



DNA barcoding, pollen and seed characteristics for documentation of four monocot Mediterranean endemic plant species in Egypt

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Abstract

Endemic taxa are critical constituents in the flora of most parts of the world. Recently, most of these taxa have become exposed to extinction. This study aims to increase understanding of four monocot Mediterranean threatened endemic taxa in Egypt (*Allium mareoticum* Bornm. & Gauba, *Bromus aegyptiacus* Tausch, *Muscari salah-eidii* (Täckh. & Boulos) Hosni, *Pancratium arabicum* Sickenb.) by description of their pollen and seed and authentication of DNA barcoding using *rbcL* and *matK* genes. Twenty field visits were conducted from March 2017 till April 2023 including different localities through Mediterranean area for covering the presence of these species. Characterization of pollen grains and seeds were examined using a light (LM) and scanning electron microscopes (SEM). DNA barcoding was carried out for documentation of the four species. Maturase K (*matK*) and ribulose-bisphosphate carboxylase (*rbcL*) were used as primers for PCR amplification and gene sequencing. The two genes were successful in identifying *Allium mareoticum*, *Bromus aegyptiacus*, and *Muscari salah-eidii* at the genus level and *Pancratium arabicum* at both species and genus levels for *rbcL* marker, and the genus level for *matK* marker. This study is considered a leading study for new barcodes of (*A. mareoticum*, (*B. aegyptiacus*, and *M. salah-eidii* which were initially made available to the NCBI.

Keywords Endemic plants · Egyptian flora · DNA barcoding · *rbcL* · *matK* · Pollen grains · Seeds

Introduction

An endemic species is that restricted to a particular geographic region due to factors such as isolation, or in response to abiotic conditions. Endemism information is critical for balancing conservation strategies (Lima et al. 2020). Because endemic taxa have a more restricted geographical range, they are used as surrogates to determine conservation priorities (Mehrabian et al. 2020). El-Khalafy et al. (2021a) recorded 41 endemics in Egypt, belonging 20 families and 36 genera including five monocot taxa. Medicinal plant use,

over-collecting, over-grazing, urbanization, and quarrying have resulted in low vegetation percentage and extinction of species in most areas of Egypt, because of excessive human activities (Hughes and Eastwood 2006).

Although the current extinction rates are nearly determined, extinction determination remains critically important for improving the accuracy of prediction and estimation (Ladel 2019). Lack of knowledge about taxon distribution is obvious all over the world (Meyer et al. 2016; Sporbert et al. 2019), because of bias in collection of species, inappropriate sampling techniques, and lack of research resources and facilities (Ceballos et al. 2015).

Endemic taxa play an important role in the composition of many countries' flora. These taxa become threatened with extinction in recent years (El-Khalafy et al. 2021b). El-Khalafy et al. (2024) assessed the four studied taxa as threatened species depending on IUCN criteria, *Bromus aegyptiacus* has been designated as critically endangered, *Allium mareoticum*, *Pancratium arabicum* as endangered, while *Muscari salah-eidii* was thought to be extinct. *Allium mareoticum* is bulbous geophyte which is oblong-ovoid. The

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stem length ranges from 12 to 25 cm. Leaves are 1–2 mm wide, linear filiform, shorter than the inflorescence. Inflorescence is umbel, globose and dense. Perianth segments are white with a pinkish or green mid-vein. It inhabited sandy and rocky places on calcareous hills in Mediterranean coastal land of Egypt. *Bromus aegyptiacus* is annual herb that reaches 40 cm in length. The branches are ascending and usually shorter than the spikelets. The lower glumes are lanceolate, 3 nerved and ciliate at the margins. The upper glumes are elliptic-ovate, 7 nerved and ciliate at the margins. Awn is subapical, straight, terete or triquetrous. It inhabited cultivated areas as a weed (Boulos 2009). *Muscari salah-eidii* is perennial bulb. Its length ranges from 30 to 40 cm. Leaves are usually solitary, swollen and fleshy in the upper part. The inflorescence is raceme with many flowers which are cylindrical, blue with green-veined perianth tube and black perianth-teeth. It inhabited sandy soil in Rafah (Boulos 2009). *Pancratium arabicum* is perennial bulb. Leaves have conspicuous papery membranous sheath at the base. Flowers are 6–11 per umbel. It inhabited coastal sand dunes (Boulos 2009).

It is crucial to document endemic species using molecular features. Afiah et al. (2014) used the RAPD-PCR technique to identify 14 endemic plants from Saint Katherine Protectorate, Sinai by their genetic fingerprints. Distance analysis of DNA sequences represents another excellent tool to compare closely related species. El-Banhawy et al. (2020a) demonstrated the utility of using DNA barcoding to accurately identify and place the genus *Euphorbia* in Egypt. DNA barcoding is considered an accurate, rapid, cost-effective, and time-efficient tool in many biodiversity studies for species identification (Hebert and Gregory 2005; Hollingsworth 2008; Hosein et al. 2017). It contributes significantly to growing efforts to preserve biodiversity (Von Crautlein et al. 2011), detection of new taxa (Bell et al. 2012), use in large taxonomic groups (Ojeda et al. 2014) and phylogenetic trees of plant communities (Joly et al. 2014), and species conservation (Yesson et al. 2011). Also, the finding of cryptic species has multiplied quickly and seems to be a major contributor to the increase in DNA barcode study publications (De Salle and Goldstein 2019). Vijayan and Tsou (2010) stated that *matK* and *rbcL* two-locus standard barcodes (chloroplast genes), had been proposed for use in identifying plant species. *rbcL* has a higher amplification success rate than *matK* and is the most well-characterized plastid coding region in GenBank (Kang et al. 2017). *rbcL* is commonly used to document endangered plant species (Alaklabi et al. 2014; Rajaram et al. 2019), endemic species (Amandita et al. 2019; Fouad et al. 2022; Shamsou and Fouad 2019), and species richness (Amandita et al. 2019; Worthy et al. 2019).

Palynology is a fascinating science with a multidimensional approach that covers almost all branches of botanical science and is regarded as a useful tool in taxonomic and evolutionary studies. Many successful trials have taken pollen characters as a basis for classification; it is an important morphological parameter for the identification of plants (Erdtman 1952; Takhtajan 1980; Burçak 2022). Pollen morphology helped botanists to reconstruct the past assemblages of plants and identify periods of environmental change (Yao et al. 2017). Seed morphology is also considered a powerful tool for the classification of various plant groups. The seed morphology was significant and useful in understanding the taxonomic relationship between species of plants (Barthlott 1981; Kanwal et al. 2012; El-Ghamery et al. 2018; El-Khalafy et al. 2021c).

