Acta Botanica Brasilica 29(1): 129-142. 2015.
doi: 10.1590/0102-33062014abb3705

Seed coat diversity in some tribes of Cucurbitaceae: implications for taxonomy and species identification

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Received: August 2, 2014. Accepted: October 8, 2014

Abstract: To evaluate their diagnostic value in systematic studies, seed coat morphology for 16 taxa from 11 genera of Cucurbitaceae were examined using stereomicroscopy and scanning electron microscopy. The taxa included representatives of the tribes Benincaseae, Bryonieae, Coniandreae, and Luffeae in order to evaluate their diagnostic value in systematic studies. Macro- and micromorphological characters of their seeds are presented, including shape, color, size, surface, epidermal cell shape, anticlinal boundaries, and periclinal cell wall. The taxonomic and phylogenetic implications of seed coat micromorphology were compared with those of the available gross morphological and molecular data. Seed character analysis offered useful data for evaluating the taxonomy of Cucurbitaceae on both intrageneric and tribal levels. Monophyly of the tribes Bryonieae, Coniandreae, and Luffeae was supported. Moreover, these analyses supported previous biochemical and phylogenetic data, indicating that distinct lineages are present within the tribe Benincaseae, that this tribe is not monophyletic, and that the subtribe Benincasinae is highly polyphyletic. A key is provided for identifying the investigated taxa based on seed characters.

Keywords: Cluster analysis, PCO, scanning electron microscopy, seed coat, tribal classification, UPGMA

Introduction

Cucurbitaceae is a widespread family of 118–122 genera and 900 species (Simpson 2010) of monoecious or dioecious herbs and erect shrubs. The family is mainly distributed in tropical and subtropical regions with relatively few species reaching the temperate regions of the world. The aerial parts of all species are sensitive to frost. The family also includes a few shrub species (e.g., *Acanthosicyos horridus*) and lianas with climbing or trailing, woody perennial stems. The family includes economically important food crops such as *Citrullus lanatus* and Nakai (watermelon), which represents approximately 10% of the world vegetable production (Vossen *et al*. 2004), *Cucumis melo* (melons), *Cucumis sativus* (cucumber), *Cucurbita pepo,* and other *Cucurbita* species (squash and pumpkins). *Luffa* are used as sponges, and *Lagenaria siceraria*, bottle gourd, are used as vessels in African and Asian cultures (Whitaker & Davis 1962; Erickson *et al*. 2005; Clarke *et al*. 2006). The leaves and shoots of many species are finally boiled and eaten as a vegetable in both Africa and Asia (Okoli & Onofeghara 1984), and many species play a role in folk medicine.

Hooker (1867) divided Cucurbitaceae into 68 genera and eight tribes. These tribes are Cucumerineae, Abobreae, Elaterieae, Sicyoideae, Gomphogyneae, Gynostemmeae, Zanonieae, and Fevilleeae. According to Jeffrey (1980; 1990), Cucurbitaceae is subdivided into two well-defined subfamilies, Zanonioideae and Cucurbitoideae, and eight tribes represent various degrees of circumscriptive cohesiveness. The subfamily Cucurbitoideae has the most important cucurbit crops, such as *Cucumis*, *Cucurbita*, and *Luffa*. Eleven tribes have been more recently recognized with 125 genera and approximately 800 species (Jeffrey 2005; Wilde & Duyfjes 2006 a; b; c; Kocyan *et al.* 2007). However, Schaefer & Renner (2011) recognized 97 genera and 940–980 species subdivided into 15 tribes. A new classification system (Schaefer & Renner 2011) resulted in the replacement of many subtribes and the elevation of others to the tribal rank. For instance, subtribe Luffinae, previously a member of Benincaseae, was elevated to the tribal level.

The tribe Benincaseae, currently with two subtribes (Benincasinae and Cucumerinae) and 35 genera, is the largest tribe of Cucurbitaceae. Molecular phylogenetic analysis by Decker-Walters *et al.* (2004) revealed a deviation from the former morphological classification (Jeffrey 1990). However, the phylogenetic classification of the tribe Benincaseae was examined by Kocyan *et al*. (2007), who demonstrated that Benincaseae is not monophyletic and that subtribes Benincasinae and Cucumerinae are highly polyphyletic.

Achigan-Dako (2008) investigated the phylogenetic analysis of 68 species and subspecies of the tribe Benincaseae-

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seae based on 127 nuclear rDNA internal transcribed spacer region (ITS) sequences and showed that the subdivision of the tribe into the subtribes Benincasinae and Cucumerinae was not supported. Moreover, they supported the elevation of Luffinae to the tribal rank.

Rizk (2001) studied the morphology and cytotaxonomy of 27 Egyptian taxa of cultivated varieties and landraces belonging to three genera of Cucurbitaceae (*Cucumis*, *Cucurbita*, and *Luffa*), six species, and five subspecies. He found that fruit characters were a good taxonomic tool when combined with other vegetative characters at the varietal level.

During the last decades, SEM has been applied to the morphological study of seeds and small fruits. Micromorphology and ultrastructural data have provided useful information concerning the evolution and classification of seed plants and have played an important role in the modern synthetic systems of Angiosperms (Heywood 1971; Abdel Khalik & Maesen 2002).

Seed morphology provides a number of characteristics that are potentially useful for species identification, phylogenetic inference, and character-state evolution (Jobst *et al.* 1998; Johnson *et al.* 2004; Attar *et al.* 2007; Moazzeni *et al.* 2007; Mostafavi *et al.* 2013). Observations of many plant groups have shown that seed morphology and anatomic features are rather conservative, and hence taxonomically important (Barthlott 1981; 1984; Werker 1997; Abdel Khalik 2010; 2013; Abdel Khalik & Hassan 2012; Hassan & Abdel Khalik 2014).

Data are rather limited on the seed morphology of representatives from the different tribes of Cucurbitaceae, and a few seed shapes are unique, which may permit the assignment of fossil seeds to particular genera.

The aims of the present study were 1) to investigate the range of variability of seed characters in 11 genera (16 species) from some Cucurbitaceae tribes, 2) to use cluster analysis to elucidate the usefulness of seed characters to distinguish these tribes, and 3) to assess whether these results correspond to the systematics of the genera as proposed by Walters (1989), Jeffrey (2005), Achigan-Dako (2008), and Schaefer & Renner (2011).

2. Material and methods

2.1. Seed material

Sixteen taxa belonging to 11 genera of Cucurbitaceae were analyzed. Some of the investigated seeds were collected from mature plants in Egypt and others were taken from herbarium specimens. A list of voucher specimens and localities is provided in Table 1. Only mature seeds were taken for investigation. The dried seeds were first examined by dissecting microscope (Olympus type BH-2), and then 5–10 seeds from each taxon were selected to cover the range of variation. Seeds were mounted on stubs with double-sided adhesive tape and sputter-coated with gold for 5 min in an S150A sputter coater (Edwards Ltd., England). The specimens were examined using a SEM-JEOL JXA840A Electron Probe Microanalyzer (JEOL Ltd, Japan), at accelerating voltages of 20–25 kV. All photomicrographs were taken at the SEM laboratory, National Research Centre, Dokki, Cairo, Egypt. The terminology used to describe seed shape, cell shape, and seed coat ornamentation followed that of Barthlott (1981; 1984), Abdel Khalik & Maesen (2002), and Teppner (2004).

2.2. Selection of coding characters

The principles for selecting coding characters were the independency of the characters and their stability within the taxa analyzed (Stuessy 1990; Davitashvili & Karrer 2010). Seeds provide several qualitative and few quantitative characters. Our focus was on the qualitative characters of seed micromorphology that were easy to detect. One quantitative character (character 6) was treated statistically as a qualitative characters.

