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# A Phylogeny of Euphorbieae Subtribe Euphorbiinae (Euphorbiaceae) Based on Molecular Data

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**Phylogenetic relationships within Euphorbiinae were inferred from our analysis of the 3′ end of the chloroplast gene *ndhF.* A sampling of that subtribe covered 88 species; 3 closely related species from the subtribes Anthosteminae and Neoguillauminiinae and the tribe Hippomaneae were included as outgroups. A phylogenetic assessment was carried out using the parsimony approach. The relationships revealed via these *ndh*F data supported the monophyly of subg. *Esula*, subg. *Chamaesyce*, subg. *Euphorbia*, and subg. *Lacanthis*. However, the polyphyly of subg. *Agaloma*, subg. *Lyciopsis*, and subg. *Eremophyton* also was strongly suggested. The African succulent Euphorbiinae can be divided into primarily two independent groups: 1) spiny succulents, which form a strongly supported clade with three subclades (subg. *Euphorbia*, subg. *Lacanthis*, and *Monadenium+Synadenium*); and 2) non-spiny succulents, which consist of sect. *Meleuphorbia*, sect. *Medusae*, sect. *Anthacantha*, sect. *Trichadenia*, sect. *Pseudeuphorbium*, sect. *Treisia*, and sect. *Pseudacalypha*. In the *ndh*F tree, the subg. *Esula* clade is placed as a sister to the rest of the Euphorbiinae. Thus, the origin of the *Euphorbia* s.l. should be sought within the herbaceous species of subg. *Esula*. The core North American endemic *Euphorbia* groups -- *Agaloma*, *Chamaesyce*, and *Poinsettia* -- are monophyletic and independent of the South American subg. *Agaloma*. Instead, they are derived from the African *Euphorbia* subg. *Lyciopsis* and *Eremophyton*. The Eurasian subg. *Esula* clade forms two subclades, which are concordant to sect. *Esula* and sect. *Tithymalus*.**

*Keywords*: euphorbiaceae, *ndh*F data, phylogeny, subtribe euphorbiinae

Approximately 2000 species of the subtribe Euphorbiinae are distributed worldwide; its herbaceous members, however, are primarily found in Eurasia and America. The largest proportion of succulent *Euphorbia* (ca. 500 species) occurs in the drier regions of southern and eastern Africa (Carter, 1988; Govaerts et al., 2000). The subtribe includes the species with a true cyathium, i.e., having a completely fused involucre with glands on the rim. Because of this unique structure, most species of Euphorbiinae have been treated as members of the genus *Euphorbia* s.l.

The naturalness of the subtribe has been supported in recent phylogenetic analyses based on data both morphological (Park and Elisens, 2000; Park and Backlund, 2002) and molecular (Steinmann and Porter, 2002). However, the classification within this subtribe remains problematic, and until now, only a few taxa, e.g., *Euphorbia*, *Monadenium*, *Synadenium*, *Elaeophorbia*, and *Pedilanthus*, have been segregated as independent genera (Carter, 1988). Although most of these are monophyletic, they have been nested within the *Euphorbia* s.s. in recent phylogenetic studies. Thus, the recognition of these groups as genera has resulted in the *Euphorbia* s.s. becoming a paraphyletic group (Park and Elisens, 2000).

Generic systems within the subtribe can be traced back to treatments by Haworth (1812), Rafinesque (1836), and Klotzsch (1859), who divided it into 15, 25, and 18 genera, respectively. Nevertheless, most recent classification systems in *Euphorbia* systematics do not accept these, but rather have adopted the subgeneric and sectional system as proposed by Wheeler (1943), Boissier (1862), and Pax and Hoffman (1931). Recently, however, Gilbert (1987), Park (1996), and Steinmann and Porter (2002) have criticized most of the sections and subgenera previously proposed. Thus, a strong phylogenetic signal is crucial to the establishment of a robust classification system within Euphorbiinae and to understanding their evolution.

