

Dicliptera aegyptiaca (Acanthaceae), A New Species from Egypt Supported by Morphological Characters and *rbcl*-based DNA Barcoding

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DICLIPTERA *aegyptiaca*, a new species from Red Sea Coast, Egypt, is described and illustrated. Diagnostic and morphological characters that distinguish it from its allied species *D. paniculata* and an identification key for the two species are provided. The new species differs from *D. paniculata* by having an unbranched stem, a congested inflorescence with dwarf axes 1.5–5mm long; subsessile cymules with peduncles 0.5–1mm long. *rbcl*-DNA barcoding is presented for this new taxon for the first time. Phylogenetic tree revealed barcode clusters for the two *Dicliptera* species and recognized significant interspecific variation between them. *D. aegyptiaca* clearly formed one clade strongly supported with a bootstrap value of 100%. Based on characters of morphology, pollen and seeds, the new species was recognized as belonging to the genus *Dicliptera*. On the other hand, DNA barcoding reflected clustering of all *Dicliptera* spp. in a large clade while *D. aegyptiaca* formed a non sister clade showing the utility of DNA barcoding for species identification rather than taxonomy.

Keywords: *Dicliptera aegyptiaca*, DNA barcoding, Egypt, Morphology, New species.

Introduction

The genus *Dicliptera* Juss. comprises about 175 species (Daniel, 2009), distributed in tropical and subtropical countries of Asia, Africa and America. Within Acanthaceae, it is placed in the Diclipterinae clade of the “Justicioid” lineage with *Hypoestes* as a sister to *Peristrophe* (McDade et al., 2000 a). In the absence of fruits, it is difficult to distinguish between *Peristrophe* and *Dicliptera* s. str. (Balkwill, 1996 and Darbyshire & Vollesen, 2007). Based on the mechanism of capsule dehiscence, Balkwill (1996) and Balkwill et al. (1985, 1986, 1996) maintained that the two taxa should remain separate, where the capsule is elastic in *Dicliptera* and inelastic in *Peristrophe*. In their work, Darbyshire & Vollesen (2007) transferred all recognized *Peristrophe* from Tropical East Africa to *Dicliptera* citing inconsistencies of capsule types and the presence of intermediate forms. The most recent molecular data on the “Justicioid” lineage, Kiel et al. (2017) support Darbyshire & Vollesen’s treatment as sampled species of *Dicliptera* and

Peristrophe are together monophyletic.

Dicliptera (inclusive of *Peristrophe*) is characterized by angled stem, opposite leaves, inflorescence panicle-like cymose, with 2–3 inflorescence units (cymules), umbellately arranged, and a resupinate bilabiate corolla. Fruit-capsule clavate, 4-seeded.

In Egypt, the genus *Dicliptera* was represented by only one species *D. paniculata* (*Peristrophe paniculata*), a rare species known only from Gebel Elba and Red Sea Coast of Egypt (Täckholm, 1974; El Hadidi & Fayed, 1994/ 95; Boulos, 2002, 2009; Shamso, 2010, 2013 and El-Gazzar et al., 2015). As part of the revisionary study of the genus *Dicliptera* in Egypt, Shamso (2010) recorded *Dicliptera paniculata* with a wide range of variation in many morphological characters. Most examined specimens represented *D. paniculata*, while some other specimens did not match and were probably an undescribed taxon. Their identities were confirmed by referring to various regional floras

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(Clarke, 1901; Andrews, 1956; Hein, 1966; Abedin et al., 2000; Hedrén, 2006; Ensermu Kelbessa, 2006 and Darbyshire et al., 2010, 2015) and much other relevant literatures (Balkwill, 1996; Balkwill & Getliffe Norris, 1989; Balkwill et al., 1985, 1986, 1988, 1996; Ensermu Kelbessa, 2003; Darbyshire & Vollesen, 2007 and Al Hakimi et al., 2017). The specimens were also compared with herbarium specimens and images available in virtual herbaria [Kew Herbarium Catalogue (<http://apps.kew.org/herbcat/navigator.do>), African Plant Database (www.tropicos.org) and JSTOR Global Plants (<https://plants.jstor.org/>)]. It is suggested that these specimens do not match with any of the described species of *Dicliptera* (incl. *Peristrophe*) and so are described here as a new species.