2.3. Analysis of seed data

Twelve characters were measured for each species. Two types of analyses were performed with NTSYS-pc 2.02k software (Applied Biostatistics Inc., Setauket, New York, USA). Firstly, we performed a cluster analysis using the average taxonomic distance and UPGMA clustering (procedures SIMINT, SAHN, and TREE). To reduce the effects of different scales of measurement for different characters, the values for each character were standardized with procedure STAND according to the formula: yI, STD = (yi – AVGyi)/STDyi, where the default value in NTSYS-pc (STAND) for yi = the value to be standardized, AVGyi = the average of all values for the character, and STDyi = the standard deviation. The cophenetic correlation coefficient between the distance matrix and the tree matrix was calculated to examine the goodness of fit of the cluster analysis to the distance matrix (procedures COPH and MXCOMP). Secondly, a principal coordinates analysis (PCO) was performed, using the product-moment correlation as a coefficient. The procedure SIMINT was used to calculate the distance matrix based on STAND data, and the procedures EIGEN, PROJ, and MXPLOT were used to perform the PCO.

3. Results

The seed morphological characters for the studied taxa of the family Cucurbitaceae are summarized in Table 2, and the stereomicroscopy and SEM images are presented in Figs. 1–4.

3.1. Seed color

The color of seeds is highly diagnostic and of systematic interest among taxa. The seed color varied from black in *Luffa acutangula* and *L. cylindrica*; black to brown in

Table 1. List of taxa used in the study. A comparison of the most traditional (Walters, 1989) and a recent phylogenetic classification based on molecular data (Jeffrey, 2005; Achigan-Dako 2008; and Schaefer & Renner 2011).

N	Taxa	Voucher	Walters, 1989	Jeffrey, 2005	Achigan-Dako and Blattner (2008)	Schaefer and Renner (2011)	Present study
1	*Acanthosicyos horridus* Welw. ex Hook.f.	Kew garden, Millennium seed bank, serial number: 238530 (K)	-	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group F
2	*Bambekea racemosa* Cogn.	Congo, Louis 5.862 (K).	-	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Coniandreae	Group A
3	*Benincasa hispida* (Thumb.) Cogn.	Tonga, Crosby 71 (K).	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group F
4	*Bryonia alba* L.	Turkey, Vezmis, Near the village Amasya, Baytop 4670 (E).	-	Tribe: Bryonieae	Tribe: Bryonieae	Tribe: Bryonieae	Group E
5	*Bryonia cretica* L.	Kew garden, Millennium seed bank, serial number: 186380 (K).	-	Tribe: Bryonieae	Tribe: Bryonieae	Tribe: Bryonieae	Group E
6	*Bryoniadioica* Jacq	Algeria, Beni Saf,Davis 51496 (E)	-	Tribe: Bryonieae	Tribe: Bryonieae	Tribe: Bryonieae	Group E
7	*Citrullus colocynthis* (L.) Schrad.	Egypt, St. Katherine, Heneidak s.n. (Suez Fac. Sci. Herb)	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group E
8	*Citrullus ecirrhosus* Cong.	Kew garden, Millennium seed bank, serial number: 105064 (K).	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group C
9	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	Kew garden, Millennium seed bank, serial number :140661 (K).	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group C
10	*Diplocyclos palmatus* (L.) C. Jeffrey	Nepal, Sankhuwasabha, Koshi Zone, Long et al. 41 (E).	-	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group C
11	*Lagenaria sphaerica* (Sond.) Naud	South Africa, Ingwaruma, KwaZulu-Natal, Pooley 509 (E).	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group B
12	*Lemurosicyos variegata* (Cogn.) Keraudren	Madagascar, Du Puy et al., M891 (K)	-	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group B
13	*Luffa acutangula* (L.) Roxb.,	Egypt, Cairo gardens, Shabetai Z6943 (CAIM)	Tribe: Benincaseae Subtribe: Luffinae	Tribe: Luffeae	Tribe: Luffeae	Tribe: Sicyoeae	Group D
14	*Luffa cylindrica* (L.) Roem.	Egypt, Sohag, abdel Khalik s.n. (SHG)	Tribe: Benincaseae Subtribe: Luffinae	Tribe: Luffeae	Tribe: Luffeae	Tribe: Sicyoeae	Group D
15	*Nothoalsomitra suberosa*	Australia, Queensland, Telford et al., 9007 (K).	-	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Sicyoeae	Group E
16	*Ruthalicia longipes* (Hook.f.) C.Jeffrey	Nigeria, Onochie FHI 34320(K).	-	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae Subtribe: Benincasinae	Tribe: Benincaseae	Group F

Table 2. Seed morphological characters of the studied taxa in the Cucurbitaceae.

N	Taxon	Seed colour	Seed shape	Seed surface	Seed size (long x wide) mm	Outer of epidermal cell shape	Anticlinal cell wall boundaries	Periclinal cell wall
1	*Acanthosicyos horridus* Welw. ex Hook.f.	Cream	Ovoid	Testa thick, hard, wingless	10-12 x 6-7	Isodiametric, 5-6 gonal cells	Straight, raised; smooth to fine folds	Flat; micro-papillate
2	*Bambekea racemosa* Cogn.	Yellowish brown	Ovoid	Smooth, wingless, arillate	5-6 x 3-4	Irregular, polygonal cells	Straight to slightly sinuous, slightly raised; folded	Flat to concave; smooth to fine folds
3	*Benincasa hispida* (Thumb.) Cogn.	Yellowish brown	Oblong	Flat, smooth, with narrow ridge (wing)	10-15x 5-8	Elongate in one direction	Straight, raised; smooth to fine folds	Flat; smooth to fine folds
4	*Bryonia alba* L.	Brown	Ovoid	Flat, smooth, with narrow ridge (wing)	3.6-4 x 2.7-3	Irregular, polygonal cells	Straight to slightly sinuous, raised; folded	Concave; smooth to fine folds
5	*Bryonia cretica* L.	Orange-brown	Ovoid	Flat, smooth with narrow ridge (wing)	5-6 x 4-6	Irregular, polygonal cells	Undulate, raised; smooth to fine folds	Flat to concave; smooth
6	*Bryonia dioica* Jacq	Light brown	Obovoid	Flat, smooth with narrow ridge (wing)	3.8-4.2 x 3.2-3.4	Isodiametric, 5-6 gonal cells	Straight to slightly sinuous, raised; smooth to fine folds	Flat; smooth to fine folds
7	*Citrullus colocynthis* (L.) Schrad.	Yellowish brown	Oblong-obovoid	Flat, smooth , wingless	6.2-10 x 3.6-5	Irregular, polygonal cells	Undulate, raised; smooth to fine folds	Flat; smooth to fine folds
8	*Citrullus ecirrhosus* Cong.	Black to brown	Obovoid	Flat, smooth , wingless, arillate	6.2-9x4-6	Irregular, polygonal cells	Undulate, raised; smooth to fine folds	Flat; micro-papillate
9	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	Black to brown	Obovoid-oblong	Flat, smooth with narrow ridge (wing), arillate	7-10 x 5-7	5-6 gonal cells to elongate in one direction	Straight, raised; smooth to fine folds	Flat; micro-papillate
10	*Diplocyclos palmatus* (L.) C.Jeffrey	Brown	Pyriform	Slightly scorbiculate at the middle, strongly winged, arillate	5-6 x4-4.8	5-6 gonal cells to elongate in one direction	Straight, raised; smooth to fine folds	Flat; smooth to fine folds
11	*Lagenaria sphaerica* (Sond.) Naud	whitish-yellow	Oblong-obovoid	Flat, compressed, pointed at one end, smooth, with narrow ridge (wing), arillate	6-7 x 4-5	Isodiametric, 5-6 gonal cells	Straight, raised; smooth to fine folds	Flat to slightly convex; smooth to fine folds
12	*Lemurosicyos variegata* (Cogn.) Keraudren	Yellowish brown	Oblong	Flat, test hard and with dentate margin, arillate	9-10 x 5-5.8	Irregular, polygonal cells	Straight to slightly sinuous, slightly raised; smooth to fine folds	Flat to slightly convex; folded
13	*Luffa acutangula* (L.) Roxb.,	Black	Oblong-obovoid	Flat, compressed, rugose, wingless	11-13x7-8	Irregular, polygonal cells	Undulate, raised; folded	Flat; smooth to fine folds
14	*Luffa cylindrica* (L.) Roem.	Black	Ovoid	Flat, compressed, smooth, with narrow ridge (wing)	10-14 x 7-10	Irregular, polygonal cells	Undulate, raised; folded	Flat; folded
15	*Nothoalsomitra suberosa* (F.M.Bailey) Telford	Brown	Ovoid	Flat, smooth, wingless	11–13 x 7–9	Irregular, polygonal cells	Straight to slightly sinuous, raised; smooth to fine folds	Flat to concave; smooth
16	*Ruthalicia longipes* (Hook.f.) C.Jeffrey	Yellowish brown	Oblong	Flat, compressed, slightly sculptured, with narrow wing	10-12 x 5-6	Elongate in one direction	Straight to slightly sinuous, raised; folded	Flat, striate