Molecular phylogenetic examinations of the *Euphorbia* s.l. have been newly undertaken. For example, Steinmann and Porter (2002) have used ITS and *ndh*F sequence data to evaluate Euphorbieae, while Ritz et al. (2003) have conducted phylogenetic analyses of the subsect *Meleuphorbia* based on ITS and four noncoding cpDNA regions. Likewise, Haevermans et al. (2004) have determined the ITS sequences for the *Euphorbia* subg. *Lacanthis* species.

In the current study, we used *ndh*F data to conduct a phylogenetic analysis of 88 Euphorbiinae species. Our objective was to elucidate the relationships and test the monophyly of traditional taxa within Euphorbiinae. The resulting phylogenetic framework provides a strong guideline for establishing a new classification system for this problematic subtribe.

## MATERIALS AND METHODS

We investigated 88 species representing most of the subgenera and sections of the subtribe Euphorbiinae. Three closely related species from subtribes Anthosteminae and

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Appendix: Euphorbiinae taxa, their representative species, voucher information, and GenBank accession for phylogenetic study using *ndh*F.

Neoguillauminiinae and tribe Hippomaneae were included as outgroups, based on the results of an earlier phylogenetic analysis of the subfamily Euphorbioideae (Park and Backlund, 2002; see also Appendix).

Total DNA was extracted from silica gel-dried leaves collected from either natural populations or plants cultivated in a greenhouse. Micro-isolation methods (Loockerman and Jansen, 1996) were used, as modified from the 2X CTAB protocol of Doyle and Doyle (1987).

PCR was performed with a reaction volume of 50 μL that contained 0.1 uL, 1.0 uL, and 2.0 uL of an unquantified DNA template, 0.2 μM (0.5 μL of 20 μM stock) of each primer, 5 μL of 10X buffer, 2.5 mM of $MgCl_2$ (5 μL of 25 mM stock), 0.5 μL dimethyl sulfoxide (DMSO), 0.4 μL of Taq polymerase, and 0.2 mM (1 uL of 10 mM stock) of each dNTP, all then brought up to the final volume with $ddH_2O$.

Previous sequencing of *Euphorbia* with *ndh*F (Steinmann and Porter, 2002) has demonstrated that its 5′ end is problematic when attempting to amplify that gene. Therefore, we focused on the 3′ end, which includes about 1.0 kb of the coding sequence. Amplifications used Primer 972 (Olmstead and Sweere, 1994) and Primer 2110Ri (Steinmann and Porter, 2002). The first cycle comprised 3 min of denaturation at 94°C, 1 min annealing at 50°C, and 2 min of extension at 72°C. This was followed by 34 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; then termination by a final extension cycle at 72°C for 15 min. The PCR products were separated by 1% agarose gel electrophoresis at 70 volts for 25 min to examine the quality of the DNA. They were then purified on QiaQuick spin columns (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Sequencing reactions for *ndh*F were conducted with Primers 972, 2110Ri, and two internal primers -- 1318 (Olmstead and Sweere, 1994) and 1603RK (ACATAGTATT(G/A)TCGGATTCCCGCGG). Each cocktail contained 1 uL of the purified PCR product, 5 μL of the 1:20 diluted primer, 1.2 μLof 5x buffer, 10.8 μL of distilled water, and 2 μL of Big Dye. The cleaned DNA was dried for 30 min in a SpeedVac centrifuge, and was subsequently resolved by electrophoresis on a 5% polyacrylamide gel, using an automatic DNA sequencer (MJ Research).

The resultant sequences for each sample were edited via the Sequencher version 4.2 program (Gene Codes, Ann Arbor, MI). All sequences for the 3′ *ndh*F gene were initially aligned in Clustal X (Thompson et al., 1997), then manually adjusted. Maximum parsimony analyses were undertaken with PAUP version 4.0b10 (Swofford, 2002), with gaps being treated as missing data. We employed a heuristic search with 100 replicates, and used random sequence additions to find multiple islands of equally parsimonious trees, a TBR branch swapping algorithm, and MULTREES optimization. Each nucleotide position was treated as if it were unordered. The consistency index (CI) and retention index (RI) were calculated, and a strict consensus tree was generated. Bootstrap analysis (Felsenstein, 1985) that involved a simple addition with 1000 replicates was conducted using Winclada ver. 1.00 in order to evaluate the stability of the different branches in the trees we obtained.

