Epidermis single layered, cutinised and cuticularised. Stomata absent in both adaxial and abaxial surface of leaf. Non-glandular, uniseriate trichomes present in the abaxial epidermis. Mesophyll heterogenous and differentiated into palisade and spongy parenchyma; both densely filled with chloroplasts. Palisade single layered; spongy parenchyma 3-4 layered; cells irregular. Palisade cells shorter towards margin; Vasculature in the form of central shallow 'C' shaped arc. Vessels in patches are separated by parenchymatous cells. Phloem found towards the abaxial epidermis and traversed by the parenchymatous patches. Bundle sheath parenchymatous.

Root: T.S. of root circular in outline. Both extrastelar and intrastelar thickening present. Periderm consists of narrow phellum, phellogen and phelloderm. Vascular cambium produced from pericyclic region. Pith absent. It produced secondary xylem to the outer side and phloem to the inner side. Distinct growth rings are seen. Vessels scattered, and found in groups. Phloem with scattered patches of stone cells. Cortical cells horizontally stretched and irregular.

Comparative Anatomy:-

Anatomical features of the species studied revealed the structural diversity of this genus. Taxonomic utility of th anatomical characters in this genus is reported elsewhere (Gales and Toma, 2006; Cutler et al., 2007; Awordine et al., 2009). The most important anatomical characters with discriminating power are leaf trichomes, nature and type of stomata, Kranz syndrome, stem epidermis, nature of cortex and hypodermis, sclereids and fibers in secondary xylem and nature of pith. Species specific variation of these characters is summarized in Table 2. The comparative data was used for making an artificial key for the identification of the species studied and is presented below.

Table 2:- Comparative anatomy of *Euphorbia* species studied.

Species	Leaf			Stem				
	Trichomes	Stomata	Kranz syndrome	Epidermal cells/hairs	Cortex	Hypodermiss	Sclereids/fibres	Pith
<i>E. heterophylla</i>	Non-glandular/multicellular	Anomocytic	Absent	Sub-rectangular/absent	Heterogeneous	Collenchymatous	Absent	Hollow
<i>E. hirta</i>	Glandular/multiseriate	Anomocytic	Present	Undulated/multiseriate	Homogeneous	Chlorenchymatous	Present	Large/parenchymatous
<i>E. microphylla</i>	Absent	Anomocytic	Present	Regular/absent	Homogeneous	Parenchymatous	Absent	Large/parenchymatous
<i>E. milii</i>	Absent	Paracytic & Anisocytic	Absent	Arched/absent	Homogeneous	Chlorenchymatous	Present	Large/parenchymatous
<i>E. tirucalli</i>	Absent	Paracytic, Anisocytic, twin stomata present.	Absent	Variously/Uniseriate	Homogeneous	Chlorenchymatous	Present	Small/parenchymatous
<i>E. pulcherrima</i>	Non-glandular/uniseriate	Absent	Absent	Undulated/absent	Heterogeneous	Chlorenchymatous	Absent	Hollow

Artificial key based anatomical characters for the identification of *Euphorbia* species.

- 1a. Bundle sheath cells shows Kranz anatomy(2)
- 1b. Bundle sheath normal (3)
- 2a. Glandular trichomes present*E. hirta*
- 2b. Glandular trichomes absent, hypodermis parenchymatous..... *E. microphylla*
- 3a. Stomata absent*E. pulcherrima*
- 3b. Stomata present..... (4)
- 4a. Stomata anomocytic..... *E. heterophylla*
- 4b. Stomata paracytic and anisocytic..... (5)
- 5a. Twin stomata present, leaves amphistomatic*E. tirucalli*
- 5b. Twin stomata absent, midrib bundles 'C' shaped.....*E. milii*

Molecular Analysis:-

Two gene regions, one chloroplast and one nuclear, were selected to study the phylogenetic interrelationships between six species of *Euphorbia* collected. The chloroplast region (*matK*) was chosen as a maternally inherited marker that contains a good representation of genetic variation. Nuclear gene region (*ITS*) offers a biparental view of genetic change and provide higher resolution of the relationships between species. Sequences were not retrieved for two species (*E. milii* and *E. pulcherrima*) for *ITS* and one species for *matK* (*E. heterophylla*). This may be due to the possible polymorphisms at the primer binding sites or due to multiple products (that warrants cloning) or due to the lack of homogenization of *ITS* loci in these species.