Citrullus ecirrhosus and *C. lanatus*; brown in *Bryonia alba*, *Diplocyclos palmatus*, and *Nothoalsomitra suberosa*; whitish-yellow in *Acanthosicyos horridus* and *Lagenaria spaerica*; and yellow to brown in the rest of the species.

3.2. Seed shape

Seed shape in the tribe Benincasinae can be categorized as follows: pear shaped in *Diplocyclos palmatus* (Fig. 3C); obovoid in *Bryonia dioica* (Fig. 2C); oblong in *Benincasa hispida*, *Lemurosicyos variegata*, and *Ruthalicia longipes* (Figs. 1E, 3G, and 4G); oblong-obovoid in *Citrullus colocynthis*, *C. lanatus*, *Lagenaria spaerica*, and *Luffa acutangula* (Figs. 2E, 3A and E, and 4A); and ovoid in *Acanthosicyos horridus*, *Bambekea racemosa*, *Bryonia alba*, *Bryonia cretica*, *Luffa cylindrica*, and *Nothoalsomitra suberosa* (Figs. 1A, C, and G; 2A; and 4C and E).

3.3. Seed surface

The seed sculpture of the studied taxa showed great variation. They varied tremendously from scrobiculate (pitted; i.e., a surface covered with hollows) in *Diplocyclos palmatus* (Fig. 3D); rugose in *Luffa acutangula* (Fig. 4B); thick (slightly sculptured) in *Acanthosicyos horridus*, *Lemurosicyos variegata*, and *Ruthalicia longipes* (Figs. 1B, 3H, and 4H); and smooth in the rest of the taxa (Figs. 1C, E, and G; 2A, C, E, and G; and 3A).

3.4. Seed ridge

Seed ridges showed great variation among the studied taxa. They included seeds with the following characters: strong ridges in *Diplocyclos palmatus* (Fig. 3C); dentate margins or ridges in *Lemurosicyos variegata* (Fig. 3G); no ridges in *Acanthosicyos horridus*, *Bambekea racemosa*, *Citrullus colocynthis*, *C. ecirrhosus*, *Luffa acutangula*, and *Nothoalsomitra suberosa* (Figs. 1A and C, 2E and G, and 4A and E); and narrow ridges in the rest of the species (Figs. 1E and G, 2A and C, 3A, and 4C and G).

3.5. Seed arillate

The outer integument covering a seed after fertilization is called an arilloid jacket. The arilloid jacket is present in *Bambekea racemosa*, *Citrullus ecirrhosus*, *C. lanatus*, *Diplocyclos palmatus*, *Lagenaria spaerica*, and *Lemurosicyos variegata* (Figs. 1C; 2G; 3A, C, E, and G), or absent in the rest of the taxa (1A, E, and G; 2A, C, and E; 3G; 4A, C, E, and G).

3.6. Seed size

Seed dimensions varied significantly among the examined taxa. The biggest seeds (10–15 mm × 5–8 mm) were measured in *Acanthosicyos horridus*, *Benincasa hispida*, *Luffa acutangula*, *L. cylindrical*, *Nothoalsomitra suberosa*, and *Ruthalicia longipes*; the smallest seeds (3.6–4 mm × 2.7–3.4 mm) were measured in *Bryonia alba* and *Bryonia dioica*. The rest of the species had slightly bigger seeds (5–10 mm × 4–5.5 mm) (Table 2).

3.7. Shape of outer epidermal cells

Outer epidermal cells can be of considerable diagnostic value for systematics. The outer epidermal cells varied from elongate in *Benincasa hispida* and *Ruthalicia longipes* (Figs. 1F and 4H), isodiametric to 5–6 gonal in *Acanthosicyos horridus*, *Bryonia dioica*, and *Lagenaria spaerica* (Figs. 1B, 2D, and 3F), 5–6 gonal to elongate in *Citrullus lanatus* and *Diplocyclos palmatus* (Figs. 3B and D), and irregular to polygonal in the rest of the taxa (Figs. 1D and H; 2B, F, and H; 3H; 4B, D, and F).

3.8. Anticlinal cell wall boundaries

Anticlinal cell wall boundaries are mostly well developed. There are three types of anticlinal cell wall boundaries: the first type is undulate in *Bryonia cretica*, *Citrillus ecirrhosus*, *Luffa acutangula*, and *L. cylindrical* (Figs. 2B; 4B, D, and H); the second type is straight in *Acanthosicyos horridus*, *Benincasa hispida*, *Citrullus lanatus*, *Diplocyclos palmatus*, and *Lagenaria spaerica* (Figs. 1B and D; 3B, D, and F); the third type is straight to slightly sinuous in the rest of the taxa (Figs. 1D and H, 2D and F, 3H, and 4F and H). Based on the relief of these cell wall boundaries there are two types of boundaries: slightly raised in *Bambekea racemosa* (Fig. 1D) and raised in the rest of the taxa (Figs. 1–4, except 1D).

3.9. Periclinal cell wall

The curvature of the outer periclinal cell wall can be a good diagnostic character. There are four different shapes for this cell wall: flat to convex in *Lagenaria spaerica* and *Lemurosicyos variegata* (Figs. 3F and H); flat to concave in *Bambekea racemosa*, *Bryonia cretica*, and *Nothoalsomitra suberosa* (Figs. 1D, 2B, and 4F); concave in *Bryonia alba* (Fig. 1H); flat in the rest of the taxa (Figs. 1B and E; 2D, F, and H; 3B and D; 4B, D, and H). The sculpture of the outer cell wall greatly varied among the studied taxa. There were five different shapes for the surface of the outer cell wall: smooth in *Bryonia cretica* and *Nothoalsomitra suberosa* (Figs. 2B and 4F); folded in *Lemurosicyos variegata* and *Luffa cylindrical* (Figs. 4B and D); striate in *Ruthalicia longipes* (Fig. 4H); micro-papillate in *Acanthosicyos horridus*, *Citrullus ecirrhosus*, and *C. lanatus* (Figs. 1B, 2H, and 3B); and smooth to fine folds in the rest of the taxa.

