Bothalia 14, 3 & 4: 857–863 (1983)

Evolution of succulent *Euphorbia* as interpreted from latex composition

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ABSTRACT

The morphology of starch grains and the gas-liquid chromatographic profile of triterpenes derived from latex of the nonarticulated laticifer of succulent African *Euphorbia* were examined for their applicability to interpret phylogenetic relationships of this genus. Several trends in starch grain morphology and triterpene composition were evident in the 38 examined taxa. Rod shaped grains, interpreted to be conservative, occurred in only a few taxa in several dwarf groups. Grains of osteoid shape prevailed in most taxa. Highly osteoid grains possessing lobed ends represented the most complex form and were present in some taxa endemic to Madagascar. Triterpene profiles which contained from 2 to 14 or more compounds were derived from all taxa. Each taxon possessed a characteristic profile, or identifying fingerprint. The composition of the profile differed quantitatively and qualitatively among taxa. Taxa with few triterpenes, tentatively interpreted as primitive, occurred in dwarf forms, whereas Madagascan taxa tended to possess high numbers of triterpenes reflective of specialization. This study supports the interpretation that laticifer starch grain morphology and triterpene composition, both gene mediated stable markers, can be employed to determine and correlate phylogenetic relationships between taxa of this complex genus.

RÉSUMÉ

L'ÉVOLUTION DES EUPHORBES SUCCULENTES INTERPRÉTÉE À DE LA COMPOSITION DU LATEX

*La morphologie des grains d'amidon et le profil chromatographique gaz liquide de triterpènes du latex des laticifères non articulés d'*Euphorbia *succulents africains ont été examinés pour leur possibilé d'application dans l'interprétation des relations phylogénétiques de ce genre. Plusieurs tendances dans la morphologie du grain d'amidon et la composition du triterpène furent évidentes dans les 38 taxa examinés. Des grains en forme de bâtonnets, interprétés comme primitifs furent observés dans quelques taxa seulement de plusieurs groupes nains. Des grains à forme ostéoïde prédominèrent dans la plupart des taxa. Des grains fortement ostéoïdes possédant des bouts lobés représentèrent la forme la plus complexe et furent présents chez certains taxa endémiques de Madagascar. Les profils de triterpènes qui contenaient de 2 à 14 composés ou plus furent obtenus de tous les taxa. Chaque taxon possédait un profil caractéristique, ou empreinte digitale d'identification. La composition du profil différait quantitativement et qualitativement parmi les taxa. Les taxa avec peu de triterpènes, interprétés provisoirement comme primitifs, se rencontraient dans les formes naines, tandis que les taxa malgaches avuient tendance à posséder des nombres élevés de triterpènes tradvisant une spécialisation. Cette étude prouve que la morphologie du grain d'amidon du laticifère et la composition du triterpène, toutes deux indicatrices stables des gènes, peuvent être employés pour déterminer et mettre en corrélation les relations phylogénétiques entre les taxa de ce genre complexe.*

INTRODUCTION

The genus *Euphorbia* is characterized by the presence of the nonarticulated laticifer which contains specialized components in its protoplasm, or latex, that can be utilized to interpret the evolution of this cell type as well as taxonomic relationships within the genus. Previous studies have shown that starch grains within laticifer plastids possessed a characteristic morphology for a taxon and morphological groups within this genus (Mahlberg, 1973, 1975; Biesboer & Mahlberg, 1981). Several investigations also have indicated that the triterpene profile and composition of latex differed for groups of taxa (Ponsinet & Ourisson, 1965, 1968; Anton, 1974; Biesboer, 1979). In a recent study it was emphasized that a taxon including cultivars possessed a characteristic triterpene profile which was genetically stable under diverse conditions (Biesboer, 1979; Biesboer, D'Amour, Wilson & Mahlberg, 1982).

Mahlberg (1975) emphasized the potential importance of utilizing the laticifer and its various components to interpret phylogeny at the species level. Laticifers in *Euphorbia* synthesize and accumulate many specialized products such as modified starch grains and triterpenes which appear to represent useful markers for such studies.