Nucleotide diversity: The *matK*, chloroplast gene region included 869 aligned base pairs of which 72 sites were polymorphic. For the nuclear loci, *ITS* comprised of 652 aligned base pairs and included 209 polymorphic sites. Nucleotide diversity parameters calculated i.e., S, Eta, k, pi and theta were high for *ITS* loci (pi = 0.204; Theta = 0.2249) and low for *matK* (pi = 0.040; Theta = 0.0426). A test of selection and neutrality, Tajima's D was calculated to assess the evolutionary processes influencing each locus. The test revealed a negative values for *ITS* (D = -0.95

and statistically significant ($P < 0.001$) whereas mat *K* yielded a positive value ($D = 0.402$), but statistically not significant (Table 3).

Clustering analysis: Nuclear and chloroplast sequences were used separately to build an UPGMA dendrogram using MEGA.5 software and were given in Figure 1. Among the four species used for *ITS* dendrogram, *E. microphylla* and *E. hirta* were showed a close clustering. Whereas, *E. heterophylla* exhibited a distant relationship to all other three species. Bootstrap analysis supported the close clustering of *E. microphylla* and *E. hirta* with 100 % support. *E. tirucalli* entered as a sister cluster to the microphylla-hirta cluster.

Table 5:- Nucleotide diversity parameters calculated using mat *K* and *ITS* sequences

Name of loci	No. of sequence	No. of sites	No. of polymorphic sites (S)	Total no. of mutations (Eta)	Average no. of nucleotide differences (k)	Nucleotide diversity (Pi)	Theta (per seq.)	Theta (per site)	Tajima's D
mat <i>K</i>	5	869	72	77	35	0.04	36.96	0.04	0.402*
<i>ITS</i>	4	652	209	254	126	0.20	138.5	0.22	-0.95^
						*Not significant, >0.10			
						^Statistical significance, $P < 0.001$			

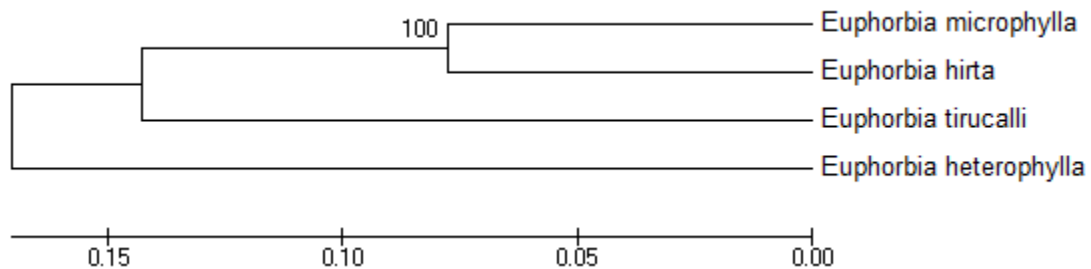


Figure 1:- UPGMA dendrogram based on *ITS* sequence. Value at the node indicates bootstrap support.

UPGMA dendrogram based on mat *K* sequences is presented in Figure 2. Akin to the *ITS* based dendrogram, *E. microphylla* and *E. hirta* exhibited a close phylogenetic relationships with a 100 % bootstrap support. *E. milii* and *E. tirucalli* were also close clustered with a bootstrap support of 69%. *E. pulcherrima* entered as a sister group to the microphylla-hirta cluster.