3.10. Cluster and Principal coordinates analysis (PCO)

The results for the cluster and principal coordinates analyses are presented in Figs. 5–8. In the UPGMA dendrogram and PCO plots (first three principal coordinates axes

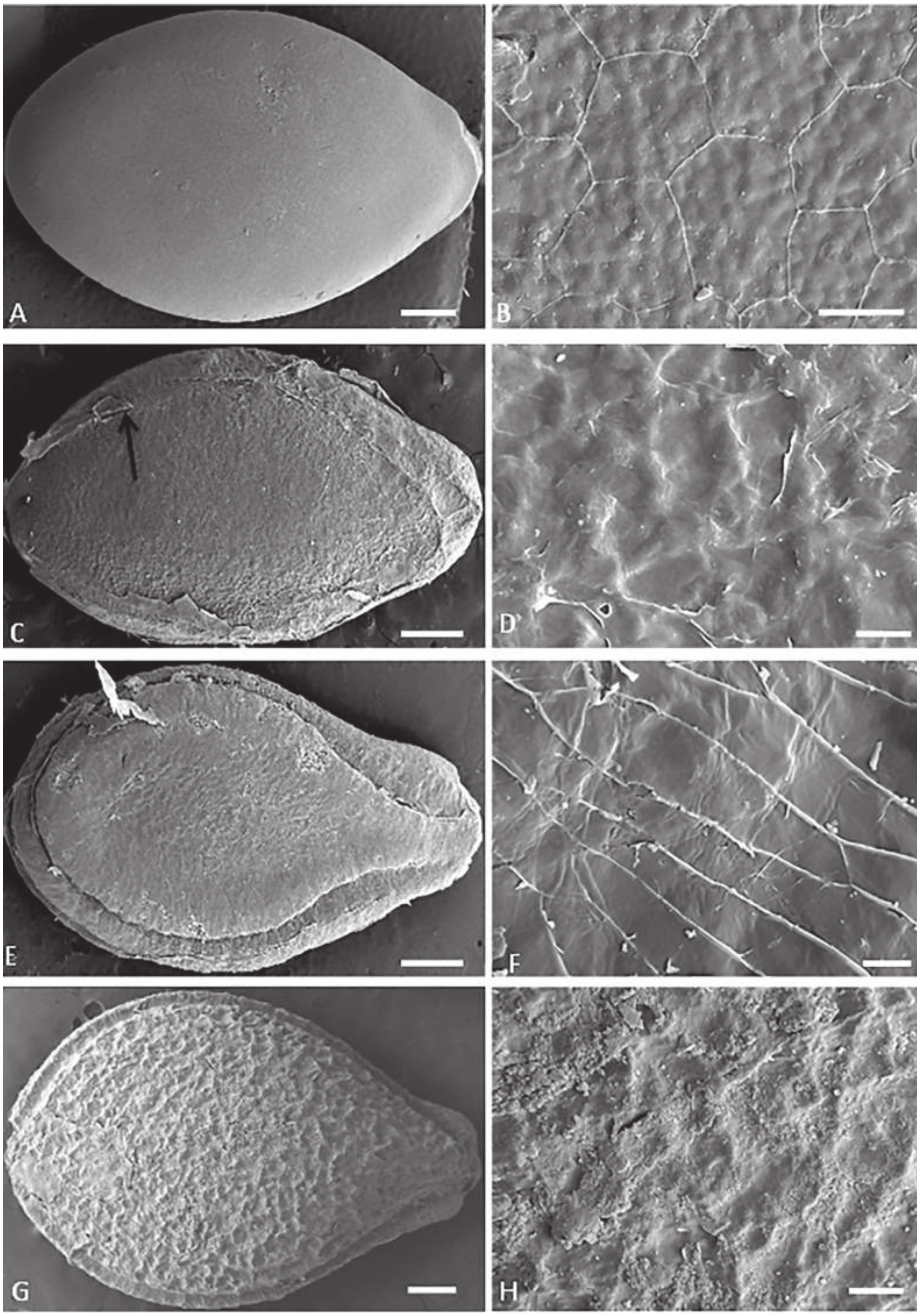

Figure 1. SEM photographs of seeds. A and B *Acanthosicyos horridus*. C and D *Bambekea racemosa.* E and F *Benincasa hispida.* G and H *Bryonia alba*. A, C, E, and G entire seed; B, D, F, and H enlargement of seed coat. Scale bars: 500 µm in A, C, E and G; 50 µm in B; 20 µm in D; 25 µm in F and H. The arrow refers to the arilloid jacket.

was 56.8% of the total observed variation. Plots 1/2, 1/3, and 2/3 together show six groups (Figs. 6-8). The main characters explained the separation between groups (characters with high factor loading were > 0.6). Six major branches and groups (A–F) with approximately 44% similarity were distinguished. 1) Branch A included only *Bambekea racemosa* (tribe Coniandreae). This branch showed the largest distance from all other branches. 2) Group B included *Lagenaria spaerica* and *Lemurosicyos variegate* (tribe Benincaseae). 3) Group C contained *Citrullus ecirrhosus*, *C. lanatus*, and *Diplocyclos palmatus* (tribe Benincaseae). 4) Group D comprised *Luffa acutangula* and *L. cylindrica* (tribe Luffeae).

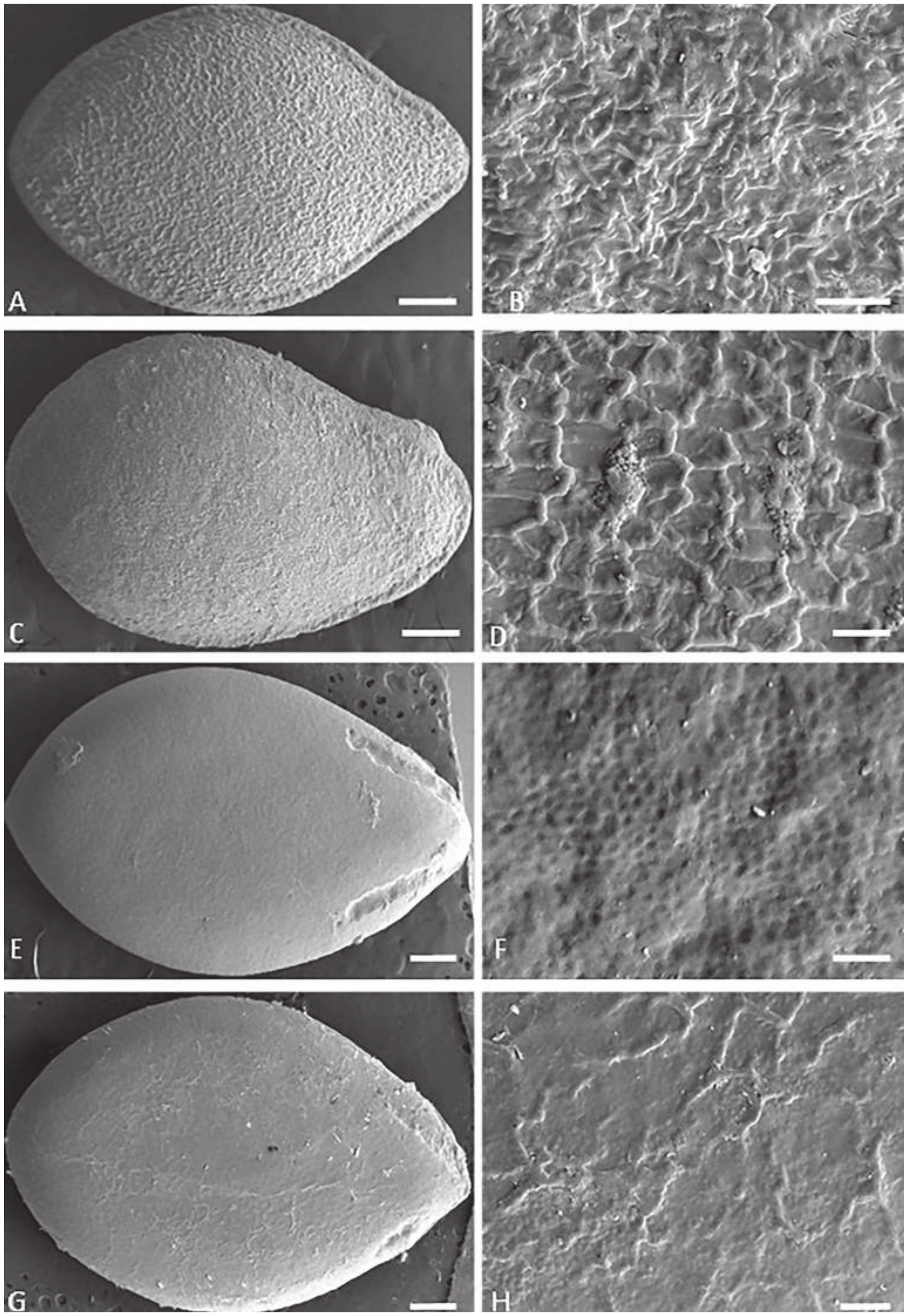

Figure 2. SEM photographs of seeds. A and B *Bryonia cretica*. C and D *Bryonia dioica*. E and F *Citrullus colocynthis*. G and H *Citrullus ecirrhosus*. A, C, E, and G entire seed; B, D, F, and H enlargement of seed coat. Scale bars: 500 µm in A, C, E, and G; 100 µm in B and H; 50 µm in D and F.