## RESULTS

In all, 91 species were examined for *ndh*F -- 88 from the ingroup and 3 from the outgroup. As aligned, the 3′*ndh*F gene was approximately 1000 bp long. Parsimony analyses with 311 parsimoniously informative characters generated 1535 trees with 969 steps, a CI of 0.5937 (excluding uninformative characters), and an RI of 0.8659. Fig. 1 presents the strict consensus tree of those 1535 trees along with bootstrap values. A search strategy with 100 random replicates designed to identify multiple islands of trees found 4 islands for the 1535 most parsimonious trees in 89 replicates.

This strict consensus supported the monophyly of subg. *Esula*, and positioned it as a sister to a weakly supported clade that consisted of all the remaining species. Although two major clades of the African succulent *Euphorbia* group were recognized, their herbaceous members were not monophyletic but were dispersed at the bases of the North American and South African clades. The succulent South African clade, composed of sect. *Meleuphorbia*, sect. *Medusae*, sect. *Treisia*, sect. *Pseudeuphorbium*, and sect. *Anthacantha*, as well as a species from subg. *Trichadenia*, were nested within the subg. *Eremophyton* and a part of the subg. *Trichadenia* groups.

Within the next African clade, the well-supported subg. *Lacanthis* clade was positioned as a sister to a weakly supported clade containing species of the subg. *Euphorbia*, *Monadenium*, and *Synadenium*.

The strict consensus tree also supported the monophyly of the New World *Euphorbia* group (subgs. *Chamaesyce* + *Poinsettia* + core *Agaloma*), with a 83% bootstrap value, and was nested in the polytomous subgs. *Lyciopsis* and *Eremophyton* species.

Our parsimony analyses using the *ndh*F data strongly supported the monophyly of subg. *Lacanthis*, subg. *Euphorbia*, subg. *Esula*, and subg. *Chamaesyce*. However, subg. *Agaloma*, subg. *Eremophyton*, and *subg. Lyciopsis* were not supported as monophyletic groups. In addition, *Synadenium* and subg. *Poinsettia* were nested in *Monadenium* and core *Agaloma*, respectively.

A basal split occurred between the two major lineages within this subtribe (Fig. 1). The subg. *Esula* clade, the first diverging lineage of subtribe Euphorbiinae, could be subdivided into the well-supported sect. *Esula* (100% bootstrap support) and sect. *Tithymalus* clades (99% bs). The clade of *Synadenium*+*Monadenium* was well-supported and was a sister of subg. *Euphorbia* in the *ndh*F tree. *Poinsettia* was nested in the core *Agaloma* clade while *Chamaesyce* branched off from the base.

## DISCUSSION

### The Most Basal Clade in Euphorbiinae

Several authors have proposed traditional hypotheses among the basal *Euphorbia* groups, as determined by their morphological characteristics. The majority have stated that members of subgenus *Esula* are the most primitive in the genus *Euphorbia* s.l. For example, Webster (1967) has