Documentation and conservation of endemics are essential, so the current research sought to increase understanding of four monocot endemic taxa (*Allium mareoticum*, *Bromus aegyptiacus*, *Muscari salah-eidii* and *Pancratium arabicum*) by examination of pollen and seed morphology of these taxa using LM and SEM and authentication of DNA barcoding using *rbcL* and *matK* genes.

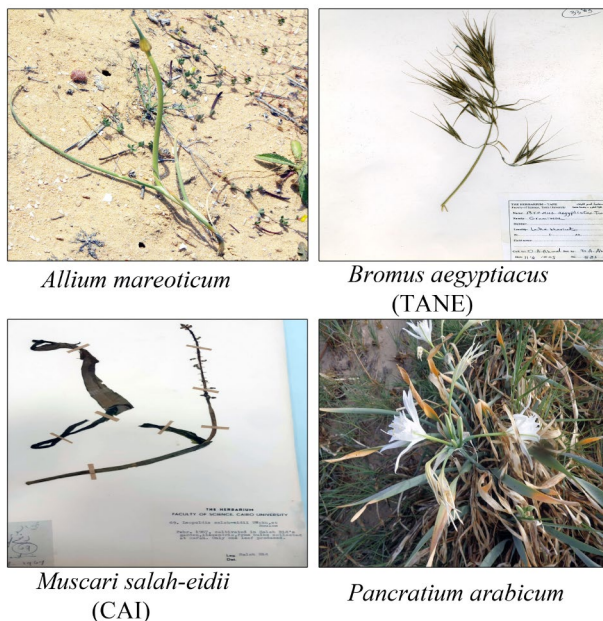
Materials and methods

Field trips and sample collection

Field visits have been investigated between March 2017 and April 2023 including different areas in Mediterranean and Nile sectors during spring season (e.g. Alexandria, Sidi Abdel-Rahman, El-Dabaa, El-Zaytona, Ras El-Hekma, Matrouh, Omayed, Tanta, Kafrelsheikh, Cairo, El-Fayoum, El-Sharqia, Menofia, Qalyobia, Baltim, Gamasa, and Rosetta). These trips aimed to record the presence of the studied species in its natural habitats, for recording their coordinates and threats. In addition to review most studies related to research area, scientific literatures, and visiting of different Egyptian herbaria (e.g., Cairo University Herbarium (CAI), Tanat University Herbarium (TANE), Alexandria University Herbarium (ALEX) and Agriculture Museum Herbarium (CAIM). In each location, specimens of the taxa were collected from different sites. In addition, flower and seed samples representing the recorded taxa were collected. Using ArcGIS 10.4.1, the distribution of each species was designed in a grid map depending on different locations. Identification and synonyms followed Täckholm (1974), Boulos (1999–2005, 2009) and updated names follow the website of Plants of the World Online (<https://powo.science.kew.org/>). The scientific names of the four endemic species, along with their families, life forms, distribution, their IUCN status, as well as their photos are

Table 1 List of the studied endemic species with their life forms, arabic names, families, and their IUCN status

Plant species	Life form	Arabic name	Family	IUCN status
<i>Allium mareoticum</i> Bornm. & Gauba	Geophyte-helophyte	Toom-Basal	Amaryllidaceae	EN B1ab (i, iii, v) + 2ab (i, iii, v); CR C2a(i)
<i>Bromus aegyptiacus</i> Tausch = <i>Bromus japonicus</i> var. <i>aegyptiacus</i> (Tausch) Asch., Schweinf. & Muschl. <i>Bromus patulus</i> var. <i>aegyptiacus</i> (Tausch) Asch. & Schweinf.	Therophyte	Abo Kantila	Poaceae	CR B1ab (i, ii, iii, iv, v); C2a(i)
<i>Muscari salah-eidii</i> (Täckh. & Boulos) Hosni = <i>Leopoldia salah-eidii</i> Täckh. & Boulos	Geophyte-helophyte		Asparagaceae	Extinct
<i>Pancratium arabicum</i> Sickenb.	Geophyte-helophyte	Sawsan Bosil	Amaryllidaceae	EN B1ab (i, ii, iii, iv, v) + 2ab (i, ii, iii, iv, v); C2a(i)

**Fig. 1** The four monocot endemic plants in the present investigation. TANE: Tanta University Herbarium and CAI: Cairo University Herbarium

shown in Tables 1 and Fig. 1. Three distinct species were gathered from their respective habitats (*Allium mareoticum*, *Bromus aegyptiacus*, *Pancratium arabicum*), while *Muscari salah-eidii* was examined at CAI due to its rarity in its own habitat.

Pollen and seed characteristics

Pollen grain description

The target species' flowers were collected and fixed in 70% alcohol. Mature anthers were allowed to dry before being carefully opened with sharp needles and sputtered onto glass slides for LM analysis. Pollen grains (five to ten pollen grains per species) were examined, measured and photographed using Canon power-shot A470, 7.1 mega

pixels. The polar axis (P) and equatorial diameter (E) were measured, pollen shape, size and aperture type were also assessed. For SEM analysis, non-acetalized pollens were transferred onto a stub and covered with a thin small of gold in a sputtering chamber, then photographed using JEOL JSM-IT100 SEM (Institute of Nanoscience and Nanotechnology, Kafrelsheikh University). The used descriptions followed Erdtman (1952), Punt et al. (2007) and Hesse et al. (2009).

Seed description

Mature seeds (three to five seeds) were examined using stereomicroscope and photographed by a digital camera (Canon power-shot A470, 7.1 mega pixels). Stage micrometer and image J software was employed for measurements and calibration. Seed color, width, length, and hilum position were examined by stereomicroscope. The mature seeds were mounted onto SEM stubs, covered with gold and photographed using a JEOL JSM-IT100 SEM. The used terminology followed Barthlott (1981) and Stearn (1992).

Energy descriptive X-ray technology (EDX)

The non-acetolyzed pollens and mature seeds were examined using a JEOL JSM-IT100 scanning electron microscope with an EDX detector at the Institute of Nanoscience and Nanotechnology, Kafrelsheikh University, coated with a very thin film of gold. The beam was focused on pollen and seed at 20 KV and analyzed for approximately 20 s and the machine dead time was not included. The count rate per second (CPS) reached 1845.

DNA barcoding

Sample collection and genomic isolation and purification

After collection, fresh leaves (0.1 gm) were put in silica gel beads for lab analysis for preserving leaf material in the field

for future DNA extraction. In case of *Muscari salah-eidii*, this study depends on herbarium specimen (0.2 gm) from Cairo University herbarium (CAI), because it is believed to be extinct in the wild. Total genomic DNA was extracted and purified from leaves through GeneJET Genomic DNA purification Mini Kit (Thermo Scientific) according to the manufacturer's protocol. The extracted DNA concentration and quality were estimated by running on 1% agarose gel electrophoresis, using a DNA molecular weight ladder 1k bp DNA marker (PeqGold 1k b, Peqlab, GMH). The method for DNA quality confirmation has been by preparing a 1% solution of agarose, allow to cool for 2 min, then add 2.5 µl of ethidium bromide, Cast a jel using a supplied tray and comb, run the gel for 30 min. at 100 V, expose the gel to UV light and photograph, and finally confirm DNA quality by the presence of a highly resolved high molecular weight band indicates good quality DNA, while the presence of a smeared band indicates degradation.