The succulent *Euphorbia* of Africa and adjacent islands represent a large assemblage of taxa of diverse habit. Their evolution remains as yet unclear, although they appear more highly specialized than nonsucculent leafy forms of this genus. Webster (1967) suggested that the subgenus *Euphorbia* may have originated from the subgenus *Esula*, whereas Kuzmanov (1964) maintained that woody forms of subgenus *Esula* were derived from primitive forms of the subgenus *Euphorbia*. The succulent forms have been classified primarily on morphological features associated with their habit (Berger, 1907; White, Dyer & Sloane, 1941; Jacobsen, 1954) since it has been difficult to utilize floral characters to distinguish between species. Yet in Jacobsen's book, which combines the works of Berger as well as White, Dyer, and Sloane, there are taxa of different habit included within most of the 27

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different groups of succulent *Euphorbia* recognized by him.

The purpose of this study is to examine whether the laticifer markers, both starch grain morphology and triterpene profile, can be utilized to distinguish between taxa of succulent *Euphorbia* and provide insight into their phylogeny. Representative taxa of nearly all groups from Jacobsen (1954) are examined for these two character markers.

MATERIALS AND METHODS

Latex was collected from succulent plants at the Botanical Garden of the University of Heidelberg. The shoot was scarified within several centimetres of the apex, and the latex exudate was collected both onto microscope slides for examination of starch grains and into vials containing acetone (analytical grade) for extraction of triterpene components.

For starch analysis, drops of exuded latex on microscope slides were stained with one drop of aq IKI, spread uniformly over the slide surface, air dried at least 24 hr, and mounted in resin for a permanent record. Three slides were prepared from more than one specimen when available in the succulent collection. Fifteen mature starch grains (those of large size) on each slide were recorded for shape and measured for length and width at the midregion as well as end of the grain. Tabulated information represent calculations from at least one slide of a specimen and standard deviations were determined for these data (Mahlberg, 1975).

For gas-liquid chromatography the acetone supernatant containing triterpenes was filtered into a fresh vial and evaporated to dryness over nitrogen. The residue was resuspended in 1 ml spectranalysed grade acetone to make a stock solution. One or 3 µl quantities of stock were transferred into fresh tubes and evaporated over nitrogen to dryness. These residues were resuspended in 0,5 ml spectranalysed grade acetone containing 0,5 mg/ml Δ^4-androsten-3,17-dione (androstendione) as an internal standard. One µl of each sample was injected onto the chromatographic column. Analyses were performed on a Hewlett-Packard 5710A gas-liquid chromatograph equipped with a flame ionization detector and operated by programming from 240–310 C at 4°/minute. Nitrogen was used as the carrier gas (20 ml/min flow rate). The injection port temperature was 250°C; the detector temperature was 300 or 350°C. Glass columns (2 mm ID × 2,43 m) were treated with 5% dimethyldichlorosilane in toluene and packed with 3% OV–1 on 100/120 mesh Supelcoport. Individual compounds were quantified on a Hewlett-Packard 3380A integrator with data on detected peaks expressed as area percent.

Specific compounds were identified from known and mass spectral analyses of extracts from particular species (Biesboer *et al.*, 1981; Mahlberg, unpublished) and from the identified retention times for several hydrocarbon compounds employed as external standards, including β–amyrin, campesterol, cycloartenol, ergosterol, euphol, germanicol, sitosterol, stigmasterol, and tirucallol.

The coinjection technique utilizing external standards also was employed to identify compounds for a taxon.