5) Group E was divided into two subgroups: a subgroup with *Bryonia dioica* and *Citrullus colocynthis* (tribe Benincaseae), and a subgroup with *Bryonia alba*, *Bryonia cretica* (tribe Benincaseae) and *Nothoalsomitra suberosa* (tribe Luffeae). 6) Group F included *Acanthosicyos horridus*, *Benincasa hispida*, and *Ruthalicia longipes* (tribe Benincaseae).

Some genera and tribes showed intra variability among themselves. In general, UPGMA and PCO indicated that seed morphology followed the currently applied tribal classification of Cucurbitaceae by Jeffrey (2005), Achigan-Dako (2008), and Schaefer & Renner (2011).

Key to the identification of some tribes of Cucurbitaceae based on seed characters

1a. Large seeds 10–15 × 6-8 mm 2
1b. Small seeds 3.5–10 × 2.7-5.5 mm 7
2a. Seed without ridge 3
2b. Seed with narrow ridge 5
3a. Seed whitish-yellow; thick; sculpture of periclinal wall micro-papillate .... *Acanthosicyos horridus*
3b. Seed black to brown; smooth or rugose; sculpture of periclinal wall smooth to fine folds 4
4a. Seed black; oblong-obovoid; rugose; anticlinal boundaries, undulate .... *Luffa acutangula*
4b. Seed brown; ovoid; smooth; anticlinal boundaries, straight to slightly sinuous .... *Nothoalsomitra suberosa*
5a. Seed black; ovoid; epidermal cell irregular to polygonal cells .... *Luffa cylindrica*
5b. Seed yellowish brown; oblong; epidermal cell elongate in one direction 6
6a. Seed thick; sculpture of periclinal wall striate .... *Ruthalicia longipes*
6b. Seed smooth; sculpture of periclinal wall smooth to fine folds .... *Benincasa hispida*
7a. Seed size 3.6–6 × 2.7–5 mm 8
7b. Seed size 6.1–10 × 3,6–5.5 mm; 12
8a. Seed without ridge .... *Bambekea racemosa*
8b. Seed with ridge 9
9a. Seed smooth; with narrow ridge; not arillated 10
9b. Seed scorbiculate; strongly ridge; arillated .... *Diplocyclos palmatus*
10a. Seed size 3.6–4.2 × 2.7–3.4 mm; brown to light brown; anticlinal boundaries straight to slightly sinuous 11
10b. Seed size 5–6 × 4–6 mm; orange-brown; anticlinal boundaries undulate .... *Bryonia cretica*
11a. Seed ovoid; brown; epidermal cell irregular to polygonal cells; periclinal cell wall concave .... *Bryonia alba*
11b. Seed obovoid; light brown; epidermal cell isodiametric, 5-6 gonal cells; periclinal cell wall flat .... *Bryonia dioica*
12a. Seed without ridge; anticlinal boundaries undulate 13
12b. Seed with ridge; anticlinal boundaries straight to slightly sinuous 14
13a. Seed black to brown; arillated; sculpture of periclinal wall micro-papillate .... *Citrullus ecirrhosus*
13b. Seed yellow brown; not arillated; sculpture of periclinal wall smooth to fine folds .... *Citrullus colocynthis*
14a. Seed oblong; thick; with dentate margin; epidermal cell irreguklar, polygonal cells .... *Lemurosicyos variegata*
14b. Seed oblong-obovoid; smooth; with narrow ridge epidermal cell isodiametric, 5-6 gonal cells to elongate in one direction 15
15a. Seed black to brown; periclinal cell wall flat; micro-papillate .... *Citrullus lanatus*
15b. Seed whitish-yellow; periclinal cell wall flat to slightly convex; smooth to fine folds .... *Lagenaria sphaerica*

4. Discussion

Several authors have tried to provide an acceptable system to classify the family Cucurbitaceae into subfamilies, tribes, and subtribes (Hooker 1867; Jeffrey 1980; 1990; Wilde & Duyfjes 2006 a; b; c; Kocyan *et al.* 2007; Schaefer & Renner 2011). These studies were based on morphological characters, such as life forms, leaves, flowers, fruits, seeds, and pollen grains. In the present study, we used a number of seed characters based on details of the seed coat surface. UPGMA provided an insight into the degree of similarity among the species and showed whether they formed groups or clusters, indicating the range of variation within and among tribes. PCO indicated which characters were important on the axes, which were the most significant based on the highest factor loading (Table 3), and hence clarified which characters caused the separation between groups and were useful to distinguish taxa. Generally, our results confirmed congruence between the UPGMA clustering and PCO analyses, and suggested six groups. In general, the results showed that different patterns of seed morphology were helpful in distinguishing various species and to confirm the tribe and subtribe classifications as proposed by Schaefer & Renner (2011), Jeffrey (2005), and Achigan-Dako (2008).

4.1. Tribes classification

4.1.1. Tribe Coniandreae Endl. ex M. Roem (1846) (groups A)

According to the cluster and PCO analysis, *Bambekea racemosa* (tribe Coniandreae) showed the largest distance from all other groups, and was distinct from the others by having wingless seed; yellowish brown; straight to slightly sinuous, slightly raised, anticlinal cell wall boundaries; and by seed size. *Bambekea racemosa* corresponded to the previously recognized position within tribe Benincaseae subtribe Benincasinae (Jeffrey 2005; Achigan-Dako 2008). However, Schaefer & Renner (2011) treated this species as sited in tribe Coniandreae based on 14 DNA regions from the three plant genomes: the mitochondrial *nad1 b/c* intron