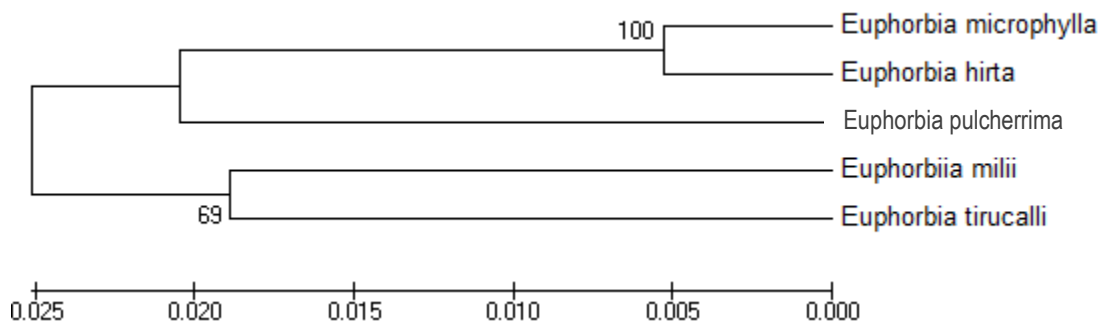


Figure 2:- UPGMA dendrogram based on mat*K* sequence. Values at the node indicate bootstrap support.

The clustering pattern observed in this study is corresponded well with the subgenus identity of the species (Table 1). The close clustering of *E. hirta*, *E. microphylla* and *E. pulcherrima* observed in the ITS based dendrogram is reflecting their subgenus identity, *Chamaesyce* (Horn et al., 2012). A unique feature of the *Chamaesyce* clade within *Euphorbia* is the predominance of C4 photosynthesis, which is both a physiological and anatomical system generally associated with plants adapted to warm, arid conditions (Sage et al., 2011). Notably, it is the only plant lineage at or below the level of genus that has all known photosynthetic types: C3, C4 and CAM (Webster et al., 1975), plus a C2 system that represents an early stage of C3 to C4 transition (Sage et al., 2011). Kranz syndrome is noticed in both *E. hirta* and *E. microphylla* in the present anatomical characterization, but not in *E. pulcherrima* and *E. heterophylla* belongs to the same subgenus. These two species are C3 plants. Hence ITS dendrogram essentially reflecting the physiology and subgenus classification of the genus. Monophyly of species belong to *Chamaesyce* revealed in this study is also reported earlier (Steinmann and Porter, 2002; Horn et al., 2012). Similar is the situation of *E. milii* and *E. tirucalli*, which were entered in to the same cluster in matK based dendrogram. Both of these species belong to the same sub genus, *Euphorbia* (Dorsey, 2013).

Summary and Conclusions:-

The major findings and conclusions drawn from the study are summarized below: 1. Expeditions across the study area identified six species of *Euphorbia*, namely *E. hirta*, *E. microphylla*, *E. heterophylla*, *E. milii*, *E. tirucalli* and *E. pulcherrima*. These species were belong to the two subgenera identified earlier, *Chamaesyce* and *Euphorbia*. *E. hirta*, *E. microphylla* and *E. heterophylla* are found in nature as wild whereas *E. milii*, *E. tirucalli* and *E. pulcherrima* are ornamentals. *E. hirta* and *E. heterophylla* exhibit a weedy nature throughout the study area. 2. Herbarium specimens were prepared and submitted at Bishop Moore College herbarium. 3. Anatomical characterization revealed the vast structural diversity existed in the studied species. Anatomical variations in the genus are correlated well with the diverse physiological and environmental adaptations. *E. hirta* and *E. microphylla* exhibited the Kranz anatomy, i.e. wreath shaped bundle sheath cells, as an adaptation for C4 photosynthesis. 4. Among the anatomical characters, leaf characters such as nature and type of stomata, nature of trichomes and stem characters like nature of hypodermis and cortex etc. are of high taxonomic significance. An artificial key based on anatomical characters, for the identification of the studied species was prepared and presented. 5. Chloroplast loci, matK and nuclear loci, ITS based molecular analysis of the genus revealed the phylogenetic interrelationships of the species studied. Nuclear diversity parameters showed elevated levels of diversity for nuclear loci when compared to the less evolving chloroplast loci. 6. Clustering pattern in the UPGMA dendrogram is mostly in accordance with the sub generic identity of the species. Species belong to the subgenera: *Chamaesyce*, *E. hirta*, *E. heterophylla*, *E. microphylla*, *E. pulcherrima* were clustered together whereas species belong to the subgenera: *Euphorbia*, *E. milii* and *E. tirucalli* were clustered distinctly 7. This preliminary work provided a blue print of morphological, anatomical and molecular variations exhibited by the genera, *Euphorbia* in the studied locality.