DNA barcoding provides an accurate, rapid and time-efficient tool for species identification utilizing short DNA sequences as internal species tags (Hebert, 2003 and Hebert & Gregory, 2005). The CBOL plant working group (2009) recommended employing *rbcL* and/or *matK* in barcoding of land plants. Compared to *matK*, *rbcL* has high amplification success rate; it is the most characterized plastid coding region in GenBank (Newmaster et al., 2006 and Kang et al., 2017). *rbcL* was used for identification of cryptic species by several research groups (Miwa et al., 2003; Costea & Stefanovic, 2009; Liu et al., 2013 and Ardiyani et al., 2017).

The aims of this study are to confirm the occurrence of the new taxon *Dicliptera aegyptiaca* using macro- and micro-morphological data and DNA barcodes, to provide the validating description of the new taxon and to provide a key to the Egyptian species of *Dicliptera* for easy recognition of the taxa in Egypt.

Materials and Methods

Plant materials

The present study was based on examination of several specimens belonging to *Dicliptera paniculata* kept in the major Egyptian herbaria [Cairo University Herbarium (CAI), the Agricultural Research Centre, Flora and Phytotaxonomy Herbarium (CAIM), National Research Centre, Plant Systematic Herbarium (CAIRC) and Sohag University Herbarium (SHG)]. The new taxon is accompanied by English description (ICBN, 2018: article 39.2).

Pollen and seeds examination

Samples of pollen and seeds were taken from mature anthers and capsules, respectively. For light microscopy (LM), anthers were boiled for a few minutes in water, macerated in a few drops of an aqueous 10% solution of KOH on a clean slide, then stained with Safranin (1% Safranin solution in 50% ethanol), mounted in glycerin jelly and observations were made with a Sterico research microscope under (E 40, 0.65) using a 16x eye piece, and the seeds were examined with the aid of a dissecting microscope. For SEM studies, both pollen and seeds were mounted onto stubs with double sided adhesive, and then these stubs were sputter-coated with gold (Ion-sputter JFC-1100). After coating, they were examined using JEOL JSM 5400LV scanning electron microscope at 15KV, at the Electron Microscopy Unit, Assiut University. The description of pollen follows the terminology of Hesse et al. (2009) and seed coat terminology follows Barthlott (1981).

DNA barcoding

Each of *Dicliptera paniculata* and the new species *Dicliptera aegyptiaca* were represented by two herbarium specimens. From each specimen, about 20mg were collected and ground under liquid nitrogen using a mortar and pestle until a fine powder was produced. DNA was extracted using a Qiagen DNeasy kit (Valencia, California, USA) as outlined by manufacturer's protocol with few modifications for herbarium tissues. To the AP1 buffer DDT (Melford Laboratories, UK) at 0.12mg/ml and Proteinase K (Sigma) at 0.04mg/ml were added and shaken with ground tissue for 60min at 65°C (de Vere et al., 2012). *rbcL* amplifications were performed following CBOL Plant Working Group (2009) with the specific primers: 5'-ATGTCACCACAAACAGAAAC-3' and 5'-TCGCATGTACCTGCAGTAGC-3' in a reaction mixture containing 25 µl PCR Master Mix (Bioline), 1µl of each primer and 20-50ng genomic DNA; the volume was completed to 50µl with sterile distilled water. The amplification protocol was 95°C for 2min followed by 34 cycles of 94°C for 1min, 55°C for 30sec and 72°C for 1min, then final extension for 7min at 72°C. Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) then sequenced with Big-dye terminator chemistry in 3130xl Genetic Analyzer (Life Technologies, California, USA) by following the standard manufacturer's protocol. Forward and reverse sequences were assembled using Codon Code

