biology and life sciences forum

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Proceeding Paper

Comparative Taxonomic Study of *Balanites aegyptiaca* (L.) Delile (Zygophyllaceae) †

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† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Abstract: *Balanites aegyptiaca* or Desert Date (Zygophyllaceae) is widely distributed in arid and semi-arid regions in Africa and South Asia. The current study aims to identify the infraspecific variation between *B. aegyptiaca* native to Egypt and Saudi Arabia. Detailed macro- and micromorphological analyses of leaflets, petioles, stems, spines, and fruits were performed using a light and Scanning Electron Microscope (SEM). Statistical analysis was performed by using the relevant R- software packages. Leaflet shape and apex, leaflet length/width ratio, leaflet indumentum density, and the petiole length were recorded. Based on these traits, the present study suggests the existence of one variety, "*B. aegyptiaca* var. *aegyptiaca*" in Makkah, Saudi Arabia. At the same time, two varieties were recorded in Egypt "*B. aegyptiaca* var. *aegyptiaca* and *B. aegyptiaca* var. *tomentosa*".

Keywords: anatomical study; *Balanites aegyptiaca*; macro- and micromorphology; SEM; stomata; R-software; Zygophyllaceae

Citation: Hamada, F.A.; El-Banhawy, A.; Ellmouni, F.Y.; Al-Juhani, W.; Makharita, R.R.; Nour, I.H. Comparative Taxonomic Study of *Balanites aegyptiaca* (L.) Delile (Zygophyllaceae). *Biol. Life Sci. Forum* **2022**, *11*, 72. https://doi.org/10.3390/IECPS2021-12060

Academic Editor: Carmen Arena

Published: 10 December 2021

1. Introduction

Balanites aegyptiaca (L.) Delile, commonly known as 'Desert Date', is a prickly evergreen tree up to 15 m high. It is a vital source of food and medicine, and it has an active role in conserving and renewing degraded vegetation cover in arid areas [1]. *Balanites aegyptiaca* is native to semi-arid regions, widely distributed in most African countries [2,3].

The genus *Balanites* Delile has undergone numerous nomenclatural and taxonomic changes. Alpinio first described the Desert Date in 1592 [4] under *Agihalid*. In 1753, Linnaeus recognised the species *Balanites aegyptiaca* as *Ximenia aegyptiaca* L. [5]. Delile renamed the genus *Balanites*, which means the fruit in Latin [6]. Hegnauer and Cronquist placed *Balanites* in the family Zygophyllaceae based on the similarities between flavonoids of *Balanites* and those found in the other members of the Zygophyllaceae [7,8]. Sands treated *Balanites* in a separate monogeneric family called Balanitaceae [9]. Boesewinkel supported the separation of the family Balanitaceae according to the unique characters of the ovule and seed [10]. Information on the anatomy, embryology, taxonomy, and pollen morphology was described by Singh [11]. In addition, molecular data of the chloroplast and nuclear markers (*rbcL*, *trn*L-F and ITS) supported the genus placement within Zygophyllaceae rather than Balanitaceae [12,13].

Sands carried out a taxonomic revision on the genus *Balanites*, and divided the polymorphic *B. aegyptiaca* into five varieties: *aegyptiaca*, *ferox*, *quarrei*, *pallida*, and *tomentosa*. He recognized these varieties based on spine length, leaflet shape, indumentum density of leaflet and flower, pedicel length, and the number of flowers per inflorescence [2,3]. In Egypt, Maksoud and El Hadidi recognized eight flavonoids; six from the vegetative parts and two from the fruits of *B. aegyptiaca* [14]. Amer et al. performed a biosystematics study on eight Egyptian populations of *B. aegyptiaca* var. *aegyptiaca*, and recognized three ecotypes: Nile valley, Sahelian and Xerotype based on some morphological traits, physico-chemical soil pattern, and RAPD markers [3]. In the Flora of Egypt and the Flora of Saudi Arabia, no taxonomic ranks were verified below the species level [15,16]. The current study aims to evaluate the infraspecific variation among *B. aegyptiaca* populations native to Egypt and Saudi Arabia, and to identify these infraspecific taxa using macro-and micromorphological traits.