Sources of materials. Living specimens in the Botanical Garden at the University of Heidelberg were represented by: *E. acrurensis* N.E. Br; *E. aggregata* Bgr. 8995; *E. albertensis* N.E. Br. 31936; *E. balsamifera* Ait. 41180; *E. bubalina* Boiss. 9505; *E. capuronii* U. & L. M1790; *E. clavarioides* Boiss. 47015; *E. davyi* N. E. Br. 46753; *E. decaryi* Guill. 21984; *E. delphinensis* U. & L.; *E. didiereoides* Denis M1534; *E. echinus* Hook. f. & Coss. 41179; *E. ecklonii* Hassl. 46767; *E. fasciculata* Thunb. 33505; *E. fiherenensis* Poiss. 11957; *E. francoisi* Leandri 22105; *E. globosa Sims* 5281; *E. groenewaldii* Dyer 39417; *E. hamata* Sweet 3590; *E. maleolens* Phill. 3011; *E. melanohydrata* Nel 32131; *E. neohumberti* Boit. 9086; *E. nyikae* Pax 23284; *E. obesa* Hook. f. 7599; *E. pentagona* Haw. 17397; *E. persistens* Dyer 47115; *E. piscatoria* Ait. 32423; *E. pseudotuberosa* Pax 33506; *E. sudanica* Chev. 19948; *E. susannae* Marl. 45790; *E. trapaeifolia* Chev. 19623; *E. trichadenia* Pax 46995; *E. unispina* N.E. Br. 4643; *E. valida* N.E. Br. 4926; *E. woodii* N.E. Br. 8708; and *E. xylophylloides* Brongn. 8948. Specimens grown at Indiana University included *E. tirucalli* L. 102 and *E. viguieri* Denis 47.

RESULTS

All taxa examined in this study yielded copious quantities of latex from laticifers for these analyses. The latex color was typically white, although in some species it was tinted yellow. Latex protoplasm is derived from the nonarticulated laticifer which is distributed throughout most tissues of the axis in *Euphorbia*.

Starch grains were abundant in exudate derived from the 38 taxa examined in this study. Comparison of their morphological form showed that most taxa possessed osteoid-shaped starch grains as the mature grain form, whereas others possessed rod-shaped grains (Table 1). The rod type or the modified spindle form were present in only a few taxa such as *E. trichadenia* Pax (group 7) and *E. ecklonii* Hässl. (group 8). The degree of osteoidy can be recognized upon comparison of the width at the midregion and end of the grain. The ends of grains of taxa in several groups as 6, 8, 9, 18, are only slightly larger than the midregion and appear somewhat osteoid in form. For other taxa the end of the grain is considerably enlarged as in groups 4, 15, 17, 19, 22, and may measure greater than twice the width of the midregion as in groups 1, 2, 12, 20, and the Madagascan groups.

Starch grain length, expressed as an average size for the larger grains present in the sample, differed among examined taxa (Table 1). Their length ranged from short grains, 21,6±3,4 µm in *E. unispina* N.E. Br. (group 25), to very long grains, 46,6±9,9 µm in *E. ecklonii*. The short starch grains as in *E. unispina* (21,6 µm) and *E. delphinensis* U. & L. (22,3±3,2 µm, group 20) represented a distinct size class which differed significantly from larger grains in latex of

TABLE 1.—Starch grain and triterpene (sterol) characters of *Euphorbia* taxa, classified according to Jacobsen (1954)