Acknowledgements:-

The authors are thankful to the Principal, Bishop Moore College, Mavelikkara for his encouragement and support. The first author thankfully acknowledges the receipt of financial support from KSCSTE, Thiruvananthapuram, Kerala in the student project scheme (File No. 7/SPS - 59/2016/KSCSTE dated 23-09-2016).

References:-

- Aditya, S. (2010): A revision of geophytic *Euphorbia* species from India. *Euphorbia world*, 6(1): 18-21.
- Aldhebiani, A. and Jury, S. (2013): Anatomical studies on the genus *Euphorbia* L. Saudi Arabia (Subgenera: *Tirucalli*, *Ermophyton*, *Esula* and *Chamaesyce*). *International Research Journal of Plant Science*, 4:168-191.
- Awordine, D., Nwoye, D., Jayeola, A., Olagoke, A. and Ogundele, A. (2009): Taxonomic significance of foliar epidermis in some members of Euphorbiaceae family in Nigeria. *Journal of Botany*, 4(1): 17-28.
- Balakrishnan, N.P. and Chakrabarty, T. (2007): The family Euphorbiaceae in India: a synopsis of its profile, taxonomy and bibliography. Bishen Singh Mahendra Pal Singh, Dehra Dun, 500p. ISBN 812110579X.
- Beg, M.J. (2015): Medicinal plants wealth of the family Euphorbiaceae in Azamgarh district (UP). *Indian J. Sci. Res.*, 11(1): 149-52.
- Berlyn, G.P. and Miksche, J.P. (1976): Botanical microtechnique and cytochemistry. Eames: Iowa State University.
- Bruyns, P.V., Klak, C. and Hanáček, P. (2011): Age and diversity in Old World succulent species of *Euphorbia* (Euphorbiaceae). *Taxon*, 60(6): 1717-1733.