2. Materials and Methods

2.1. Plant Materials and Statistical Analysis

Sixteen samples of *B. aegyptiaca* were collected from Egypt and Saudi Arabia from their natural habitats. The collected specimens were deposited at the herbarium of Suez Canal University, Ismailia, Egypt (SCUI). The macromorphological traits were investigated for three to five replicates of each sample. Twenty-two quantitative and qualitative characters were measured for the stem, leaflet, and fruit; the description follows Sands [2] and Smith [17]. The coordinates of the collection localities were recorded using a Handy GPS, then listed in Table A1. Analysis of variance (ANOVA) was performed and followed by a Post Hoc Tukey Honestly Significant Difference (HSD) test. The data matrix used for the cluster analysis was based on 16 quantitative characters. Hierarchical clustering with Euclidean distance was conducted after being scaled and standardized for the data matrix [18].

2.2. Anatomical Study and Scanning Electron Microscope (SEM)

For the anatomical study, three fresh samples of each stem, leave and petiole were fixed in FAA solution and then preserved in 70% ethyl alcohol until sectioning. The samples were dehydrated gradually in a series of ethyl alcohol, and then the paraffin method of Johansen [19] was applied. The sectioning was performed with Reichert–Jung, Depew, NY 14043 USA rotary microtome, with a thickness of 10 to 12 µm. The sectioning was done at the same regions for the stem, leaflets, and petioles of all sample replicate. Sections stained with 2% crystal violet and 2% Erythrosin B (saturated in clove oil) then permanently mounted with Canada balsam.

For Scanning Electron Microscope (SEM), the stem, spine, leaflet, and fruit of *B. aegyptiaca* were fixed onto cupper stubs with double-sided adhesive tape and were coated for 5 min with gold in a polaron JFC-1100E coating unit. The samples were investigated and photographed by using JEOL JSM-IT200, Japan. Thirteen micromorphological characters of the stem were investigated, and twelve quantitative and qualitative traits were studied for leaflet abaxial (AB) and adaxial (AD) surfaces. In addition, sixteen quantitative and qualitative characters were described for the fruit micromorphology. The trichomes were counted in an area of 5 µm × 5 µm, and the micromorphological description followed Barthlott [20], Weryszko-chmielewska and Chernetskyy [21], Koch and Barthlott [22], and Khokhar et al. [23].

3. Results

3.1. Vegetative and Fruit Macromorphology

Balanites aegyptiaca is a spiny tree or shrub, and its height ranges from 1.5 to 4 m with different trichomes' densities. The density of spines per branch varies within the same individual tree and among different trees. Spines were unbranched or rarely branched in samples 11 (Edfu- Marsa Alam, Red Sea, Egypt) and 16 (Aswan, Egypt). The spine length

ranged from 0.42 to 4.7 cm, while the spinule length ranged from 0.16 to 0.84 cm, with an average size of 0.56 cm.

Leaves were spirally arranged and stipulate; the stipules were always caducous and varied in length from 0.156 to 0.28 cm. An interpetiolar stipule was noted for all samples with an average length of 0.528 cm. The leaves were bifoliate and subsessile or petiolate, where petiole subtends the spines. The petiole length varied from 0.10 to 0.99 cm, and the petiolule length ranged 0.06 to 1.60 cm.

The leaflet colour varied from yellowish-green or pale to dark green. The leaflet size was 0.89–6.40 × 0.33–3.55 cm, and its area ranged from 0.21 to 14.85 cm^2 with an average of 2.86 cm^2. Most of the examined samples showed elliptic leaflets. Other leaflet shapes were recorded as follows: three samples represented the narrowly elliptic leaflets; sample 12, (Edfu- Marsa Alam, Red Sea, Egypt) and samples 15 and 16, (Aswan, Egypt). Ovate leaflets were recorded in sample 7 (Wadi el-Gemal National Park, Egypt). The orbicular leaflet was recognized in sample 10 (Edfu- Marsa Alam, Red Sea, Egypt). Finally, the suborbicular leaflet was recorded for two samples (samples 1 and 2 collected from Makkah, Saudi Arabia). The leaflet apex was generally obtuse, except sample 10, which was rounded, and samples 3, 7, 9, 11, 13, 15 and 16 attained acute apices; meanwhile, the leaflet base was cuneate.