PCR amplification and gene sequencing

Green taq (DreamTaq) master mix (Fidelity: 1 X) (Thermo Scientific) was employed for gene amplification depending on the manufacture protocol applying a thermal cycler (Creacon, Holand). Two locus standard barcodes, *matK* and *rbcL* chloroplast genes were used as primers (Table 2). The thermal cycler consisted of denaturing step at 94 °C for 2 min followed by 35 cycles at 94 °C for 1 min, an annealing step at 48 °C for 1 min and 30 s, and an extension step at 72 °C for 1 min and 30 s; the final extension step took 10 min at 72 °C. PCR products were loaded onto a 1.5% (w/v) agarose gel, stained with ethidium bromide, electrophoresized (75 V, 150 mA), and viewed on a UV plate. For DNA purification, the Gene JET PCR Purification Kit (Thermo Scientific) was used. ABI PRISM® 3100 Genetic Analyzer was applied for PCR products and performed by MacroGen In. Seoul, Korea (<https://www.macrogen.com/>). The GeneJET method has been carried out as follows: add a 1:1 volume of Binding Buffer to completed PCR mixture, if the DNA fragment is ≤ 500 bp, add a 1:2 volume of 100% isopropanol, centrifuge for 30–60 s, add 700 µL of Wash Buffer to the GeneJET purification column, centrifuge for

30–60 s, discard the flow-through and return the purification column to the collection tube, centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any remaining wash buffer, place the GeneJET purification column in a clean 1.5-mL microcentrifuge tube, add 50 µL Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min, and finally, discard the GeneJET purification column and store the purified DNA at -20 °C.

All sequences were submitted to the GenBank with accession numbers: *rbcL*; *Allium mareoticum* (OP562091); *Bromus aegyptiacus* (OP562092); *Muscari salah-eidii* (OP562093); *Panocratium arabicum* (OP562094) and *matK*; *A. mareoticum* (OP562017); *B. aegyptiacus* (OP562018); *M. sala-eidii* (OP562019); and *P. arabicum* (OP562020).

Data analysis

Totallab analysis software (www.totallab.com, Ver.1.0.1) was used to analyze data from a gel documentation system (Geldoc-it, UVP, England). Individual sequences were aligned against the NCBI Gene bank nucleotide database to confirm their identity, using the Online NCBI blast (<https://blast.ncbi.nlm.nih.gov/>). The Genetic distances and Multi-alignments were computed by the Pairwise Distance method using ClusteralW software analysis (www.Clus-teralW.com). Nucleotide sequences were also compared with the most similar at NCBI (<https://www.ncbi.nlm.nih.gov/>). Mega x was employed for creating phylogenetic trees according to blast results (Kumar et al. 2018). Phylogenetic tree was constructed depending on construct/Test UPGMA tree. The phylogeny test was Bootstrap method, and the substitution method was Maximum Composite Likelihood.

Results

Pollen and seed description

Pollen morphological characters (LM & SEM) were illustrated in Table 3 and some of the specific structures (microphotographs) were arranged and illustrated in Fig. 2. The

Table 2 Primers of DNA barcoding used in PCR amplification of the four studied endemic species

No	Species	Primer	
		<i>rbcL</i>	<i>matK</i>
1	<i>Allium mareoticum</i>	5-ATTCGACTACGCCGGAGCGGCGGAC-3 3-TGCGAGCGACGAGCACTCGGGCGC-5	5-CCAGGCATCGACGACGCGA-3 3-TTTCGAGCGCGACGGGCAG-5
2	<i>Bromus aegyptiacus</i>	5-AGCGATCAGCGCGCGGGGTAAC-3 3-AACGACTCGCGGATTACGACGCG-5	5-AATTTACGATCHATTCAATTCMATWTTTCC-3 R-3-AGTTYTARCACAAGAAAGTCGAARTATATA-5
3	<i>Muscari salah-eidii</i>	5-TCGCATGTACCYGCAGTTGC-3 3GATTTGCGCTGTTTCGGCTTGCTTTATAAA5	5- GAACTAGTCGGATGGAGTAG-3 3-ACTGTATCGCACTATGTATCA-5
4	<i>Panocratium arabicum</i>	5-AGCGATCAGCGCGCGCATGGCG-3 3-ACGTTTCGGACGGGGGACGGCGGA-5	5-GTACTAGCGACGCGCAAGGG-3 3-AACAGCGACTACGACTTCAG-5

polar length of the pollen grains ranged from 37.52 to 51.2 μm , while the equatorial width ranged from 25.5 to 47.95 μm . All pollen grains have a large size category and heteropolar. The shape class ranges from Perolate to prolate. Regarding the aperture, monosulcate shape is the most represented, and operculum and margo are absent in all taxa. Three different exine sculptures were described but reticulate type is the most represented.

The seed characters (LM & SEM) were illustrated in Table 4; Fig. 3. The color of the seeds ranges from light brown to black. Four shapes of seeds were described: broad ovate; lanceolate; ovate; and reniform to elliptic for *Allium mareoticum*, *Bromus aegyptiacus*, *Muscari salah-eidii*, *Pancratium arabicum*, respectively. The hilum is basal and raised in all taxa. Four different types of surface sculpture patterns were described: reticulate-verrucate; striate; ruminate and reticulate. The shape of the epidermal cell was irregular, except in *Pancratium arabicum* which was polygonal. The anticlinal and periclinal walls have been described also in Table 4.

Energy descriptive X-ray technology (EDX) for pollen and seeds

The mineral composition of pollens and seeds of the examined taxa indicated that 12 elements were represented (Table 5). The results revealed that pollen and seeds contain high amount of mineral elements. Mineral composition and relationship among them indicate the predominant minerals were Carbon (C) and Oxygen, followed by Magnesium (Mg), Potassium (K) and Calcium (Ca). On the other hand, Nitrogen (N), Silicon (Si) and Sodium (Na) are less represented (Table 5).

Molecular phylogeny samples identification

In *Allium mareoticum*, the *rbcL* and *matK* genes amplified via PCR resulted in approximately 1370 and 794 bp fragments, respectively; 800 and 606 bp fragments respectively in *Bromus aegyptiacus*; 1273 and 1245 bp fragments respectively in *Muscari salah-eidii*; and 1040 and 800 bp fragments respectively in *Pancratium arabicum* (Fig. 4).