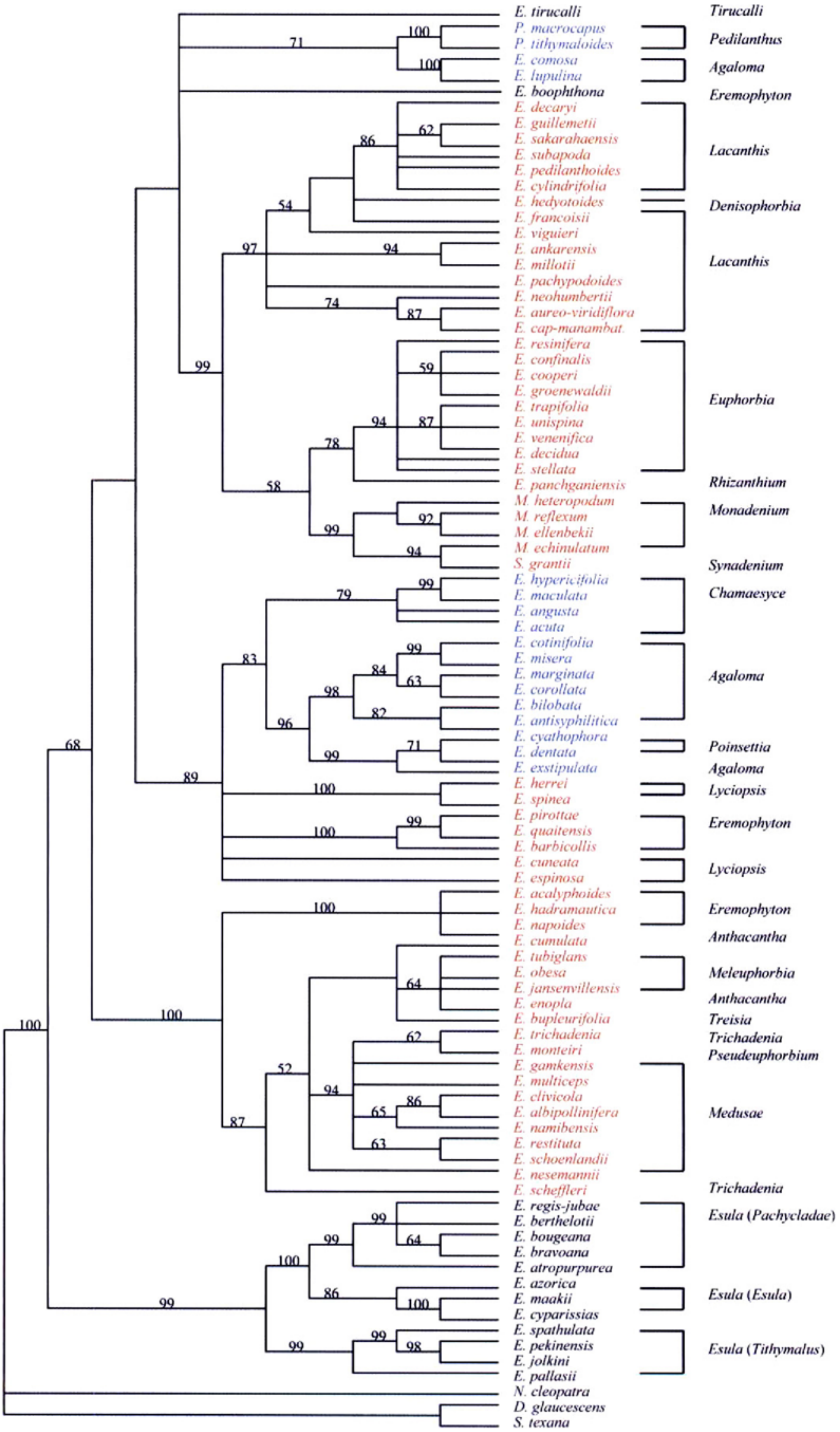

**Figure 1**. Strict consensus tree for 1535 most equally parsimonious cladograms of subtribe Euphorbiinae based on *ndh*F data. Subgeneric and sectional names of *Euphorbia* s.l. are represented. Numbers above branches denote bootstrap values >50%. Red species from African origins; blue species from New World; black species from Northern hemisphere.

hypothesized that the origin of the African endemic subg. *Euphorbia* is within the *Esula* sect. *Balsamis* through the progressive succulence. However, Croizat (1972) has proposed a contrasting view, i.e., that the South African *E. monteiri* (sect. *Pseudeuphorbium*) is one of the most primitive members in the entire *Euphorbia* subgroup. Plants of this species are characterized by a herbaceous upper part and a tuberous and succulent lower portion, such that it resembles a mix of subg. *Esula*, *Trichadenia*, and *Medusea*, which then finally connects this species with the latter three taxonomic groups (Croizat, 1972). Nevertheless, a more recent molecular phylogeny (Steinmann and Porter, 2002) depicts four major lineages, but does not indicate the most basal clade within subtribe Euphorbiinae. Therefore, describing the strong branching pattern among the basal *Euphorbia* remains one of the major issues in its phylogeny.