Aligner software, v. 7.1.2., the contig sequences were deposited in the GenBank database under accession numbers MH028051 and MG1990431.1 for *Dicliptera paniculata* and KU947958.1 and KU947961.1 for *Dicliptera aegyptiaca*. The available online sequences for *Hypoestes*, *Peristrophe* and *Dicliptera* in Gene bank were employed to construct a phylogenetic tree and calculate pairwise distances using Maximum Likelihood (ML) method and Cluster W in MEGA v. 6 (Tamura et al., 2013) based on Tamura 3 parameter model (Tamura, 1992) with gamma distribution. Significance was assessed using 1000 bootstrap replications (Felsenstein, 1985).

Results and Discussion

Dicliptera aegyptiaca E. Shamso sp. nov.

Type: Egypt: Wadi El Faraied, Red Sea coast 23°31'0"N; 35°19'60"E; 12/2/1961; Täckholm et al. 856 (holotype CAI).

Annual herb, 20–30cm tall, unbranched, rarely with short lateral branches above. *Stem* angled, glabrous, sparsely strigose with appressed eglandular multicellular hairs on angles and nodes, cystolith present. *Leaves* rapidly deciduous at lower nodes, blackish-green when dry. Leaf lamina narrowly ovate to lanceolate 15–45x 9–15mm, attenuate at base, acuminate apex, minutely glandular pubescent, strigose with eglandular multicellular hairs at nerves and margins. *Petiole* 2–6mm long, strigose (Fig. 1 A). *Inflorescence* congested in the axils with dwarf axes 1.5–5mm long, inflorescence of (1–) 2–3 cymules (inflorescence units), umbellately arranged, often compound, with many umbels in each axil. *Inflorescence bracts* (secondary bracts) 2, subequal, subulate, 3–5x 0.3–0.5mm, sparsely hairy, hyaline margin at lower half, apex acuminate. *Cymule* subsessile, the longest peduncle ranges from 0.5–1mm long, subtended by 2-unequal tertiary bracts, the larger one 8–13x 0.8–1mm, linear-lanceolate, apex sharply acuminate, the shorter 6–9x 0.8–1mm, linear-lanceolate, acuminate apex, pubescent with glandular and sparsely eglandular hairs and hyaline margin at lower half. Each cymule composed of 2–(–3) flowers with 2-bracteoles each, maturing sequentially. *Bracteoles* as bracts, 5–6x 0.5mm (Fig. 1 B). *Calyx* whitish green, sparsely hairy outside, tube 0.5–1mm long, lobes lanceolate, 3–4x 0.5mm attenuated to sharp acuminate apex and ciliate margin. *Corolla* bilabiate, resupinate 8–8.5mm long, densely

eglandular hairy outside, glabrous inside, tube 3–3.5mm long, lower lip tri-fid 4–5x 2.5mm, upper lip retuse to acute 5–5.5x 3mm (Fig. 1 C). *Stamens* 2, epipetalous, filaments 3–5mm long, sparsely hairy, anther-thecae superposed, 0.5x 0.3mm. *Pollen grains* prolate, medium-sized, with mean polar axis ranges from 32.1–35.8µm, mean equatorial diameter ranges from 20–21.9µm, circular-triangular in polar view, heterocolpate (tricolporate with paired pseudocolpi parallel to each colporus), both colpori and pseudocolpi of about equal length (30.7–34.6µm), colpal membrane extremely fine reticulate, endoapertures raised circular; exine sculpture foveolate to micro-reticulate (Fig. 2). *Ovary* oblong 1.5–2x 0.5mm, surrounded by a cupular disc at base, attenuate at apex, eglandular hairy; style filiform, 6–7mm long, glabrous, stigma bi-fid. *Capsule* 4-seeded, elliptic, 6–7x 2mm, densely retrorse eglandular multicellular hairs, with a short rostrum (sterile portion) 1.5–2mm long. *Seeds* discoid, 2x 2–2.2mm, notched at central white hilum, seed surface variously convoluted, indistinct reticulation, covered with minute papillae and tubercles. Tubercles dense around the edges of the seed, with sharp pointed hooks in one or two rows (Fig. 3).