Species identification depending on the two genes revealed that both genes were successful in identifying *Allium mareoticum*, *Bromus aegyptiacus*, and *Muscari salah-eidii* at the genus level, because of the publishing of these species with the two markers at the first time on databases. *Pancratium arabicum* was identified at both species and genus levels for *rbcL* marker, while the genus level for *matK* marker (Table 6).

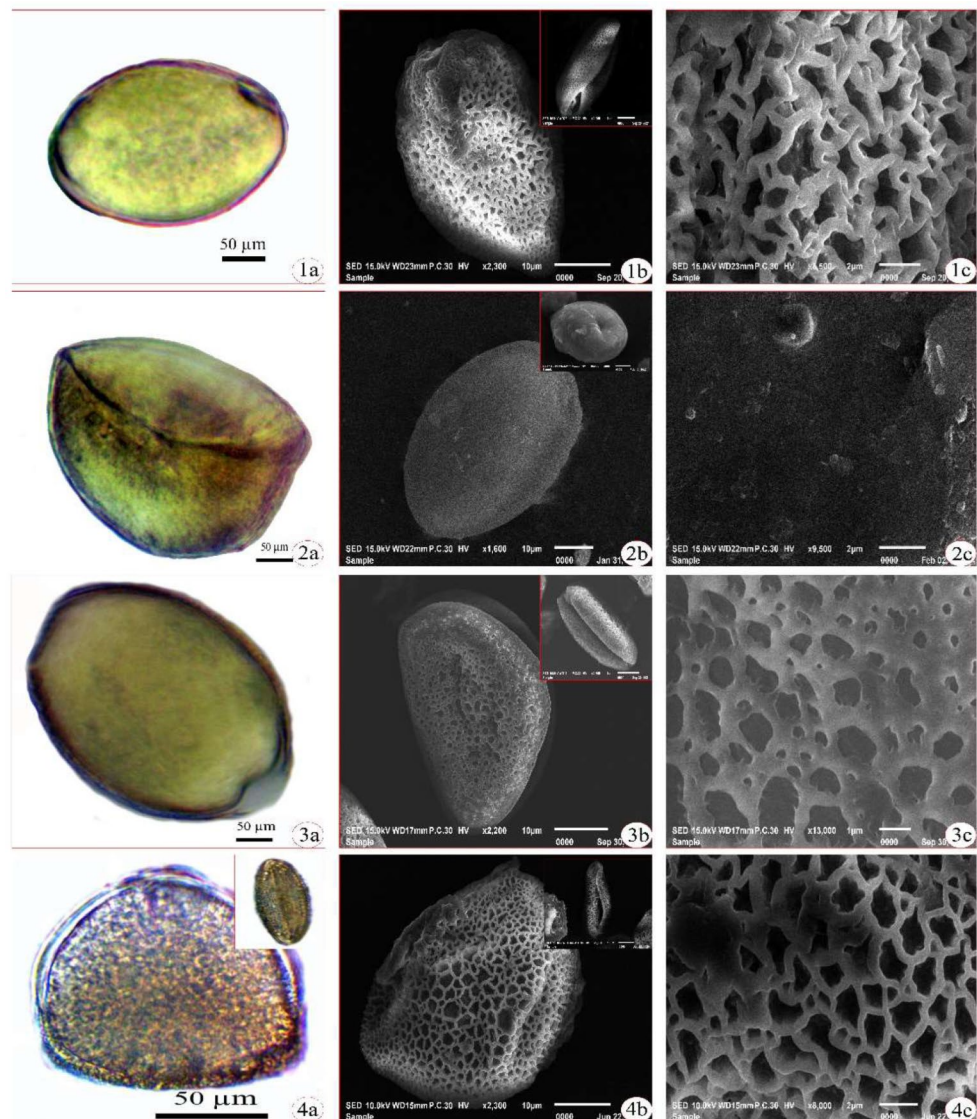
The amplified DNA was purified, sequenced, and analyzed using the online database (NCBI), then compared with

Table 3 Micro-morphological characteristics of pollen grains (LM & SEM)

Attributes Taxa	Dimension (μm)		Pollen size (P + E / 2)	Pollen shape		Polarity	Aperture Shape	Number	Exine sculpture			Tectum	Annulus	Col- lumella visibil- ity
	Polar = length (P)	Equatorial = width (E)		Amb (LM)	Shape class (P/E X 100)				Operculum	Margo				
Allium	40.765– 42.665 (41.715)	29.169– 30.121 (29.645)	Large	Elliptic	Prolate	Hetero- polar	Monosulcate (slit- like)	1	Absent	Absent	Reticulate	Indistinct	Absent	Distinct
Bromus	50.927– 51.526 (51.226)	34.828– 35.142 (34.985)												
Muscari	37.181– 37.984 (37.582)	24.916– 26.123 (25.519)	Large	Elliptic	Prolate	Heteropolar	Monosulcate (slit- like)	1	Absent	Absent	Reticu- late-fove- olate	Semi- tectate	Absent	Indis- tinct
Pancratium	49.946– 50.532 (50.239)	47.562– 48.345 (47.953)												

E = equatorial diameter or width; P = polar axis or length, LM: Light microscope, SEM: Scanning electron microscope

Fig. 2 (1–4). Micro-photographs of pollen grains of the studied taxa (LM & SEM), a: LM, b and c: SEM. **(1)** *Allium mareoticum*; prolate, monosulcate, semi-ectate, reticulate sculpture, **(2)** *Bromus aegyptiacus*; prolate, monoporate, ectate, areolate sculpture, **(3)** *Muscari salah-eidii*; prolate, monosulcate, semi-ectate, reticulate-foveolate sculpture, **(4)** *Pancratium arabicum*; peroblate, monosulcate, ectate, reticulate sculpture



other related species. Sequencing for *rbcL* and *matK* regions of *Allium mareoticum*, *Bromus aegyptiacus*, *Muscari salah-eidii*, and *Pancratium arabicum* resulted in approximately: (1370, 794); (800, 606); (1273, 1245); and (1040, 800) bp sequences (affected length of the query), respectively. The highest similarity percentages between *Allium mareoticum* and the other recorded *Allium* species reached 100% for *rbcL* with *Allium roseum* which has accession number KF997289.1, and 100% for *matK* with *Allium strictum* which has accession number JN864817.1 (Table 6). The two tested markers support the inclusion of *A. mareoticum* in the genus *Allium* and the most closely related species are *Allium roseum* using *rbcL* and *Allium strictum* using *matK* (Fig. 5a, b) which are illustrated in the phylogeny trees constructed by NCBI BLAST.