The fruit was single-seeded with a leathery or wrinkled surface, and its dimensions were 2.44–3.66 × 1.00–2.71 cm. The shape of the fruit was subspherical, ellipsoid, or ovoid.

ANOVA indicated a significant variation of morphological traits among samples with a *p*-value ($p = 6.27 \times 10^{-10}$, R-squared = 0.963). The conducted clustering Agglomerative cluster analysis Figure 1 split the samples into two clusters, differentiating samples from Egypt and Saudi Arabia. Cluster A contained all individuals collected from Saudi Arabia, with samples 11 and 10 from Egypt collected from Marsa Alam, Red Sea, Egypt. In contrast, sample 10 was divergent into a single branch. Cluster B contained only *B. aegyptiaca* collected from Egypt. The data matrix, based on 16 quantitative characters, was used for the cluster analysis and is illustrated with descriptive data in Table A2.

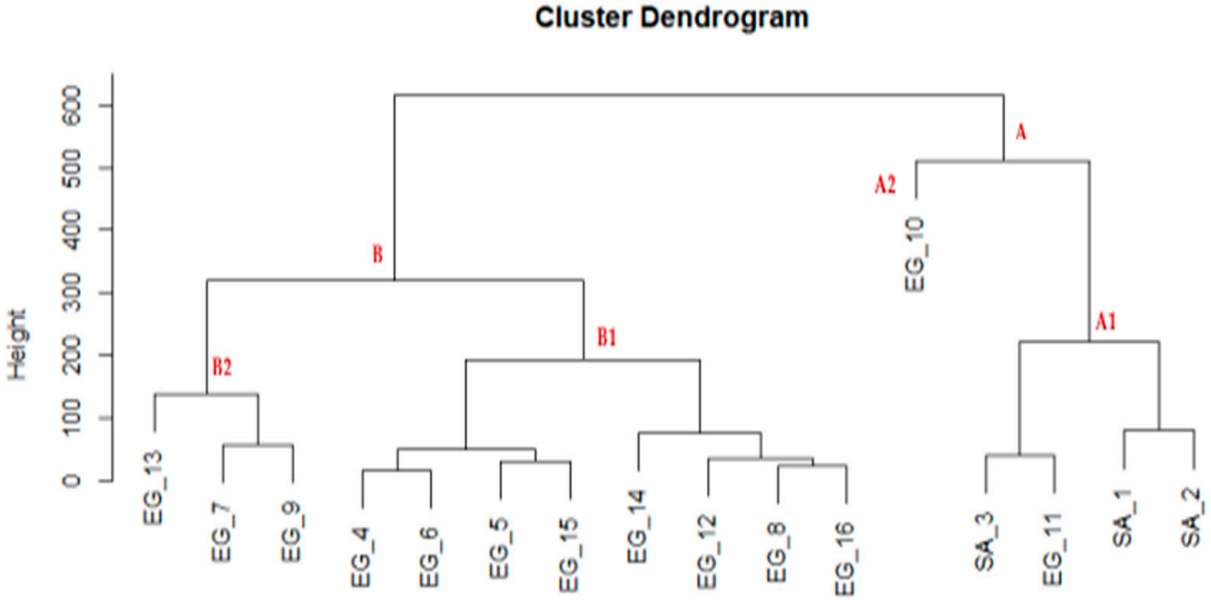

Figure 1. Agglomerative cluster analysis of *Balanites aegyptiaca* collected from Egypt (EG) and Saudi Arabia (SA) based on morphological data.

3.2. Anatomical Characterization

The anatomical characterization of samples collected from Wadi el-Gemal (samples 4, 5, 6, 7, and 8) showed unique anatomical traits compared to those from other localities. The leaflet's 'transverse section' (T.S.) showed more than one vascular bundle in the midrib re-

gion of sample 5 (Figure 2a); however, the others had only one vascular bundle (Figure 2b).