The position of subg. *Esula* as the first branching *Euphorbia* clade is supported in our *ndh*F phylogenies. This relationship has been proposed from sets of morphological data (Park and Elisens, 2000). Within the subg. *Esula* clade, the woody Canary Islands subsect. *Pachycladae*, perhaps the most primitive of the Euphorbias (Kuzmanov, 1964; Webster, 1967), forms a monophyletic group that is nested within the herbaceous members of the sect. *Esula*. Thus, the origin of *Euphorbia* should be sought within the herbaceous species of subg. *Esula* rather than within the woody members of the *Esula* sect. of *Pachycladae* as hypothesized by Kuzmanov (1964).

## Phylogenetic Relationships of African Succulent *Euphorbia*

Our results strongly suggest that the African succulent *Euphorbia* groups are not monophyletic, and that two major groups should have evolved independently.

The first clade, supported by a 100% bootstrap value, consists of six non-spiny *Euphorbia* groups: sect. *Pseudacalypha*, subsect. *Meleuphorbia*, subsect. *Medusea*, subsect. *Treisia*, subsect. *Anthacantha*, and sect. *Trichadenia*. Gilbert (1987) has defined subg. *Esula* sect. *Trichadenia* as problematic, but has included most of the above taxa based on their similar axillary inflorescences and peduncular-spined morphologies. Our molecular data strongly uphold those conclusions. Within the clade, the well-supported sect. *Pseudacalypha* of subg. *Ereomphyton* is a sister group of the remaining taxa. The monophyly of the sect. *Pseudacalypha* is congruent with the close relationships among *E. acalyphoides*, *E. napoides*, and *E. hadramautica* that are inferred by Carter (1985) based on morphology. However, other sections of subg. *Eremophyton* are dispersed throughout the tree. The African species of sect. *Eremophyton* form one of the basal groups of the petaloid-appendaged *Euphorbias* in the New World, whereas the Australian *E. boophthona* of sect. *Eremophyton* is included in the core African clade. Carter (1988) has suggested the heterogeneity of subg. *Eremophyton*.

Subsect. *Meleuphorbia*, a species that includes the melon-shaped South African *Euphorbia*, is not monophyletic but forms a clade with *E. enopla* from subsect. *Anthacantha*. Our result is in agreement with recent phylogenetic analyses by Ritz et al. (2003), who used ITS and cpDNA data and suggested that subsect. *Meleuphorbia* is not monophyletic because species from subsect. *Anthacantha* are nested in the *Meleuphorbia* clade. Furthermore, our phylogeny demonstrates that *E. bupleurifolia* from sect. *Treisia* and *E. cumulata* of subsect. *Anthacantha* are also close relatives of the species of *Meleuphorbia*. However, the relationship of sect. *Pseudeuphorbium* is unresolved, although Govaerts et al. (2000) have now assigned it to sect. *Trichadenia*. Our molecular data confirm a close relationship between them.

The second clade also is strongly supported by a 99% bootstrap value, and includes subg. *Lacanthis*, subg. *Euphorbia*, *Monadenium*, and *Synadenium*. Highly succulent stems with stipular spines characterize most of the species in this clade, which can be divided into Subclade I, comprising subg. *Lacanthis* and sect. *Denisophorbia*, and Subclade II, including subg. *Euphorbia*, the Indian species of subg. *Rhizanthium*, *Monadenium*, and *Synadenium*.