According to NCBI GenBank, alignment of *rbcL* sequence of *Bromus aegyptiacus* with other related species, revealed

98.98% similarity (more similar) with *Bromus hordeaceus* which has accession number AY395531.1, *Bromus japonicus* (MN185242.1), *Bromus inermis* (HQ600445.1), *Bromus commutatus* (MG225974.1) and *Bromus arvensis* (AY836154.1). Also, *matK* sequencing result revealed 99.4% similarity with *Bromus hordeaceus* which has accession number AY395531.1, *Bromus secalinus* (JN895869.1) and *Bromus japonicus* (MF597222.1). So, the two markers confirm the involvement of *Bromus aegyptiacus* in genus *Bromus* and the most related taxa are similar for the two markers which represent *Bromus hordeaceus* and *Bromus japonicus* (Fig. 6a, b).

Alignment of *rbcL* sequencing of *Muscari salah-eidii* with other related species recorded 99.8% similarity (more similar) with *Muscari longipes* (*Leopoldia longipes*) with accession number JX090455.1 and 99.6% with *Muscari inconstictum* (JX090474.1) and *Muscari chalusicum*

Table 4 Micro-morphological characters of the seeds (LM & SE)

Attributes Taxa	Color	Shape	Dimension (L x W) mm	Hilum	Surface sculpture pattern	Epidermal cell shape	Anticlininal wall		Periclininal wall				
				Position			Level	Elevation	Width	Shape	Surface sculpture	Elevation	Surface sculpture
<i>Allium</i>	Black	Broad ovate	1.8-2x 1.4-1.6	Basal	Raised	Reticulate - verrucate	Irregular	Depressed	Narrow	Undulate	Smooth	Raised	Verru- cate
<i>Bromus</i>	Light brown	Lanceolate	11.8-12x 1.7-1.8	Basal	Raised	Striate	Irregular	Raised	Narrow	Straight	Smooth	Depressed	Smooth
<i>Muscari</i>	Black	Ovate	0.7-0.85x 0.55-0.65	Basal	Raised	Ruminate	Irregular	Depressed	Narrow	Sinuate	Smooth	Raised	Smooth
<i>Pancratium</i>	Black	Reniform to elliptic	8.5-9x 5-5.5	Basal	Raised	Reticulate	polygonal	Raised	Narrow	Straight	Smooth	Depressed	Smooth

(L) = length; (W) = width. LM: Light microscope, SEM: Scanning electron microscope

(L) = length; (W) = width. LM: Light microscope, SEM: Scanning electron microscope

(JX090471.1). While, *matK* sequencing result revealed 98.7% similarity with *Muscari parviflorum* which has accession number MH152630.1 and 98.6% with *Muscari inconstictum* (MH152620.1). This is illustrated in the obtained phylogenetic tree constructed by NCBI BLAST (Fig. 7a, b). The two markers for the previous species support the inclusion of *M. salah-eidii* in the genus *Muscari*, where the most closely related are *Muscari longipes* and *Muscari parviflorum*.

The highest similarity of *Pancratium arabicum* is 98.2% for *rbcL* with *Pancratium arabicum* (HE603957.1) and *Pancratium maritimum* (NC063302.1) as illustrated in Table 6. While, *matK* alignment revealed 98.26% similarity with *Pancratium tenuifolium* (JQ024979.1), *Pancratium canariense* (OL690097.1), *Pancratium sickenbergeri* (FN594920.1) and *Pancratium foetidum* (FN594909.1) (Fig. 8a, b). Moreover, involvement of *P. arabicum* in genus *Pancratium*, where the most closely related species are *Pancratium maritimum*, and *Pancratium tenuifolium*.