Distribution: Confined to the southern coastal plain of the Red Sea of Egypt.

Specimens examined: Wadi El Faraied, Red Sea coast 23°31'0"N; 35°19'60"E; 12/2/1961; Täckholm et al. 856 (holotype CAI) – Gebel Hamata, Red Sea coast, 7/2/1961; Täckholm et al. 351(CAI).

Habitat: Very rare in sandy and silty moist soils.

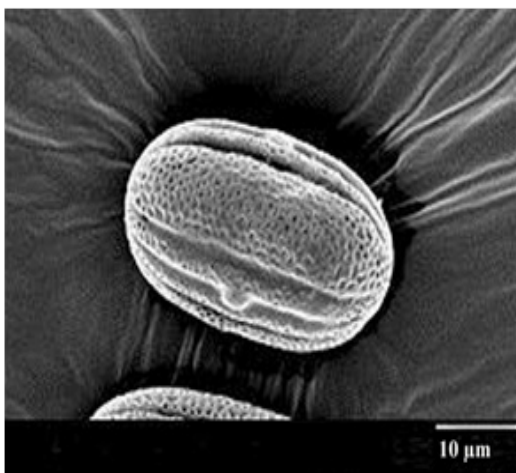
Etymology: the specific epithet *aegyptiaca* refers to Egypt where it was first discovered.

Recognition: *Dicliptera aegyptiaca* closely resembles *D. paniculata* but differs in its stem unbranched, rarely with short lateral branches above (vs basely and laterally branched), inflorescence congested at the nodes with dwarf axes 1.5–5mm long, composed of (1–) 2–3 cymules, cymule subsessile (vs inflorescence lax panicle-like, opposite at each nodes, with long axes (20–) 30–50mm long, composed of 3–4 cymules, cymule pedunculate). Pollen dimension 32.1–35.8x 20–21.9µm, foveolate to micro-reticulate sculpture (vs pollen dimension 40x 25–30µm, reticulate sculpture). (Fig. 1 and 2).



Fig. 1. *Dicliptera aegyptiaca* E. Shams (Täckholm et al. 856); A: Image of specimen showing habit, B: congested inflorescence and cymule, C: Rusupinate corolla.

A



B

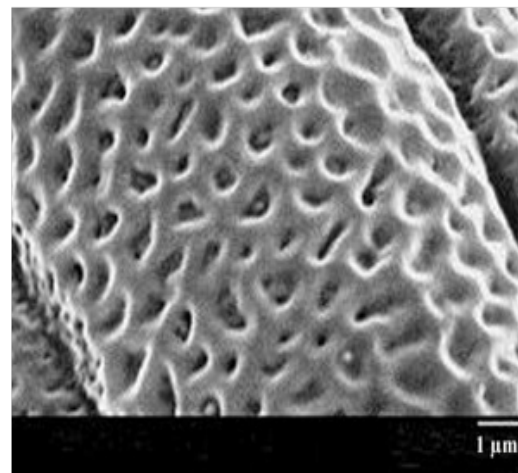


Fig. 2. SEM micrographs of pollen grains of: *Dicliptera aegyptiaca*; A: Whole grain, equatorial view, B: Exine sculpture.