Group/taxon	Starch grain: Shape	Length µm	Width, µm: Mid	End	Triterpene retention time, min
Group 1. Bushes, cylindrical leafy branches; 11 taxa					
E. balsamifera Ait.	Osteoid(b)	36.5± 3.6	7.5	17.9	
E. piscatoria Ait.	Osteoid	32.5± 3.5	6.6	8.8	
Group 2. Shrubs; branches thickened near apex; leafy; 7 taxa					
E. neohumberti Boit.	Osteoid	33.0± 4.0	5.1	12.1	
E. vigueri Denis	Osteoid	31.0± 2.7	6.0	8.8	
Group 4. Coralline shrubs; leaves soon falling; 62 taxa					
E. fiherenensis Poiss.	Osteoid	30.4± 4.9	5.2	9.6	
E. tirucalli L.	Osteoid	44.6± 2.4	7.8	13.8	
E. xylophylloides Brongn.	Osteoid	34.0± 8.5	7.0	10.4	
Group 6. Shrubs; tuberculate branches; leaves soon falling; 5 taxa					
E. hamata Sweet	Osteoid	36.3± 6.4	6.2	8.0	
Group 7. Dwarf; leafy branches from caudex; 4 taxa					
E. pseudotuberosa Pax	Osteoid	33.6± 6.4	5.4	7.5	
E. trichadenia Pax	Rod	31.7± 2.2	4.7	4.7	
Group 8. Dwarf; short leafy stem; 6 taxa					
E. ecklonii Hassl.	Rod	46.6± 9.9	6.6	7.7	
Group 9. Dwarf; herbaceous shoots; 10 taxa					
E. bubalina Boiss.	Osteoid	36.9± 2.2	5.2	5.8	
Group 11. Dwarf; tuberculate stem; thorny; 3 taxa					
E. fasciculata Thunb.	Osteoid	37.0± 5.7	8.0	15.3	
Group 12. Dwarf; short, tuberculate branches; 1 taxon					
E. clavarioides Boiss.	Osteoid	28.0± 3.1	5.0	11.4	
Group 13. Dwarf; caudex with tuberculate branches; 7 taxa					
E. woodii N.E. Br.	Spindle	28.9± 2.4	5.2	3.6	
Group 14. Dwarf; crown of tuberculate branches; 19 taxa					
E. davyi N.E. Br.	Osteoid(b)	31.8± 4.0	6.2	13.1	
E. maleolens Phill.	Osteoid	30.2± 4.6	5.5	7.6	
Group 15. Dwarf; numerous tuberculate branches; 24 taxa					
E. albertensis N.E. Br.	Osteoid	31.6± 4.6	7.1	11.5	
E. melanohydrata Nel	Osteoid(1)	33.4± 5.6	6.8	12.9	
Group 17. Dwarf; globose branches; 6 taxa					
E. globosa Sims.	Osteoid(b)	39.3± 7.5	6.9	12.0	
Group 18. Dwarf; columnar often angular branches; 9 taxa					
E. obesa Hook. f.	Osteoid	31.6± 5.0	6.7	6.6	
E. susannae Marl.	Osteoid	29.2± 2.4	5.2	7.9	
E. valida N.E. Br.	Osteoid	33.4± 8.7	6.9	8.1	
Group 19. Dwarf; shoot tuberculate, angled; thorny; 21 taxa					
E. aggregata Bgr.	Osteoid	29.3± 4.6	6.9	10.3	
E. pentagona Haw.	Osteoid	35.0± 4.2	6.6	10.8	
Group 20. Shrubs; leafy round or ribbed branches; thorny; 22 taxa					
E. capuronii U. and L.	Osteoid	27.5± 2.1	5.3	11.4	
E. decaryi Guill.	Osteoid	38.8± 2.6	5.2	14.9	
E. delphinensis U. and L.	Osteoid(b)	22.3± 3.2	6.7	13.2	
Group 21. Shrubs; fleshy leafy stems; 8 taxa					
E. sudanica Chev.	Osteoid	29.8± 7.1	10.1	15.1	
E. trapaeifolia Chev.	Osteoid	30.0± 6.3	6.9	12.5	
Group 22. Dwarf; several angled branches; thorns; 17 taxa					
E. groenewaldii Dyer	Osteoid	31.9± 2.5	6.2	14.3	
E. persistens Dyer	Osteoid(1)	30.2± 2.5	5.0	11.8	
Group 23. Trees; elongated angled stems; 3 taxa					
E. nyikae Pax	Osteoid	35.9± 6.0	5.9	11.5	
Group 24. Shrubs; branches 3–13 angled; leafy; thorns; 72 taxa					
E. acrurensis N.E. Br.	Osteoid(1)	28.6± 2.6	6.6	13.4	
E. echinus Hook. and Coss	Osteoid(1)	29.1± 4.6	7.6	12.6	
Group 25. Shrubs; branches rounded, tuberculate; thorns; 6 taxa					
E. unispina N.E. Br.	Osteoid	21.6± 3.4	5.7	7.2	
Madagascan (ankarensis) group					
E. francoisi Leandri	Osteoid(1)	32.6± 2.4	5.3	13.6	
Madagascan (perrieri) group					
E. didiereoides Denis	Osteoid(b,1)	44.2± 5.2	8.5	24.7	