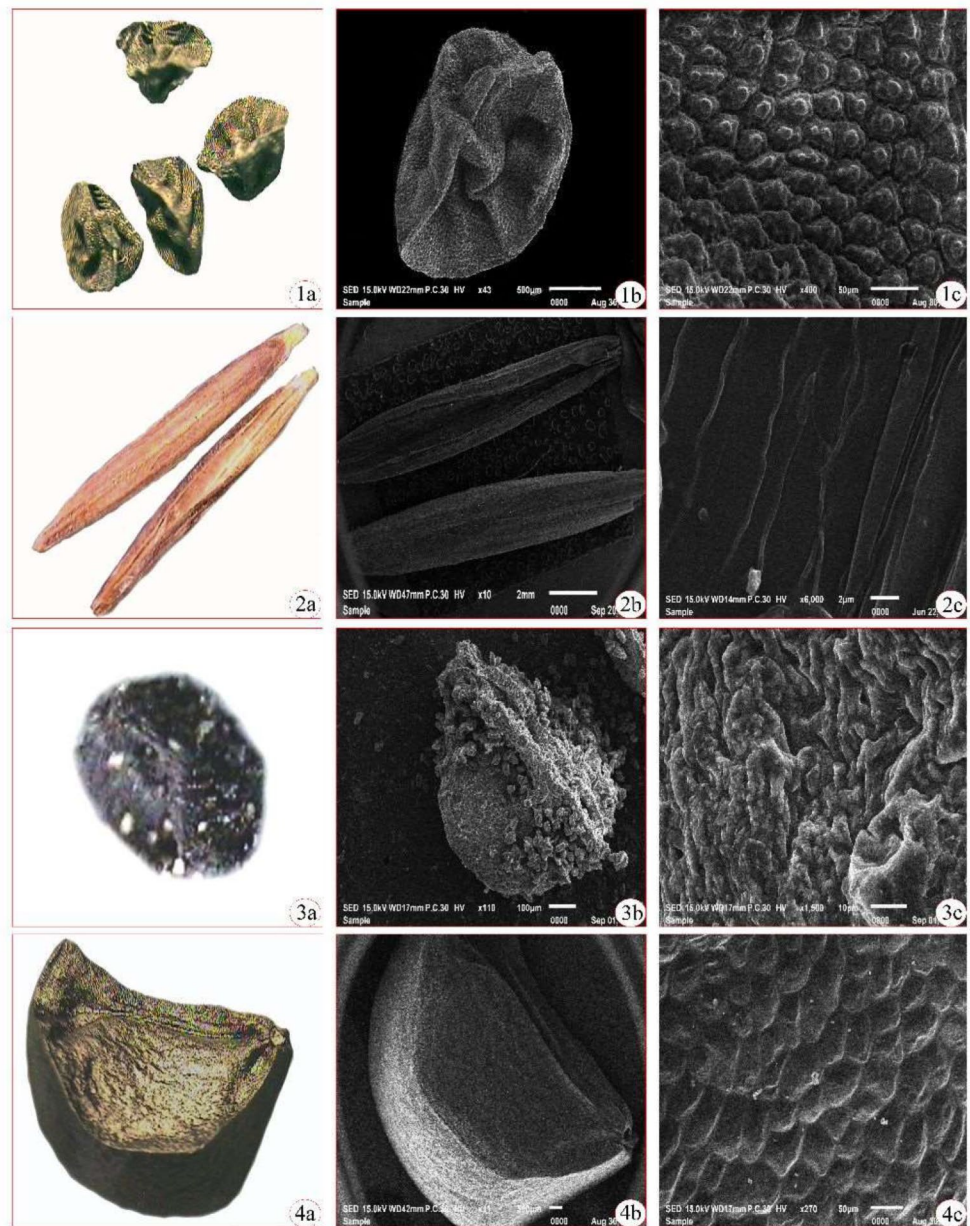
Discussion

Knowledge of biodiversity and endemism spatial patterns is critical for conservation planning (Ferrier 2002), especially in the face of rapid landscape transformation (Foley et al. 2005) and climate change (Thomas et al. 2004). Most of the endemics are threatened with over-cutting and over-collecting by local inhabitants, herbalists, and scientific researchers. No rules governing the commercial collection of wild native medicinal plants. The most serious aspect of this practice is that it typically targets rare and localized flora, causing further damage (Seif El-Nasr an Bidak 2005a).

Pollen and seed description

The morphology description of pollens is generally considered as a good taxonomic marker (Ding et al. 2005). Pollen grains are a highly recognizable object with consistent features and exine sculpture that can be help in identifying parent genera or even species. It may be also helped to solve problems of great biological interest like, the history of flora, past climates, prehistoric environments, as well as the standardization of honey and drugs (Martin 2001). Mono-sulcate apertures are most represented in monocots, and this agrees with Nour et al. (2022). The shape class of *Allium mareoticum* is prolate and has reticulate sculpture, but this disagrees with Nour et al. (2022), since they reported that (*A*) *mareoticum* has an oblate shape and rugulate-granulate exine sculpture. Exine ornamentation is reticulate in *Pancratium arabicum*, foveolate in *Muscari salah-eidii*, areolate in *Bromus aegyptiacus*. (*B*) *aegyptiacus* and *P.*

Fig. 3 (1–4). Micro-photographs of seeds of the studied taxa (LM & SEM), a: LM, b and c: SEM. (1) *Allium mareoticum*; black, broad-ovate, irregular cell shape, reticulate-verrucate surface sculpture, (2) *Bromus aegyptiacus*; light brown, lanceolate, irregular cell shape, striate surface sculpture, (3) *Muscari salah-eidii*; black, ovate, irregular cell shape, ruminant surface sculpture, (4) *Pancratium arabicum*; black, reniform to elliptic, polygonal cell shape, reticulate surface sculpture



arabicum are distinguished by their annulus which is absent in the remaining studied taxa. In general, Amaryllidaceae is a stenopalynous family in general, with pollen grains that are generally monocolpate, bilaterally symmetrical, boat-shaped, with subsilate or rugulate-foveolate, rarely reticulate tectum (Erdtman 1952).

Regarding the seed morphology, the color of the seeds was black, except in *B. aegyptiacus* which is light brown. This agreed with El-Masry (2007), Arai et al. (2011), Tugay et al. (2021), and Eroğlu et al. (2021). The seeds exhibit various shapes; reniform shape was represented in *P. arabicum*; ovate shape in *M. salah-eidii* and *(A) mareoticum*; and lanceolate in *(B) aegyptiacus*. The fascinating character is the surface sculpture pattern where three types were recorded.

Reticulate was the most represented surface sculpture as it was represented in *(A) mareoticum* and *P. arabicum*, such results agreed with El-Masry (2007), Hassan and Abdelkhalik (2014). Ruminant sculpture was represented in *M. salah-eidii*, such results agreed with Eroğlu et al. (2021) and Tugay et al. (2021). Striate sculpture was represented in *(B) aegyptiacus*, such results agreed with El-Masry (2007).

DNA barcoding

DNA barcoding is currently becoming a reliable method for species identification; a short genetic sequence from a standard part of the genome can be sufficient to verify a plant's identity (Elansary et al. 2017). DNA barcoding allows

Table 5 Element presence and their percentage in pollen and seeds of the examined taxa by EDX

Element Taxa code	C	N	O	Mg	Al	P	S	Cl	K	Ca	Si	Na
Pollen grains												
<i>Allium mareoticum</i>	59.3	23.0	16.8	0.10	-	0.20	0.34	0.08	0.15	0.07	-	-
<i>Bromus aegyptiacus</i>	75.1	-	23.0	-	1.06	-	-	0.36	-	-	-	0.57
<i>Muscari salalah-eidii</i>	59.2	18.5	21.5	0.14	-	0.08	-	0.31	0.18	0.11	-	-
<i>Pancreaticum arabicum</i>	74.6	-	24.1	0.24	-	0.36	0.20	-	0.10	0.39	0.03	-
Seeds												
<i>Allium mareoticum</i>	60.5	-	37.2	0.57	0.17	-	0.15	-	0.10	0.89	0.22	0.16
<i>Bromus aegyptiacus</i>	49.5	-	48.1	-	0.12	-	0.14	0.16	0.34	0.13	1.49	-
<i>Muscari salalah-eidii</i>	52.4	13.3	33.0	0.14	-	0.13	0.21	0.24	0.54	-	-	-
<i>Pancreaticum arabicum</i>	62.4	-	37.1	0.10	0.05	-	-	-	0.08	0.27	-	0.04

accurate identification of endemics, which is critical for conservation of natural plant genetic resources (De Groot et al. 2011). DNA barcoding is a novel system for rapid, accurate, and automatable species identification that employs short, standardized gene regions as internal species tags. As a result, the Linnaean taxonomic system will become more accessible, benefiting ecologists, conservationists, and a wide range of agencies responsible for pest control, invasive species, and food safety. In addition to assigning specimens to known species, DNA barcoding will accelerate species discovery by allowing taxonomists to quickly sort specimens and highlight divergent taxa that could be new species. By expanding their capabilities in these ways, DNA barcoding allows taxonomists to significantly expand, and eventually complete, a global inventory of life's diversity (Hebert and Gregory 2005).

Among several barcode loci, *matK* and *rbcL* were proposed as the preferred plant barcoding loci by The Consortium for the Barcode of Life (CBOL Plant Working Group 2009). Vijayan and Tsou (2010) proposed using *matK* and *rbcL* two-locus standard barcodes (chloroplast genes) to identify plant species. *rbcL* has a higher amplification success rate than *matK* and is the most well-characterized plastid coding region in GenBank (Kang et al. 2017). *rbcL* is commonly used to document endangered plant species (Alaklabi et al. 2014; Rajaram et al. 2019), endemic species (Amandita et al. 2019; Fouad et al. 2022; Shamsou and Fouad 2019), and species diversity (Amandita et al. 2019; Worthy et al. 2019). In addition, *matK* gene is ideal in size, has a high rate of substitution, a large proportion of nucleic acid variation at the first and second codon positions, a low transition/transversion ratio, and contains mutationally conserved regions. These *matK* gene features are used to determine family and species relationships. Using *matK*, angiosperms achieved approximately 90% amplification with a single pair of primers, while gymnosperms achieved 83% amplification and cryptogams 10% (Lahaye et al. 2008). Similarly, *rbcL* has been widely used for phylogenetic analysis, yielding better results at the family and class levels but with moderate discrimination power at the species level (CBOL Plant Working Group 2009; Chen et al. 2010).