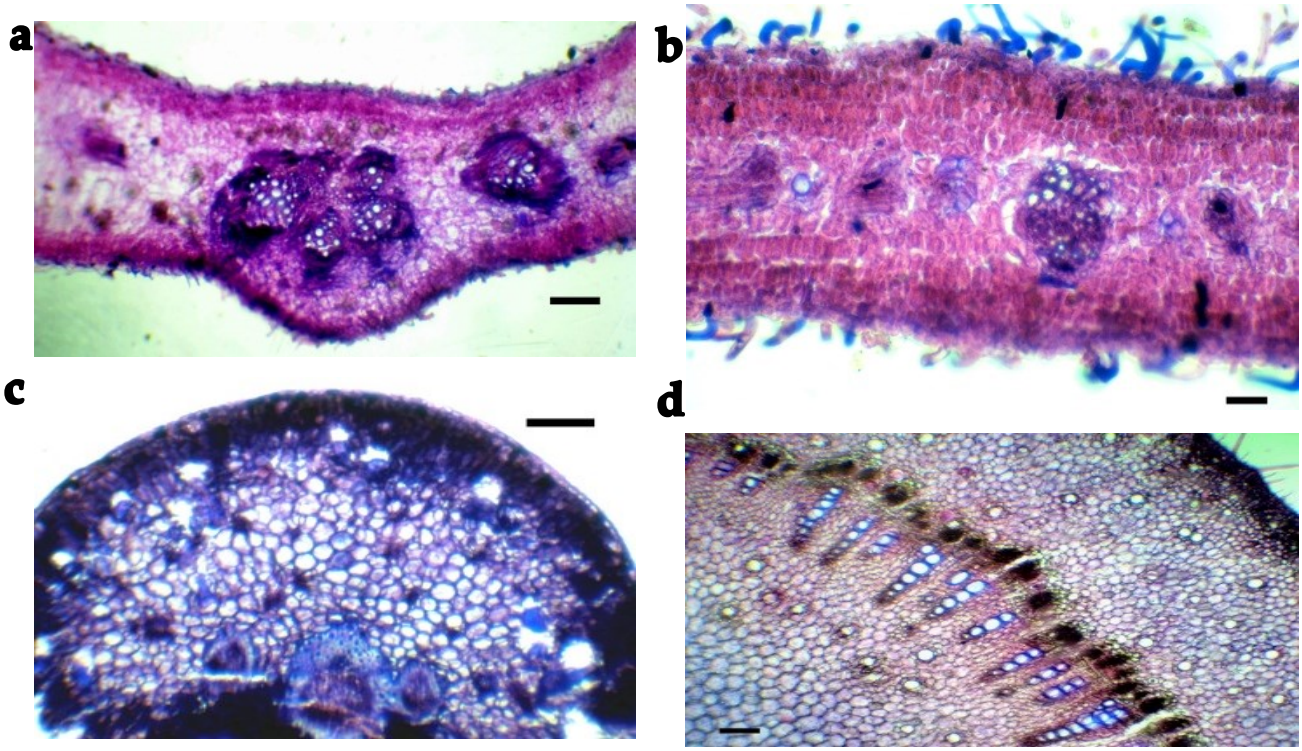

Figure 2. Light micrographs of stem, leaflet, and petiole of *Balanites aegyptiaca*. (**a**) leaflet section showing more than one vascular bundle; (**b**) leaflet section showing one vascular bundle; (**c**) stem section showing the presence of schizogenous glands in the cortex region; (**d**) petiole section showing gap cavities underneath the hypodermis within the cortex region. Scale bar: (**a**) 225 µm; (**b**) 85 µm; (**c**) 55 µm; (**d**) 125 µm.