Tentatively identified compounds include: A = campesterol; B = stigmasterol; C = ergosterol; D = euphol; E = tirucallol; F = sitosterol; G = β-amyrin; H = germanicol; I = cycloartenol.

Symbols: b, branching; 1, lobed.

other taxa. No evident relationship was apparent between length and shape; the small grains in *E. unispina* were somewhat osteoid in shape, while the small grains in *E. delphinensis* were highly osteoid. Similarly, the long grains in *E. ecklonii* were only somewhat osteoid, while those in *E. tirucalli* L. (group 4) as well as several other taxa (groups 11, 21, Madagascan) were highly osteoid.

Three general classes for grain length appeared evident (Table 1). Latex from many taxa contained starch grains approximately 30,0 to 34,0 μm long in different groups. No significant differences in length were evident between these taxa, and thus the 30,0 to 34,0 μm range represented a distinctive class for starch grains. There is also a class of grains of short length (20,0–24,0 μm) and a class of long length (40,0 μm or greater).

Even within a group, starch grain length can differ significantly between taxa (Table 1). This point is evident in group 4 in which the starch grains of *E. tirucalli* are in the long class whereas those of *E. fiherenensis* Poiss. and *E. xylophylloides* Brongn. are of intermediate length. In group 20 the starch grains of *E. delphinensis* are in the short class whereas other examined taxa possess grains of intermediate length.

Starch grain morphology also can differ within a group (Table 1). In group 18 the three taxa possessed somewhat osteoid grains as evidenced by the relationship of midregion to end width. However, in other groups the degree of grain osteoidy differed appreciably among the representative taxa (groups 1, 4, 14, Madagascan).

Starch grains also can develop branches at their midregion, as exemplified in several groups (symbol b). This trend can be observed among a number, but not all, grains in a population for a taxon. In latex from taxa possessing highly osteoid grains, the enlarged ends of some grains of the population may undergo lobing (symbol 1) to form grains of complex shapes (groups 15, 22, 24, Madagascan).

The triterpene (sterol) composition yielded characteristic profiles for each taxon (Table 1). Approximately 60 compounds, each with a specific retention time (RT), were detected in the profiles from each of the 38 examined taxa (closed circles in Table 1). Information on peak height and position for each chromatographic profile provided qualitative and quantitative data on triterpenes for a taxon as illustrated for *E. neohumberti* Boit. (Fig. 1). Few low or high molecular weight compounds other than triterpenes were present in the acetone extracts of latex. The number of triterpenes in the profile of a taxon ranged from as few as 2 in *E. groenewaldii* Dyer (group 22) to 14 in *E. didiereoides* Denis (Madagascan group).

Qualitative comparison of the complete profile from one group with that in another group showed little or no similarity (Table 1). One or several compounds may be similar between different groups. For example, euphol (RT 13,69 or compound D in Table 1) was present in taxa in a number of groups (exemplified by 4, 17, 21), but few other compounds were common between these