The *matK* and *rbcL* genes were amplified and sequenced quickly in this study. BLAST matching and phylogenetic tree analysis of the examined species using the two markers as barcodes revealed more than 95% similarity with the highly similar species. The *rbcL* and *matK* barcodes were used because of the recommendation of the Consortium for the Barcode of Life (CBOL Plant Working Group 2009), that both chloroplast genes are the core barcodes of plants. Furthermore, the results obtained with the *rbcL* and *matK* genes confirmed their utility in phylogenetic analysis within the

Fig. 4 PCR amplified *rbcL* and *matK* genes for the examined taxa. a: *Allium mareoticum*, b: *Bromus aegyptiacus*, c: *Muscari salah-eidii*, and d: *Pancratium arabicum*

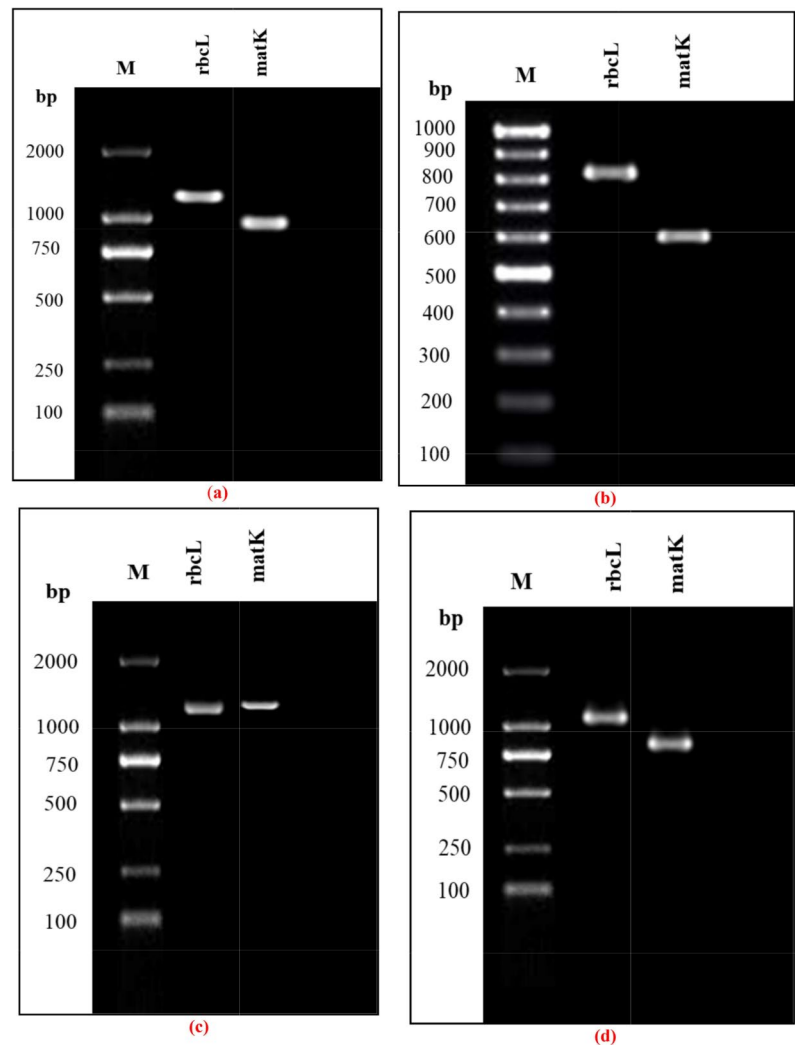


Table 6 BLAST results of the barcode sequences against NCBI databases. Query: the current study sequences, subject: NCBI published sequences

Query	Subject species	Subject ID	Total Score	Query cover	E. value	Identity %
<i>rbcL</i>						
<i>Allium mareoticum</i>	<i>Allium roseum</i>	KF997289.1	1837	99%	0.0	100
<i>Bromus aegyptiacus</i>	<i>Bromus hordeaceus</i>	AY395531.1	1050	98%	0.0	98.98
<i>Muscari salah-eidii</i>	<i>Leopoldia longipes</i> (<i>Muscari longipes</i>)	JX090455.1	2109	93%	0.0	99.83
<i>Pancratium arabicum</i>	<i>Pancratium arabicum</i>	HE603957.1	1772	97%	0.0	98.24
<i>matK</i>						
<i>Allium mareoticum</i>	<i>Allium platyspathum</i>	JN864793	593	95.5%	5e-165	100
<i>Bromus aegyptiacus</i>	<i>Bromus hordeaceus</i>	MF597209.1	1376	96%	0.0	99.34
<i>Muscari salah-eidii</i>	<i>Muscari parviflorum</i>	MH152630.1	2141	99%	0.0	98.68
<i>Pancratium arabicum</i>	<i>Pancratium tenuifolium</i>	JQ024979.1	1291	96%	0.0	98.26

family and subclass of angiosperms (Fazekas et al. 2008). The two used genes were successful in identifying *Allium mareoticum*, *Bromus aegyptiacus*, and *Muscari salah-eidii* at the genus level, and *Pancratium arabicum* at species and genus levels for *rbcL* marker and the genus level for *matK* marker. Some studies in Egypt used these two markers in identification of other endemic species. Ghareb et al.