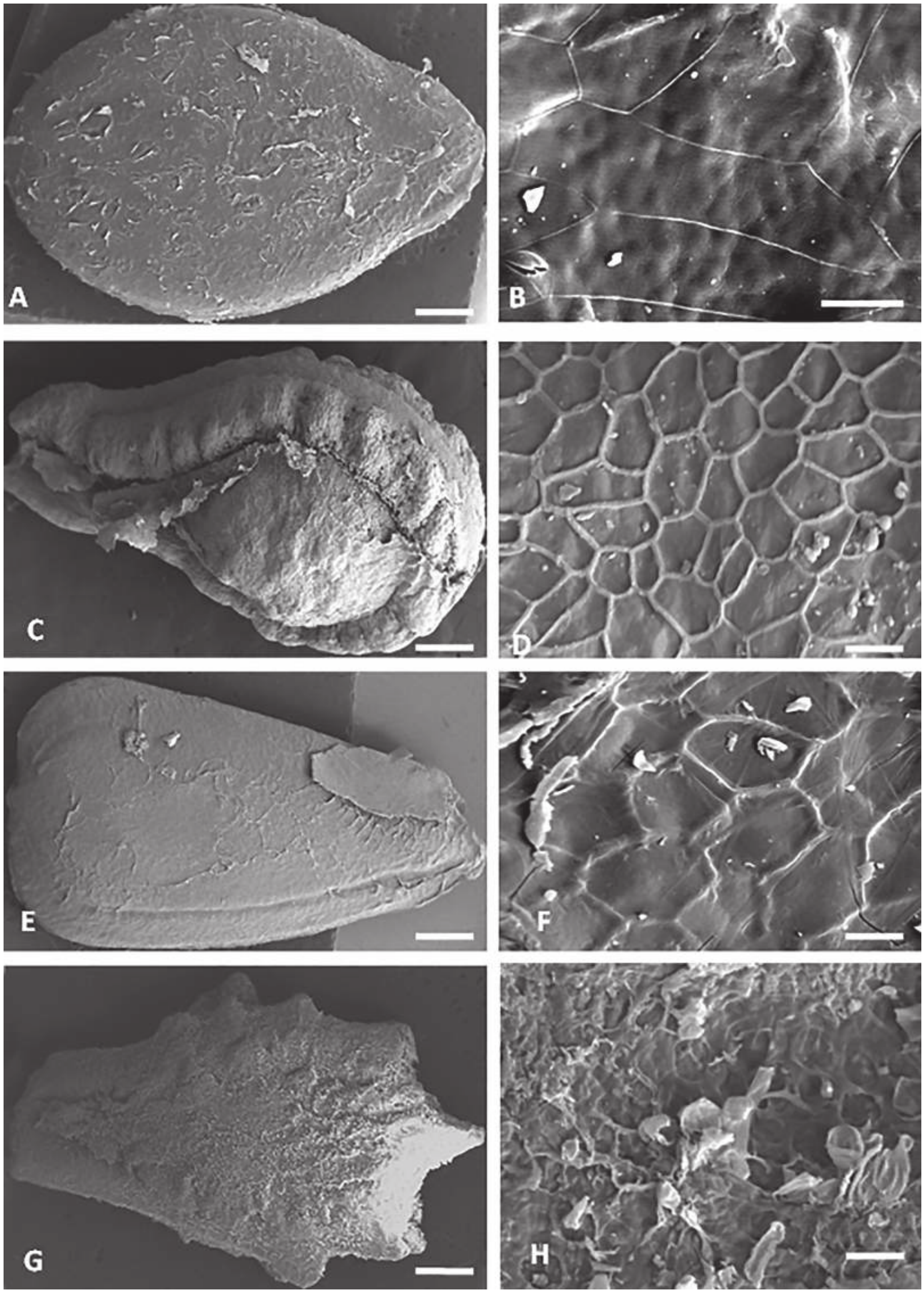

Figure 3. SEM photographs of seeds. A and B *Citrullus lanatus*. C and D *Diplocyclos palmatus*. E and F *Lagenaria sphaerica.* G and H *Lemurosicyos variegata*. A, C, E, and G entire seed; B, D, F, and H enlargement of seed coat. Scale bars: 500 µm in A, C, E, and G; 50 µm in B and D; 25 µm in F; and 43 µm in H.

and *matR* gene; the nuclear ribosomal 18S, ITS1-5.8S-ITS2, and 28S genes; and the plastid genes *rbcL*, *matK*, *ndhF*, *atpB*, *trnL*, *trnL-trnF*, *rpl20-rps12*, *trnS-trnG* and *trnH-psbA*, spacers, and introns.

Seed coat characters were used by Jeffrey (2005) to re-circumscribe Coniandreae to include 19 genera, which was strongly supported by molecular data. Coniandreae seeds lack a hypodermis, while other Cucurbitaceae have seed coats with a hypodermis including one or many layers of sclerotic cells (Singh & Dathan 2001). Based on results by Kocyan *et al*. (2007), Bambekea, Cucumeropsis, and Eu-reiandra may be sister to the Coniandreae (but this only has

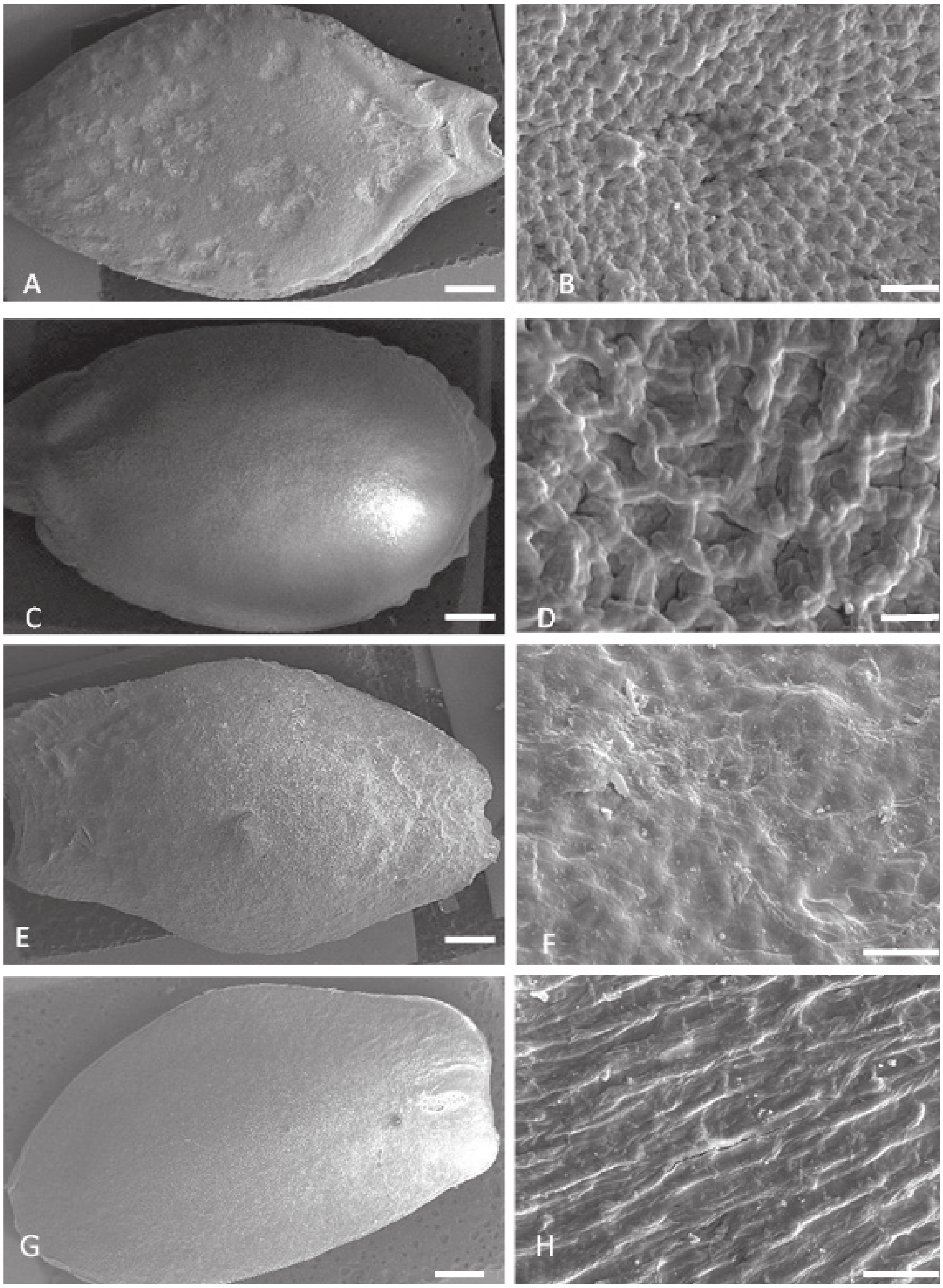

Figure 4. SEM photographs of seeds. A and B *Luffa acutangula*. C and D *Luffa cylindrica*. E and F *Nothoalsomitra suberosa*. G and H *Ruthalicia longipes*. A, C, E, and G entire seed; B, D, F, and H enlargement of seed coat. Scale bars: 500 µm in A, C, E, and G; 50 µm in B; 17 µm in D; 100 µm in F and H.

68% bootstrap support) and probably should be included in that tribe; although Jeffrey (2005) still placed them in Benincaseae. This result agrees with those of Kocyan *et al*. (2007) and Schaefer & Renner (2011) who separated it as a tribe.