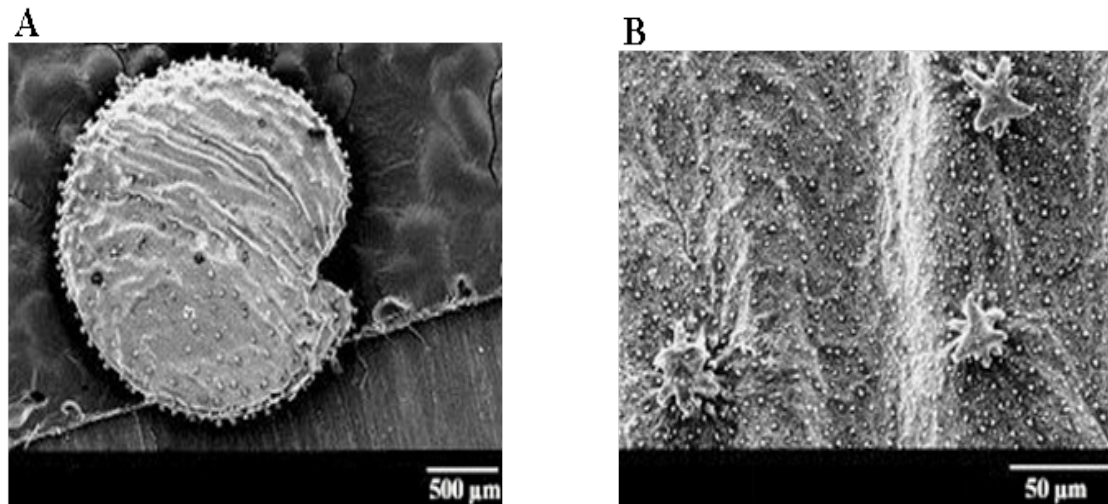


Fig. 3. SEM micrographs of seeds of *Dicliptera aegyptiaca*; A: Whole seed, B: Testa sculpture.

rbcl-based DNA barcoding provided additional support for *Dicliptera aegyptiaca* that appeared in the phylogenetic tree (Fig. 4), the tree revealed considerable interspecific variation between *Dicliptera aegyptiaca* and *D. paniculata*. The new species *D. aegyptiaca* clearly formed one clade strongly supported with a bootstrap value of 100% while *D. paniculata* along with other *Dicliptera* spp. formed a non-sister large

clade. DNA sequences therefore appear to help recognize groups of individual specimens and are useful for species recognition. The paraphyly of *Dicliptera* observed in this investigation was also recognized in other Acanthaceae genera including *Acanthus* using ITS sequences (McDade et al., 2000 b) and *Justicia* using ITS as well as plastid trnL-K, trnS-G and rps16 (Deng et al., 2016).