The palisade tissue constitutes >50% of the mesophyll layer compared to the small palisade layer <50% recorded in samples collected from other locations. Stem sections differentiated into four regions: the epidermis, the hypodermis and cortex, and the pith. The epidermis showed anticlinal cutinized epidermal cells. The cross-section was composed mainly of parenchymatous cells. Three to four rows of lamellar collenchyma hypodermis filled with tannins were present underneath the epidermis. Druses crystals spread, especially in the middle. Trichomes were non-glandular, unicellular, straight, reversed comma-shaped, in addition to little, short compound hairs in specimens of the (Edfu- Marsa Alam, Red Sea) Figure 2b. Sample 14, collected from the High Dam region, showed the presence of schizogenous glands in the cortex region Figure 2c. The anatomical traits of the petiole were alike in all samples. While sample 11, gathered from Marsa Alam Aswan Road, appears to have gap cavities underneath the hypodermis within the cortex region Figure 2d. The spine shows a unique anatomical pattern as it has no vascular bundles, and only fibre bands in the upper part of the cortex were detected. Parenchymatous loose cells occur in the pith region.

3.3. Vegetative and Fruit Micromorphology (SEM)

The vegetative organs (stem, spine, and leaflet) and the fruit of *B. aegyptiaca* were covered with unicellular non-glandular trichomes of different lengths Figure 3a; their length ranged from 28.31 to 429.21 µm. The surface of the trichome was smooth in most samples Figure 3b; while cuticular ornamentation in the form of dense striations was observed in sample 9 from Wadi el-Gemal Figure 3c. The trichomes had one lateral suture lying along the trichome length Figure 3c. Their density varied from sparse (6 trichomes per unit area) in sample 1 collected from South Al Jumum, Makkah, Saudi Arabia, to moderate in

sample 14 from Aswan (19 trichomes per unit area), and dense in sample 9 (30 trichomes per unit area).

Leaflets were amphistomatic with sunken stomata Figure 3f,g (indicated with stars) or deeply sunken Figure 3d,e,g actinocytic stomata on the stem, spine and leaflet of the studied samples. The stomata were surrounded by a ring of subsidiary cells of various numbers and shapes, where the number of cells ranged from five to eight (Figure 3d–j). For the stem and spine, the superficial stomatal pore size was 9.53–21.35 × 6.78–22.76 µm, their area ranged from 55.12 µm^2 to 126.54 µm^2, and the stomatal opening size was 3.11–15.60 × 0.18–2.02 µm. The trichome density and the number of stomata on the spine decreased gradually toward the spine's tip. For the leaflet, the superficial stomatal pore size was 8.54–16.73 × 5.02–11.98 µm, and their area was in the range of 27.06 µm^2 to 168.74 µm^2, and the stomatal opening size was 4.11–11.67 × 0.25–1.40 µm. The density of the trichome and the stomatal count on the abaxial leaflet surface was higher than that located on the adaxial surface.