4.1.2. Tribe Benincaseae Ser. (1825) (groups B, C, and F)

Walters & Decker-Walters (1991) examined eight species belonging to the tribe Benincaseae using starch gel electrophoresis and showed three major evolutionary lineages within the Benincaseae: 1) *Benincasa*, *Citrullus*, and *Lagenaria*, 2) *Bryonia* and *Ecballium*, and 3) *Luffa*. Chung *et al.* (2003) investigated the genetic relationships in Benincaseae, Cucurbiteae, Joliffieae, Melothrieae, and Sicyeae tribes of Cucurbitaceae based on consensus chloroplast simple sequence repeats (ccSSR) and established previous biochemical and morphological data that indicated distinct lineages within the tribe Benincaseae and cast doubt on the hypothesis that Benincaseae was a monophyletic tribe. Kocyan *et al*. (2007) presented a phylogenetic network and

Table 3. Seed morphological characters showing highest factor loading on the first three principal coordinates axes. The shaded numbers indicate characters with a high factor loading >0.6.

N	Characters	Principal coordinates		
		1	2	3
		Factors loading		
1	Seed shape	0.73	0.52	-0.47
2	Seed sculpture	0.37	-0.50	0.20
3	Seed wings	0.31	-0.65	0.12
4	Arillate (An exterior covering seed after fertilization)	-0.47	0.54	0.29
5	Seed color	0.86	0.53	-0.35
6	Seed size (mm) (Length x width)	0.43	0.92	-0.61
7	Epidermal cell patterns	0.55	0.11	-0.32
8	Anticlinal walls	-0.52	-0.39	0.49
9	Relief of cell wall boundaries	-0.23	0.47	0.71
10	Sculpture of anticlinal boundaries	0.44	-0.58	0.66
11	Curvature of outer periclinal cell wall	0.20	-0.76	0.29
12	Secondary cell wall sculpture	0.54	0.11	-0.27
Percentage per **PCO**		**25.9**	**17.5**	**13.4**
Percentage total variation for the first three principal coordinates amount 56.8%				

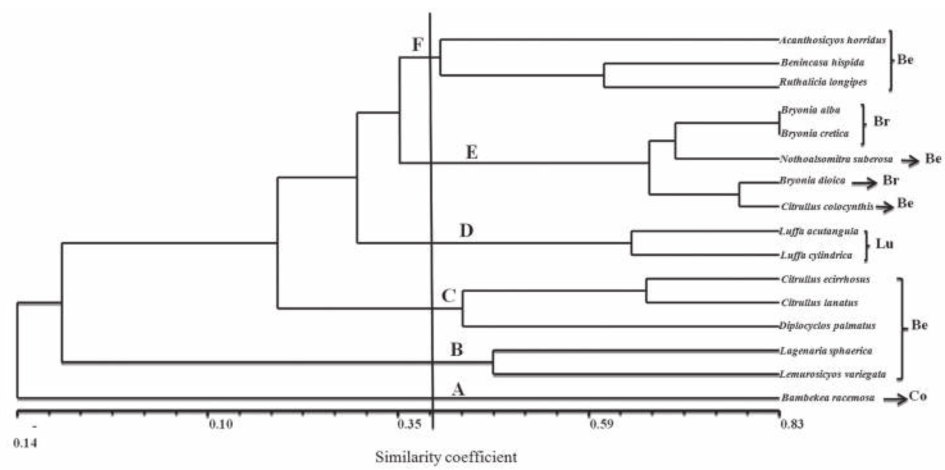

Figure 5. Dendrogram illustrating the relationships among the investigated species based on seed characters: **Be**, tribe Benincaseae; **Br**, tribe Bryonieae; **Co**, tribe Coniandreae; **Lu**, tribe Luffeae.

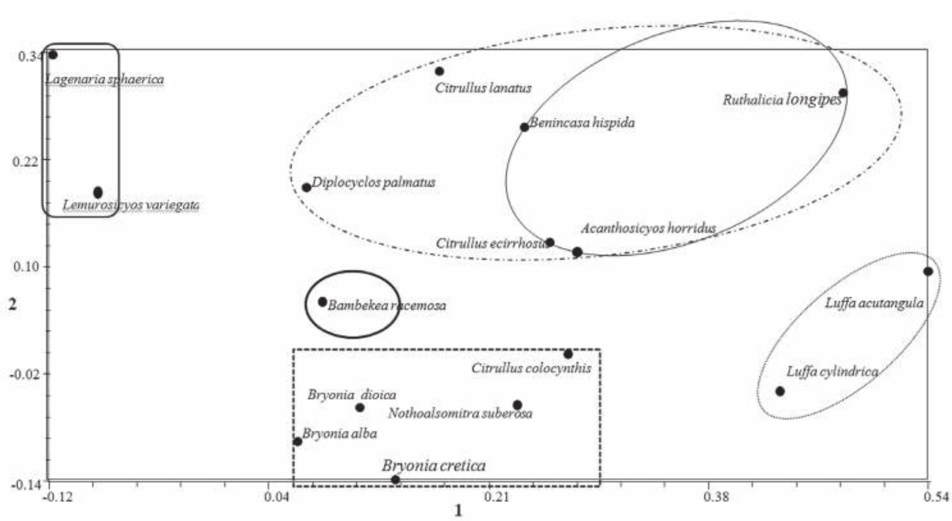

Figure 6. Scatterplot of the 16 OUTs plotted against the first principal coordinate by the second principal coordinate.

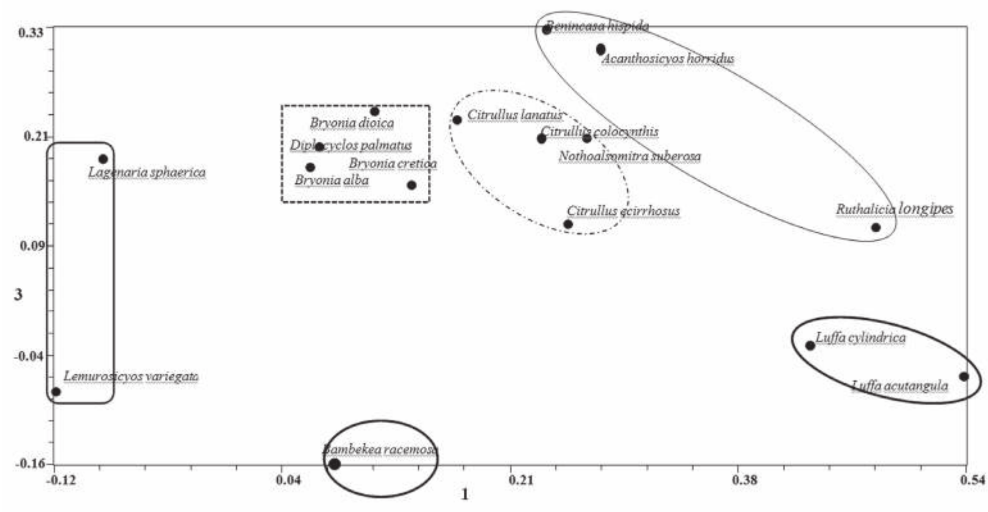

Figure 7. Scatterplot of the 16 OUTs plotted against the first principal coordinate by the third principal coordinate.