8. Bruyns, P.V., Mapaya, R.J. and Hedderson, T.J. (2006): A new subgeneric classification for *Euphorbia* (Euphorbiaceae) in southern Africa based on *ITS* and *psbA-trnH* sequence data. *Taxon*, 55(2): 397-420.
9. Cutler, D., Botha, T. and Stevenson, D. (2007): *Plant Anatomy an Approach*, Blackwell publishing.
10. Dorsey, B. (2013): Phylogenetics and morphological evolution of *Euphorbia* subgenus. *Euphorbia* (Doctoral dissertation, University of Michigan).
11. Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T. and Wilson, A. (2010): Geneious v5.1, Available from <http://www.geneious.com>.
12. Essiett, U.A., Illoh, H.C. and Udoh, U.E. (2012): Leaf epidermal studies of three species of *Euphorbia* in AkwaIbom State. *Pelagia Research Library*, 3(4): 2481-2491.
13. Frajman, B. and Schönswetter, P. (2011): Giants and dwarfs: molecular phylogenies reveal multiple origins of annual spurges within *Euphorbia* subg. *Esula*. *Molecular phylogenetics and evolution*, 61(2): 413-424.
14. Gales, R.C. and Toma, C. (2006): Comparative anatomy of the vegetative organs of some *Euphorbia* species (Euphorbiaceae juss.) from the Romanian flora. *Romanian journal of Biology-Plant Biology*, 51: 39-47.
15. Gamble, J.S. (1921): *Flora of the Presidency of Madras*, Published by: Bishen Singh, Mahendra pal Singh, Dehradun.
16. Gunawardene, N.R., Daniels, D.A., Gunatilleke, I.A.U.N., Gunatilleke, C.V.S., Karunakaran, P.V., Nayak, G.K., Prasad, S., Puyravaud, P., Ramesh, B.R., Subramanian, K.A. and Vasanthi, G. (2007): A brief overview of the Western Ghats–Sri Lanka biodiversity hotspot. *Current Science*, 93(11): 1567-1572.
17. Horn, J.W., van Ee, B.W., Morawetz, J.J., Riina, R., Steinmann, V.W., Berry, P.E., and Wurdack, K.J. (2012): Phylogenetics and the evolution of major structural characters in the giant genus *Euphorbia* L. (Euphorbiaceae). *Mol. Phylogen. Evol.*, 63: 305-326.
18. Horn, J.W., Xi, Z., Riina, R., Peirson, J.A., Yang, Y., Dorsey, B.L., Berry, P.E., Davis, C.C. and Wurdack, K.J. (2014): Evolutionary bursts in *Euphorbia* (Euphorbiaceae) are linked with photosynthetic pathway. *Evolution*, 68(12): 3485-3504.
19. Johansen, D. A. (1940): *Plant microtechnique*. New York: McGraw Hill.
20. Kakkar, L. and Paliwal, G.S. (1974): Studies on the leaf anatomy of *Euphorbia*. V. Epidermis. In *Proc. Indian Natl. Sci. Acad., Part B Biol. Sci.*
21. Librado, P., and Rozas, J. (2009): DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25: 1451-1452.
22. Luković, J., Malenčić, D., Zorić, L., Kiproviski, B., Merkulov, L. and Boža, P. (2009): Anatomical characteristics and antioxidant properties of *Euphorbia nicaeensis* ssp. *glareosa*. *Open Life Sciences*, 4(2): 214-223.
23. Manilal, K.S. and Sivarajan, V.V. (1982): *Flora of Calicut*. Published by Bishen Singh Mahendra Pal Singh, Dehradun.
24. Metcalf, C.R. and Chalk, L. (1950): *Anatomy of the Dicotyledons Vol. II*. Clarendon Press, Oxford, pp. 247.
25. Park, K.R. and Jansen, R.K. (2007): A phylogeny of *Euphorbieae* subtribe *Euphorbiinae* (Euphorbiaceae) based on molecular data. *J. Plant. Biol.*, 50: 644–649.
26. Prenner, G. and Rudall, P.J. (2007): Comparative ontogeny of the cyathium in *Euphorbia* (Euphorbiaceae) and its allies: exploring the organ–flower–inflorescence boundary. *American Journal of Botany*, 94(10): 612-1629.
27. Ramarajan, S., Murugesan, A.G. and Ganthi, S. (2015): Diversity status of the family, Euphorbiaceae in about 30 km radius of Kudankulam Nuclear Power Project area, Southern India. *African Journal of Plant Science*, 9(5): 235-238.
28. Sage, R.F., Christin, P.A. and Edwards, E.J. (2011): The C4 plant lineages of planet Earth. *Journal of Experimental Botany*, 62: 3155 – 3169.
29. Sambamurthy, A.V.S.S. (2005): *Taxonomy of Angiosperms*. Published by IK International. Pvt. LTD, New Delhi.
30. Sasidharan, S. (2006): Flowering plants of Kerala: A checklist. CD prepared by advanced societies. *Botanical Journal of Linnean Society*, 94: 79-95.
31. Steinmann, V.W. and Porter, J.M. (2002): Phylogenetic relationships in *Euphorbieae* (Euphorbiaceae) based on *ITS* and *ndhF* sequence data. *Ann. Missouri Bot. Gard.*, 89: 453–490.
32. Tajima, F. (1989): Statistical Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics*, 123: 585-595.
33. Tamura, K., Peterson, D., Peterson, N., Stecher, N., Nei, M. and Kumar, S. (2011): MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Method. *Molecular Biology and Evolution*, 28: 2731-2739.

34. Volga, V.R., Narayanan, M.R. and Kumar, N.A. (2013): Endemic trees of Western Ghats—a check list from Wayanad district, Kerala, India. International journal of plant animal and environmental sciences, 3: 197-202.
35. Webster, G.L., Brown, W.V. and Smith, B.N. (1975): Systematics of photosynthetic carbon fixation pathways in *Euphorbia*. Taxon, 24: 27–33.
36. Yang, Y., Riina, R., Morawetz, J.J., Haevermans, T., Aubriot, X. and Berry, P.E. (2012): Molecular phylogenetics and classification of *Euphorbia* subgenus *Chamaesyce* (Euphorbiaceae) Taxon, 61: 764-789.
37. Zahra, N. B., Ahmad, M., Khan, Z., Shinwari, Zafar, M. and Sultana, S. (2014): Systematic significance of anatomical characterization in some euphorbiaceous species. Pak. J. Bot., 46: 1653-1661.
38. Zhang, Z.R. (2008): Morphological and anatomical studies on young stem and leaf from cutting plantlet of *Euphorbia tirucalli*. Green pharmacy, pp.147.