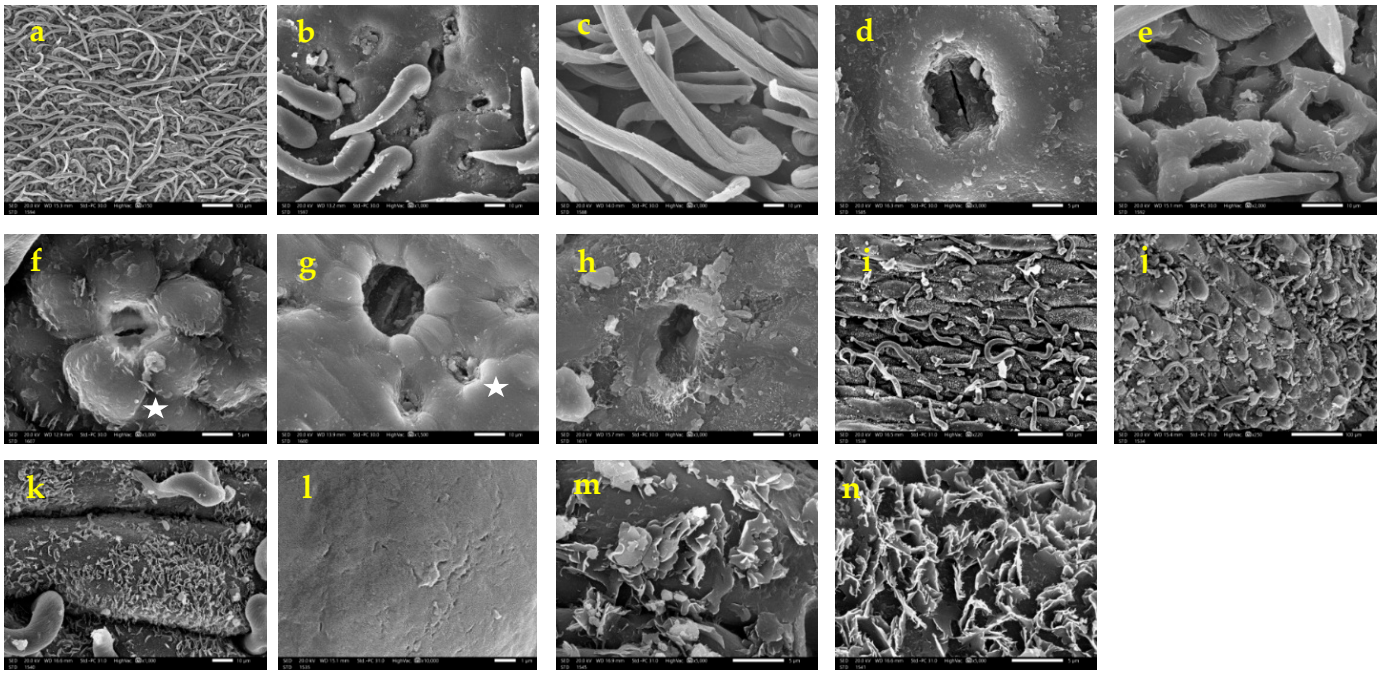

Figure 3. Scanning Electron Microscope (SEM) photomicrographs of *Balanites aegyptiaca*. (**a**) general view of the leaflet trichomes; (**b**) smooth trichome surface on the stem; (**c**) striate trichome surface with lateral suture on the stem; (**d**–**g**) sunken or deeply sunken stomata surrounded by a ring of subsidiary cells of various numbers and shapes; (**h**–**m**) fruit surface. (**h**) general view of the lenticel; (**i**,**j**) epidermal cell outline: (**i**) elongated rectangular, (**j**) elongated elliptic; (**k**) anticlinal wall, relief of cell boundary, the curvature of the outer periclinal cell walls, and the fine relief of the cell wall; (**l**–**n**) epicuticular secretions: (**l**) waxy granules, (**m**) non-entire flat crystalloids waxy platelets with sinuate margin, (**n**) non-entire flat crystalloids waxy platelets with laciniate margin. The stars indicate the sunken stomata. Scale bar: (**a**,**i**,**j**) = 100 µm; (**b**,**c**,**e**,**g**,**k**) = 10 µm; (**d**,**f**,**h**,**m**,**n**) = 5 µm; (**l**) = 1 µm.

The fruit surface of *Balanites aegyptiaca* is covered with unicellular non-glandular trichomes of length ranged from 22.16 to 232.97 µm, and it was characterized by the presence of lenticels Figure 3h. The superficial area of the lenticel varied from 54.87 to 112.50 µm^2, and its size was 9.97–14.62 × 6.52–10.13 µm. The epidermal cell size was 20.61–48.02 × 28.83–120.79 µm, and the epidermal cell area ranged from 544.99 µm^2 to 4148.23 µm^2. The epidermal cell outline was elongated rectangular in samples 9 and 14, collected from Egypt, or elongated elliptic in the sample collected from Saudi Arabia, Figure 3i,j, respectively. The anticlinal wall was straight, the relief of cell boundary was channelled, and the curvature of the outer periclinal cell wall was convex, Figure 3k. The

fine relief of the cell wall exhibited scabrate ornamentation. In addition, waxy epicuticular secretions were noticed as secondary sculpture elements on the surface with various densities Figure 3k. The epicuticular secretions were in the form of waxy granules in sample 1 Figure 3l, or non-entire flat crystalloids waxy platelets with sinuate or laciniate margin in samples 9 and 14, Figure 3m,n, respectively.