The monophyly of subg. *Lacanthis* s.s. + sect. *Denisophorbia* is consistent with previous phylogenetic analyses, based on ITS sequence data (Haevermans et al., 2004), but the placement of *E. hedyotoides* (sect. *Denisophorbia*) within the clade contradicts our analytical results. We found that sect. *Denisophorbia* is nested within *Lacanthis*, forming a strong lineage (95% bootstrap value). Two lineages are evident in Subclade II, including subg. *Euphorbia* and Indian subg. *Rhizanthium*. The second lineage contains species for *Monadenium* and *Synadenium*. The spine-shield *Euphorbia* species form a monophyletic lineage, a sister group of the endemic Indian *E. panchganiensis* of subg. *Rhizanthium*. Subg. *Euphorbia* is a well-supported lineage as predicted by Carter (1994), who has argued that it is the most natural group within the genus *Euphorbia*. Within the monophyletic subg. *Euphorbia*, three species of subs. *Spirales*, all native to western and central Africa, are monophyletic; this upholds the earlier treatment by Carter (1994). *Synadenium* is nested within the paraphyletic *Monadenium*, and the inclusion of *Synadenium* in the genus *Monadenium* is well-supported by the *ndh*F data.

## The Origin of Petaloid-appendaged *Euphorbia* in the New World

A close relationship among the petaloid-appendaged *Euphorbia* (including *Agaloma*, *Chamaesyce*, *Poinsettia*, and *Pedilanthus*) has been proposed by Wheeler (1939), Dressler (1957, 1961), and Webster (1967). Most of the taxa, except for *Poinsettia*, are characterized by glands with petaloid appendages, stipulate leaves, and ecarunculate seeds. Furthermore, a common basic chromosome number ($n=14$) indicates a close relationship among subg. *Agaloma*, *Poinsettia*, and *Chamaesyce* (Urbatsch et al., 1975).

Although our results do not resolve the question of petaloid-appendaged New World *Euphorbia* being monophyletic, we are now able to recognize two distinct clades: 1) a core North American clade consisting of *Chamaesyce*, *Agaloma*, and *Poinsettia*; and 2) a small clade containing the South American *Agaloma* (sect. *Stachydium*) and *Pedilanthus*. Thus, our results suggest that these two are independent of the South American petaloid *Euphorbia*, and that the endemic North American groups are derived from the African members of *Euphorbia* subg. *Lyciopsis* and *Eremophyton*. Recent phylogenetic analysis based on morphological and molecular data have also suggested that the North and South American *Euphorbia* have arisen independently from different Old World ancestors (Park, 1996; Steinmann and Porter, 2002). Within the core American clade, subg. *Poinsettia* is nested within the subg. *Agaloma* while subg. *Chamaesyce* is monophyletic, forming a sister group of the *Agaloma+Poinsettia* clade in the *ndh*F tree.

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### Genus *Euphorbia*

**Subgenus *Agaloma***: *E. cotinifolia* (Park 24009, EU022035), *E. misera* (Park 24010, EU022036), *E. marginata* (Park 24011, EU022037), *E. corollata* (Park 24012, EU022038), *E. bilobata* (Park 24013, EU022042), *E. antisyphilitica* (Park 24014, EU022043), *E. exstipulata* (Park 24015, EU022041), *E. comosa* (Webster 25425, AF538222), *E. lupulina* (Park 24078, EU022046).

**Subgenus *Chamaesyce***: *E. hypericifolia* (Park 24067, EU022031), *E. maculata* (Park 24068, EU022032), *E. angusta* (Park 24069, EU022033), *E. acuta* (Park 24070, EU022034).

**Subgenus *Eremophyton***: *E. boophthona* (Coveny 3054, AF538207), *E. acalyphoides* (Park 24019, EU022059), *E. hadramautica* (Park 24020, EU022091), *E. napoides* (Park 24021, EU022092), *E. pirottae* (Park 24022, EU022060), *E. quaitensis* (Park 24023, EU022061), *E. barbicollis* (Park 24024, EU022105).