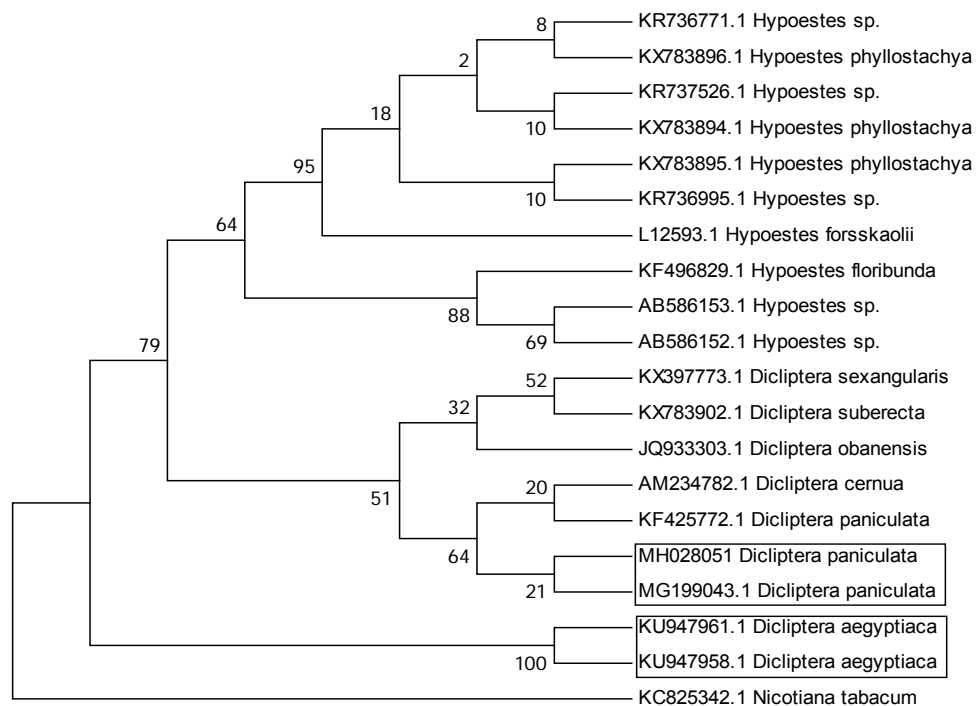


Fig. 4. Phylogenetic tree using Maximum Likelihood method based on Tamura 3 parameter model for rbcl sequences of *Hypoestes*, *Dicliptera* and *Peristrophe* spp. Bootstrap values based on 1000 replications are listed as percentages at branching points.

Key to the Egyptian species of Dicliptera

1- Stem unbranched, rarely with short lateral branches above. Inflorescence congested at the nodes with short axes, 1.5– 5mm long. Cymule sessile, peduncles 0.5– 1mm long, the larger cymule-bract 8– 13x 0.8 – 1mm, linear-lanceolate, apex sharply acuminate.....***D. aegyptiaca***

- Stem basely and laterally branched. Inflorescence in lax panicle- like, with axes (20–) 30– 50mm long. Cymule pedunculate, peduncles 10– 30mm long, the larger cymule-bract 12– 15x 1mm, oblong -linear, apex sharply acute...
.....***D. paniculata***

Conclusion

Results of the present investigation reflect a harmony between morphological features and DNA barcoding in delimiting *D. aegyptiaca* as a new species. On the other hand, appearance of *D. aegyptiaca* as an outgroup for a large clade containing taxa of *Dicliptera* and *Hypoestes* supports validity of DNA barcoding in species identification rather than taxonomy. After the introduction of this new species to science, the number of *Dicliptera* species in Egypt is raised to two.

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***Dicliptera aegyptiaca* (العائلة الأكانثية)، نوع جديد من مصر مدعوماً بالصفات المورفولوجية وتقنية الباركود للأحماض النووية باستخدام جين rbcL**

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أسفرت الدراسة عن وجود *Dicliptera aegyptiaca* كنوع جديد بمنطقة ساحل البحر الأحمر بمصر وقد تناول البحث دراسة الصفات المورفولوجية والصفات التمييزية بينه وبين النوع القريب له *Dicliptera paniculata* مع عمل مفتاح تعريفى لهما وتم عمل باركود للنوع الجديد لأول مرة باستخدام الشفرة الوراثية للجين rbcL DNA. يتميز النوع *Dicliptera aegyptiaca* بنبات ذو ساق غير متفرع ونورة ذات محور منقزم 1,5-5 مللي طول ونويرة محدودة ذات شمراخ قصير 0,5-1 مللي طول. أظهرت الشجرة التطورية للشفرة الوراثية لهذين النوعين وجود فروق بينهما وهذا اتضح من ظهور *Dicliptera aegyptiaca* كفرع منفرد مدعوماً بتكررات تأكيد بقيمة 100%. وقد أظهرت الدراسة المورفولوجية للنبات ولحبوب اللقاح و البذور إنتماء النبات الجديد لجنس *Dicliptera* بينما أظهرت الشجرة التطورية للنبات الجديد فى فرع آخر غير المخصص لباقي أنواع الجنس مما يظهر فائدة تقنية الباركود فى تعريف الأنواع أكثر من إيجاد العلاقات التصنيفية بينها.