4. Discussion

Balanites aegyptiaca is a polymorphic woody species with a wide distribution. Previously, many taxa have been described based on the floral characters of the species, which are inconsistent and inconvenient to use alone [2,3,24–28]. In the current study, an integrated taxonomic analysis was performed based on morphological data of the vegetative parts and the fruit; anatomical data of stem, spine, leaflet and petiole and the epidermal surface sculpture of stems, spines, leaflets (abaxial and adaxial surfaces), and fruits using Scanning Electron Microscope (SEM).

The conducted cluster analysis divided the studied samples into two clusters A and B. Both clusters A and B were subsequently divided into two subclusters 1 and 2, based on the petiole length, leaflet length/width ratio, leaflet shape and apex, and leaflet indumentum density. Cluster A included samples characterized by their spine length that exceeded 2 cm, and sparse leaflet indumentum, which is congruent with the description of *B. aegyptiaca* var. *aegyptiaca* reported by Sands [2]. The subcluster A1 had a suborbicular or elliptic leaflet shape with acute or obtuse apex. While the subcluster A2 (contained sample 10 collected from Edfu- Marsa Alam Road, Edfu, Red Sea, Egypt) was characterized by the presence of an orbicular leaflet shape (leaflet length/width ratio = 1.1) with rounded apex, the largest average leaflet area was 7.65 cm^2, and the longest petiole average value was 1 cm. Whereas, members of cluster B had spineless branches or shorter spines that reach up to 1.8 cm, and a moderate or dense leaflet indumentum. The subcluster B1 attained overlapped character states, whereas the subcluster B2 (containing samples 7, 9, and 13) was compatible with the description of *B. aegyptiaca* var. *tomentosa* mentioned by Sands [2].

This study suggests the presence of *B. aegyptiaca* var. *aegyptiaca* in Makkah, Saudi Arabia, while two varieties were identified in Egypt: *B. aegyptiaca* var. *aegyptiaca* and *B. aegyptiaca* var. *tomentosa*. Further confirmation is needed to identify these two varieties using molecular techniques such as molecular phylogeny, DNA barcoding, and whole-genome sequencing.

Author Contributions: Conceptualization, A.E.-B.; methodology, A.E.-B., F.A.H., F.Y.E., W.A.-J., R.R.M. and I.H.N.; validation, A.E.-B., F.Y.E., W.A.-J., R.R.M. and I.H.N.; formal analysis, I.H.N. and F.Y.E.; investigation, F.A.H., I.H.N. and F.Y.E.; resources, F.A.H., W.A.-J. and A.E.-B.; data curation, F.Y.E., I.H.N. and F.A.H.; writing—original draft preparation, I.H.N., W.A.-J., F.Y.E., F.A.H., A.E.-B. and R.R.M.; writing—review and editing, I.H.N., F.Y.E. and A.E.-B.; visualization, A.E.-B., I.H.N. and F.Y.E.; funding acquisition F.A.H., A.E.-B., F.Y.E., W.A.-J., R.R.M. and I.H.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this article.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Collection sites and sampling information of sixteen *Balanites aegyptiaca* samples collected from Egypt (EG) and Saudi Arabia (SA).