**Subgenus *Esula***: *E. regisjubae* (Park 24016, EU022047), *E. berthelotii* (Jansen 24001, EU022048), *E. bougeana* (Jansen 24002, EU022049), *E. bravoana* (Jansen 24003, EU022051), *E. atropurpurea* (Jansen 24004, EU022050), *E. azorica* (Jansen 24005, 052), *E. maakii* (Park 23001, EU022053), *E. cyparissias* (Park 23002, EU022054), *E. spathulata* (Park 24017, EU022055), *E. pekinensis* (Park 23003, EU022056), *E. jolkini* (Park 23004, EU022057), *E. pallasii* (Park 23005, EU022058).

**Subgenus *Euphorbia***: *E. resinifera* (Park 24040, EU022077), *E. confinalis* (Park 24041, EU022078), *E. cooperi* (Park 24042, EU022084), *E. groenewaldii* (Park 24043, EU022085), *E. trapifolia* (Park 24044, EU022079), *E. unispina* (Park 24045, EU022082), *E. venenifica* (Park 24046, EU022083), *E. decidua* (Park 24047, EU022080) *E. stellata* (Park 24048, EU022081), *E. panchganiensis* (Singh s.n.), *E. cumulata* (Park 24049, EU022093), *E. tubiglans* (Park 24050, EU022094), *E. obesa* (Park 24051, EU022097), *E. jansenvillensis* (Park 24052, EU022099), *E. enopla* (Park 24053, EU022104), *E. bupleurifolia* (Park 24054, EU022109), *E. monteiri* (Park 24056, EU022102), *E. gamkensis* (Park 24057, EU022096), *E. multiceps* (Park 24058, EU022098), *E. clivicola* (Park 24059, EU022101), *E. albipollinifera* (Park 24060, EU022111), *E. namibensis* (Park 24061, EU022110), *E. restituta* (Park 24062, EU022106), *E. schoenlandii* (Park 24063, EU022108), *E. nesemannii* (Park 24064, EU022103).

**Subgenus *Lacanthis*:** *E. decaryi* (Park 24025, EU022062), *E. guillemetii* (Park 24026, EU022063), *E. sakarahaensis* (Park 24027, EU022064), *E. subapoda* (Park 24028, EU022073), *E. pedilanthoides* (Park 24029, EU022074), *E. cylindrifolia* (Park 24030, EU022075), *E. hedyotoides* (Park 24031, EU022067), *E. francoisii* (Park 24032, EU022076), *E. viguieri* (Park 24033, EU022071), *E. ankarensis* (Park 24034, EU022065), *E. millotii* (Park 24035, EU022072), *E. pachypodoides* (Park 24036, EU022066), *E. neohumbertii* (Park 24037, EU022068), *E. aureoviridiflora* (Park 24038, EU022069), *E. capmanambatoensis* (Park 24039, EU022070).

**Subgenus *Lyciopsis*:** *E. herrei* (Park 24073, EU022044), *E. spinea* (Park 24074, EU022045), *E. cuneata* (Park 24075, EU022107), *E. espinosa* (Park 24076, EU022112).

**Subgenus *Poinsettia*:** *E. cyathophora* (Park 24071, EU022039), *E. dentata* (Park 24072, EU022040).

**Subgenus *Tirucalli*:** *E. tirucalli* (Park 24018, EU022028).

**Subgenus *Trichadenia*:** *E. scheffleri* (Park 24065, EU022100), *E. trichadenia* (Park 24055, EU022095).

**Genus *Monadenium*:** *M. heteropodum* (Park 24003, EU022086), *M. reflexum* (Park 24005, EU022087), *M. ellenbeckii* (Park 24006, EU022088), *M. echinulatum* (Park 24007, EU022089).

**Genus *Pedilanthus*:** *P. macrocarpus* (Park 24001, EU022029), *P. tithymaloides* (Park 24002, EU022030).

**Genus *Synadenium*:** *S. grantii* (Park 24008, EU022090).

Outgroups:
*N. cleopatra* (McPherson 17882, AF538256)
*D. glaucescens* (McPherson 15531, AF 538260)
*S. texana* (Park 24077, EU022113)