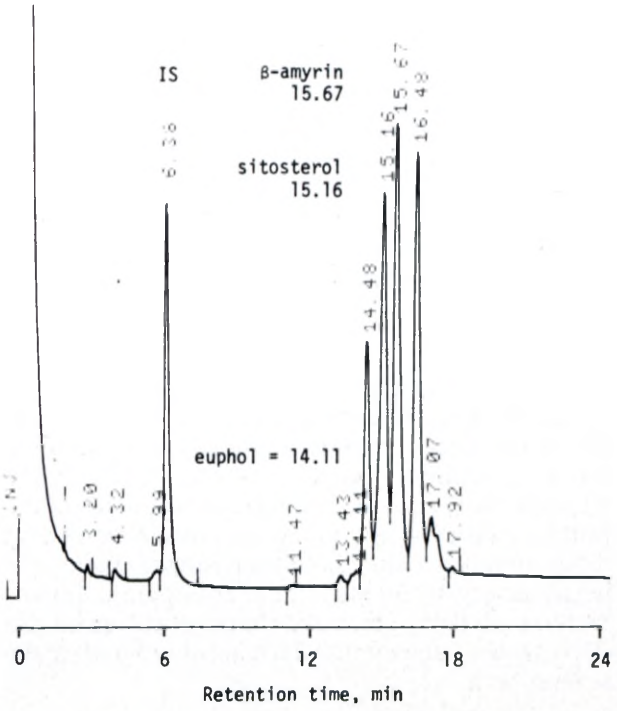

FIG. 1.—Chromatogram of *E. neohumberti* illustrating the several triterpene peaks present in the acetone extract of latex. IS is the internal standard. Euphol, sitosterol and β–amyrin are present in the latex, whereas compounds represented by the other peaks require identification.

groups. Even groups with a small number of compounds, as groups 13, 15 and 22, appeared not to have compounds with similar retention times.

Taxa within a group often contained few or no similar compounds as exemplified by groups 1, 7, 19, 24, or the Madagascan group. Taxa in several groups possessed similar compounds (as in groups 2, 4, 18, 20), yet each taxon differed by the presence or absence of one or more specific compounds. Several of these compounds have been identified (compounds are identified by letters A to I in Table 1), whereas other compounds require identification.

Taxa in several groups contained similar triterpene profiles. In group 2 the peaks with RT 14 through 18 were similar, but the taxa can be distinguished from each other by peaks in the area with RT 11 to 14. Only several components can be identified at this time including euphol (RT 13,69) and β–amyrin (RT 15,24). In group 4, represented by *E. fiherenensis*, *E. tirucalli*, and *E. xylophylloides*, euphol (RT 13,69) and tirucallol (RT 14,35) are common compounds, whereas the remaining components of the profile distinguish between these taxa.

Quantitative differences between the several components in a profile were apparent for each taxon and were evident as differences in chromatographic peak height and area. The ratio of compounds to each other, as represented by the prominent euphol and tirucallol components in *E. tirucalli* was similar for different samples.

Quantitative differences were evident for the same compound when it was present in different taxa. For example, in group 4 euphol and tirucallol were present in each of the taxa *E. fiherenensis*, *E.*

TABLE 2.—Percentage composition of sterols as components of the profile from taxa of group 4

Taxon	Euphol	Tirucallol	Unknowns		
			1	2	3
E. fiherenensis Poiss.	58.4	38.8	1.6	1.2	–
E. tirucalli L.	73.9	24.1	2.0	–	–
E. xylophylloides Brongn.	68.8	22.7	4.0	2.2	2.3

tirucalli, and *E. xylophylloides.* Euphol was the most abundant sterol in each taxon, 58,4%, 73,9% and 68,8% respectively, whereas tirucallol was present in lesser quantities, 38,8%, 24,1%, and 22,7% respectively (Table 2). The other components of the profile indicated as unknowns also occurred in different ratios within these taxa; further studies will be necessary to identify these triterpenes. Importantly, each taxon possessed characteristic quantities of particular compounds which aided to separate the several taxa.

Coinjection of different external standards with the samples from *E. obesa* Hook. f. tentatively identified sitosterol and β−amyrin among the eight components of the profile. Similar studies as well as those integrated with mass spectrometry are necessary to ascertain the identity of compounds in this and other taxa.