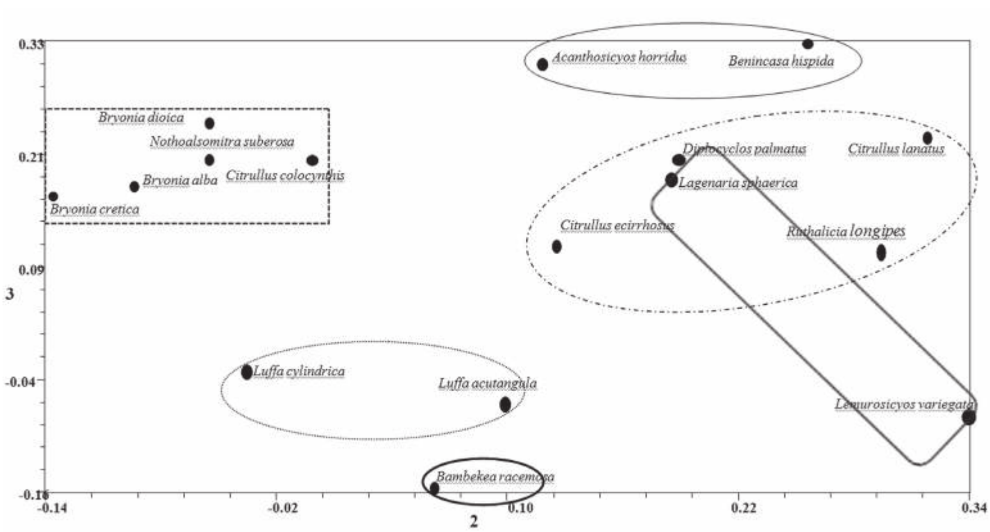

Figure 8. Scatterplot of the 16 OUTs plotted against the second principal coordinate by the third principal coordinate.

demonstrated that the tribe Benincaseae was not monophyletic and that the subtribes Benincasinae and Cucumerinae were highly polyphyletic.

Achigan-Dako (2008) investigated the phylogenetic analysis of 68 species of the tribe Benincaseae based on 127 nuclear rDNA ITS sequences and showed that the subdivision of the tribe into subtribes Benincasinae and Cucumerinae was not supported.

Our UPGMA and PCO results showed that the tribe Benincaseae was separated into four clusters and a branch. Within group B there was a close relationship with 0.50 similarity corresponding to group B including *Lagenaria spaerica* and *Lemurosicyos variegata.* Specializations in seed morphology include oblong to obovoid seeds; flat, dentate margin seed; arillated; isodiametric to polygonal epidermal cell shape; straight to slightly sinuous, raised, smooth to fine folded anticlinal boundaries; and flat to slightly convex periclinal cell walls.

Moreover, in group C (*Diplocyclos palmatus*, *Citrullus lanatus*, and *C. ecirrhosus)* there was a close relationship with about 0.45 similarity. They shared the same seed shape characters; arillated; raised, smooth to fine folds anticlinal cell wall and flat periclinal cell wall, but differed in seed wing and seed size; and their undulate to straight anticlinal cell wall.

Another branch of species was represented by *Citrullus colocynthis*, which separated from other species of *Citrullus* based on yellowish brown seed, not arillated, and smooth to fine folds in the periclinal cell wall.

Also, a branch of species represented by *Nothoalsomitra subcerosa* separated with a group of *Bryonia* based on ovoid seed shape, flat smooth seed surface, and irregular, polygonal epidermal cell shapes. Schaefer & Renner (2011) defined *Nothoalsomitra subcerosa*, *Luffa*, and other genera in the separate tribe Sicyoeae.

Inside group F, there was a close relationship with 0.43 similarity including *Acanthosicyos horridus*, *Benincasa hispida*, and *Ruthalicia longipes*, based on the yellowish brown seeds; seed size large with 10–15 mm × 5–8 mm; elongate in one direction to isodiametric epidermal cell shape; straight to slightly sinuous, raised anticlinal boundaries; and a flat periclinal cell wall.

Our results are congruent with those of Walters & Decker-Walters (1991), Chung *et al*. (2003), Kocyan *et al*. (2007), and Achigan-Dako (2008), which suggests that tribe Benincaseae is not a monophyletic group and that subtribe Benincasinae is highly polyphyletic; this is because we found the taxa from this tribe interspersed to both different clusters and with taxa from other tribes.

4.1.3. Tribe Luffeae C. Jeffrey (2005) (group D)

Inside this cluster (group D), two species of *Luffa* were recognized with 0.70 morphological similarity. These species can be clearly defined on the basis of various features: black seed, flat, compressed, seed large size (10–14 × 7–10 mm), irregular to polygonal cells epidermal, undulate, raised anticlinal cell wall boundaries and flat periclinal cell walls. *Luffa* is distinct from other members of the tribe Benincaseae in several respects. For instance, these species produce fibrous fruits with operculate dehiscence and have racemose staminate flowers with three to five stamens and free petals, solitary pistillate flowers, and three to five parted tendrils (Heiser & Schilling 1990). Likewise, all other genera of the Benincaseae have haploid chromosome numbers of 10, 11, or 12, but all species of *Luffa* have the haploid number 13 (Whitaker 1933). Heiser & Schilling (1988) investigated the phylogeny of the genus *Luffa* based on morphological characters and revealed two phyletic lines: one of them comprised *Luffa cylindrical* (*L. aegyptiaca*) and *L. acutangula*, and the other included the rest of the species. Moreover, Walters & Decker-Walters (1991) showed that three major evolutionary lineages within the Benincaseae and one major line included *Luffa*. In addition, Singh & Dathan (1998) investigated the seed coat anatomy of the Cucurbitaceae and showed that *Luffa* differed from the rest of the Benincaseae and they elevated them to tribal rank. However, Telford (1982) placed it in the Benincaseae because of its globose synandrium with strongly sigmoid thecae. Kocyan *et al*. (2007) placed *Luffa* far from Benincaseae. Generally, these results agree with those of Heiser & Schilling (1988), Walters & Decker-Walters (1991), Jeffrey (2005), Kocyan *et al*. (2007), and Achigan-Dako (2008), which suggests that tribe Luffeae is a monophyletic group.

4.1.4. Tribe Bryonieae Dumort. (1827) (group E)

Inside this cluster (group E), three species of genus *Bryonia* (*B. alba*, *B. cretica*, and *B.dioica*), and two species from tribe Benincaseae (*Nothoalsomitra subcerosa* and *Citrullus colocynthis*) were recognized with 0.70 morphological similarity. These species can be clearly defined on the basis of various features: ovoid to obovoid seed, flat and smooth seed surface, irregular to polygonal cells epidermal, although *Bryonia* differed from the other in having seed with a narrow ridge, small seed size, and a straight to slightly sinuous, raised anticlinal cell wall.

Singh & Dathan (1998) showed that *Bryonia* and *Ecballium* differed in their osteosclereids in the main mechanical layer, which were placed radially in the latter and obliquely in the former, and they segregated these two genera into distinct tribes. Current observations of seed coat can be diagnostic or indicative of phylogenetic relationships, and these results are in agreement with the phylogenetic results of Jobst *et al.* (1998), Chung *et al*. (2003), Schaefer & Renner (2011), seed coat anatomy results of Singh & Dathan (1998), isozyme by Walters *et al*. (1991) and morphology by Jeffrey (1980; 2005), which suggests that tribe Bryonieae is a monophyletic group.

Conclusions

The structure of seed coats offers a set of characters useful for the taxonomy of the Cucurbitaceae. The present study showed that seeds of Cucurbitaceae display high diversity in shape, color, size, surface, epidermal cell characters, anticlinal cell wall boundaries, and periclinal cell wall; some species even have specialized structures. Seed coat morphology also provided some evidence for intrageneric classification and corresponded with the phylogenetic results of Chung *et al*. (2003), Kocyan *et al*. (2007), Achigan-Dako (2008), and Schaefer & Renner (2011). Furthermore, current results support the monophyly of Bryonieae, Coniandreae, and Luffeae, as suggested by Chung *et al.* (2003), Jeffrey (2005), Kocyan *et al*. (2007), Achigan-Dako (2008), and Schaefer & Renner (2011). Likewise, these analyses support previous biochemical and phylogenetic data, indicating that distinct lineages exist within the tribe Benincaseae and demonstrate that the tribe Benincaseae is not monophyletic and that subtribe Benincasinae is highly polyphyletic. Finally, seed coat analysis confirmed that developmental variation in seed characters is taxonomically useful, not only because it gives us a better understanding of sculpture development, but also because it allows us to formulate the taxonomy of Cucurbitaceae on the genera and tribal levels, and it is useful for constructing an identification key.

Acknowledgments

We are grateful to the Directors and Curators of the International Herbaria (K, E and CAIM) and Kew garden, Millennium seed bank for providing us with materials.

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