ID	Collection Sites	Date	Latitude	Longitude
SA1	South Al Jumum, Makkah, Saudi Arabia	3 April 2021	21°34′27.0″ N	39°41′16.4″ E
SA 2	North Al Jumum, Makkah, Saudi Arabia	5 April 2021	21°35′56.1″ N	39°42′11.3″ E
SA 3	North Al Jumum, Makkah, Saudi Arabia	20 May 2021	21°38′51.5″ N	39°41′06.8″ E
EG4	Wadi el-Gemal National Park, Marsa Alam, Red Sea, Egypt	6 March 2021	24°38′30.88″ N	35°2′25.55″ E
EG 5	Wadi el-Gemal National Park, Marsa Alam, Red Sea, Egypt	6 March 2021	24°37′50.58″ N	35°1′43.186″ E
EG 6	Wadi el-Gemal National Park, Marsa Alam, Red Sea, Egypt	6 March 2021	24°37′37.03″ N	35°1′16.50″ E
EG 7	Wadi el-Gemal National Park, Marsa Alam, Red Sea, Egypt	6 March 2021	24°37′33.68″ N	35°1′11.06″ E
EG 8	Wadi el-Gemal National Park, Marsa Alam, Red Sea, Egypt	6 March 2021	24°41′12.8″ N	35°4′59.64″ E
EG 9	Wadi el-Gemal National Park, Marsa Alam, Red Sea, Egypt	6 March 2021	25°4′48.55″ N	34°53′12.22″ E
EG 10	Marsa Alam, Red Sea, Egypt	8 March 2021	25°04′43.3″ N	34°53′14″ E
EG 11	Edfu- Marsa Alam Road, Edfu, Red Sea, Egypt	8 March 2021	25°1′39.18″ N	33°3′13.79″ E
EG 12	Edfu- Marsa Alam Road, Edfu, Red Sea, Egypt	15 April 2021	25°1′39.40″ N	33°3′14.08″ E
EG 13	Edfu, Aswan, Egypt	15 April 2021	25°1′39.92″ N	33°3′12.54″ E
EG 14	High Dam region, Aswan, Egypt	30 April 2021	23°58′36.69″ N	32°53′50.28″ E
EG 15	High Dam region, Aswan, Egypt	30 April 2021	23°58′41.26″ N	32°53′53.52″ E
EG 16	Aswan University, Aswan, Egypt	30 April 2021	23°59′52.21″ N	32°51′37.07″ E

Table A2. Quantitative traits with descriptive data used for the agglomerative cluster analysis of *Balanites aegyptiaca*.

Variables	Mean ± StDev	SE Mean	Minimum	Q1	Median	Q3	Maximum	IQR
Leaflet length (cm)	2.693 ± 1.22	0.227	0.89	1.927	2.415	3.083	6.4	1.155
Leaflet width (cm)	1.273 ± 0.736	0.137	0.331	0.73	1	1.585	3.55	0.855
Leaflet length/width ratio	2.321 ± 0.625	0.116	1.268	1.839	2.368	2.774	3.636	0.935
Leaflet area (cm^2)	2.864 ± 3.167	0.588	0.21	1.093	2.04	3.045	14.845	1.952
Petiole length (cm)	0.3936 ± 0.2466	0.0458	0.1	0.185	0.3	0.549	0.99	0.364
Petiolule length (cm)	0.377 ± 0.571	0.202	0.06	0.064	0.092	0.71	1.6	0.646
Apex angle	114.77 ± 21.38	4.04	58.5	107.13	117	127	171	19.88
Base angle	59.21 ± 26.36	4.89	26.5	39.25	54.5	69	124.5	29.75
Stipule length (cm)	0.2072 ± 0.0546	0.0223	0.156	0.156	0.2025	0.2545	0.28	0.0985
Interpetiolar stipule (cm)	0.5594 ± 0.1715	0.0767	0.275	0.4015	0.624	0.685	0.685	0.2835
Spine length (cm)	1.711 ± 1.291	0.358	0.42	0.662	1.37	2.457	4.7	1.794
Spinule length (cm)	0.4988 ± 0.2335	0.0953	0.1662	0.3328	0.461	0.7195	0.836	0.3867
Fruit length (cm)	3.0852 ± 0.3596	0.0848	2.438	2.819	3.048	3.4275	3.664	0.6085
Fruit width (cm)	1.751 ± 0.506	0.119	0.997	1.334	1.711	1.924	2.712	0.59
Fruit length/width ratio	1.5792 ± 0.2597	0.0612	1.2142	1.3712	1.5348	1.8121	2.1509	0.4409
Fruit area (cm^2)	4.843 ± 1.88	0.443	2.59	3.521	4.229	6.137	9.49	2.616

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