DISCUSSION

Latex exudate from laticifers in *Euphorbia* contained plastids and numerous triterpenes. This laticifer type is present in only a few dicotyledonous families including Euphorbiaceae, and the unusual characters of this cell type indicate it to be of recent evolutionary origin (Mahlberg, 1975; Mahlberg & Sabharwal, 1967, 1968). These starch grains have been reported in the early literature (Gaucher, 1902; Marloth, 1913−32), as have been the presence of triterpenes (Haines & Warren, 1949). The genetical control of starch grain morphology in plastids and the patterns of triterpenes (sterols) in the laticifer represent stable traits associated with the phylogeny of a taxon (Biesboer, 1979; Mahlberg, 1982).

Starch grains from laticifers can aid to distinguish between groups of taxa in this genus. Both grain morphology and length can be utilized to evaluate interspecific relationships between taxa. Rod and spindle shaped grains, which characterize leafy taxa in several subgenera uncommon on the African continent (Biesboer & Mahlberg, 1981) are interpreted to be less specialized than osteoid grains. Progressive specialization of starch grains is associated with increased length for the several grain types. Starch grains somewhat osteoid in form, present in taxa from dwarf groups, are interpreted to be less specialized than those starch grains with much enlarged ends. This pattern of starch grain phylogeny may also reflect the trend of specialization for these taxa. Taxa in dwarf groups differed within and between groups for the features of starch grain size and form, although they appeared to represent groups intermediate in specialization among the *Euphorbia.* Grains with enlarged and lobed ends, as observed for Madagascan taxa, are interpreted to represent phylogenetically derived taxa. The occurrence of rod shaped starch grains in several African taxa requires further study. This condition may represent a secondarily derived condition involving loss of a capacity to develop the osteoid feature.

The triterpene (sterol) profile of latex was found to be quantitatively and qualitatively distinctive for a taxon and comparable to a fingerprint for the taxon. Our studies support the interpretation that these compounds represent unique biological markers which can be employed to advantage to identify and correlate phylogenetic relationships between taxa of this genus.

It has been reported that latex sterol composition of a taxon was stable under different conditions and from different cultivars of a species (Biesboer, 1979; Biesboer *et al.,* 1982). Latex triterpenes have been utilized previously to interpret relationships between taxa of *Euphorbia* (Ponsinet & Ourisson, 1965, 1968; Anton, 1974; Biesboer, 1979). These authors described different groups of *Euphorbia* reflective of their major triterpenes to provide a chemotaxonomic interpretation of this genus.

The development of gas-liquid chromatography, such as we employed, has provided a highly sensitive technique to obtain quantitative and qualitative data on the numerous components in latex. Importantly, the degree of affinity between taxa is reflective of the number of identical compounds present among the components of the profile and the relative quantity of similar components when present in different taxa. Thus, examined taxa within groups 2 and 4 represent more natural groupings than do taxa within other groups (as 7 and 24).

A high number of triterpenes in latex is interpreted to represent a derived condition, and is noted for some Madagascan taxa. The fewest number of triterpenes was noted in taxa from several dwarf groups although other taxa among the dwarf groups had a greater number of sterols as did taxa in groups containing shrubby and arborescent taxa. As a generalization both the starch grain and triterpene data support an interpretation that Madagascan taxa represent a derived condition.

Further studies are necessary on the mechanism and genetic processes controlling the features of starch grain morphology and triterpene formation in

the laticifer. Studies indicate that the numerous variations of each feature are derived by gene mutation resulting in the formation of several morphological forms of starch grain and numerous triterpene compounds without altering species survival ability. Integration of data on these two features which represent stable markers from the laticifer will provide insight into the phylogeny of this complex genus and will aid to develop more natural groupings than exist currently.

ACKNOWLEDGEMENTS

This study was aided with a Richard Merton Guest Professorship in the Institute for Systematic Botany and Plant Geography at the University of Heidelberg to P. Mahlberg. Funds for this program were provided by the Deutsche Forschungsgemeinschaft, the University of Heidelberg and the National Science Foundation (DEB 78–02045).

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