(2020a, b) succeeded in identification of endangered *Phlomis aurea* plant of Saint Katherine by *rbcL* and *matK* markers and *Silene schimperiana* by the *rbcL* marker. Hashim et al. (2021) identified eight endangered species endemic to Saint Katherine (at the species level) and the ninth species (at the genus level). *rbcL* was the most easily amplified and recognized barcode. *Rosa arabica* Crép, a species

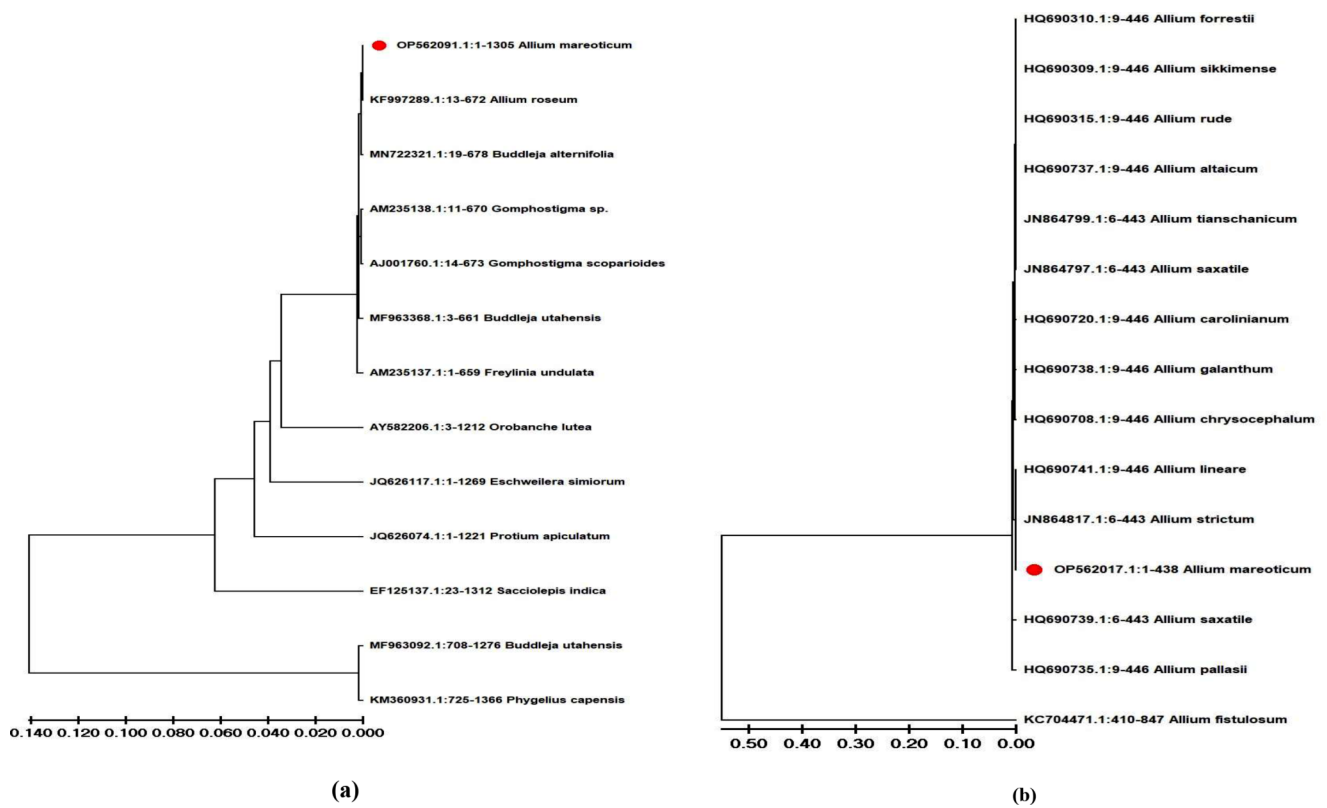


Fig. 5 Phylogenetic tree using UPGMA method based on P-distance model for *rbcL* and *matK* sequences of *Allium mareoticum*

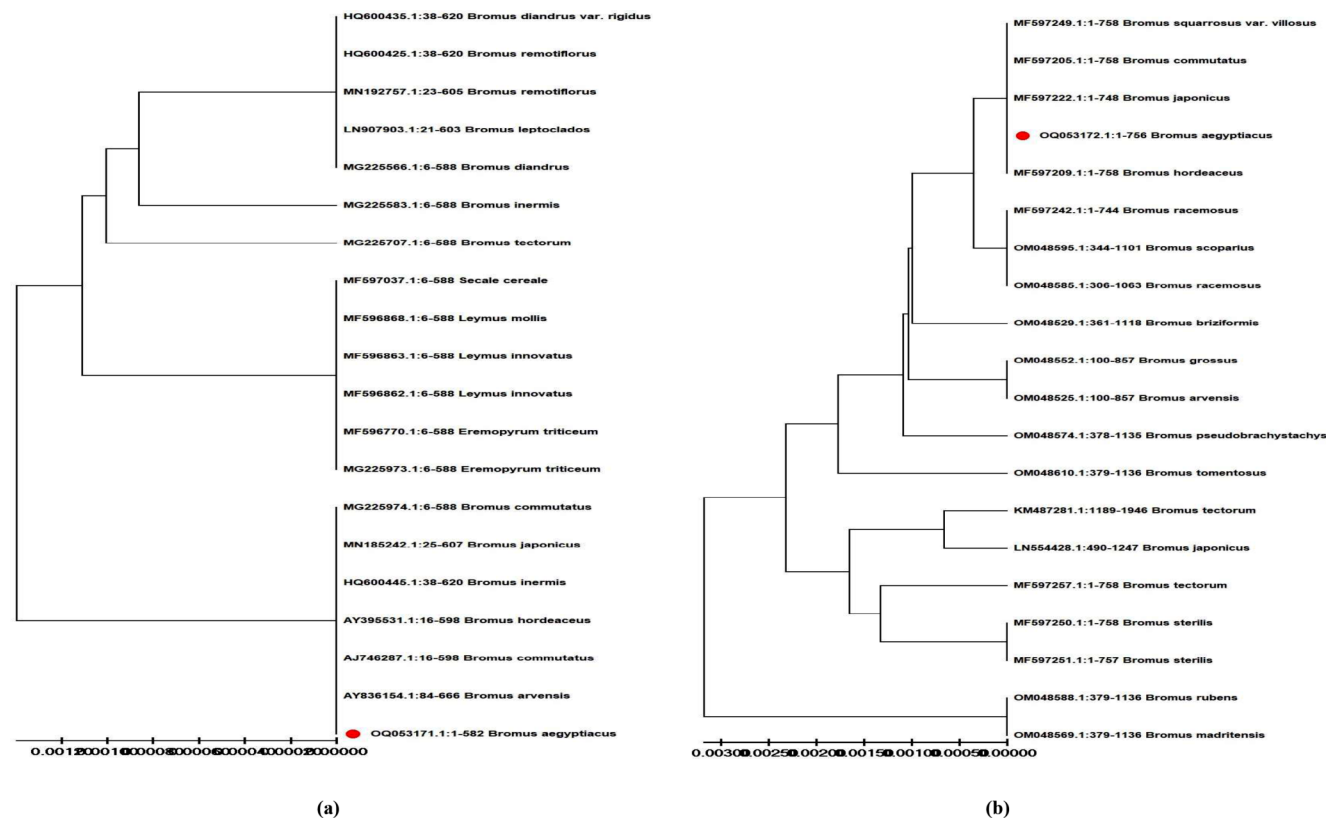


Fig. 6 Phylogenetic tree using UPGMA method based on P-distance model for *rbcL* and *matK* sequences of *Bromus aegyptiacus*

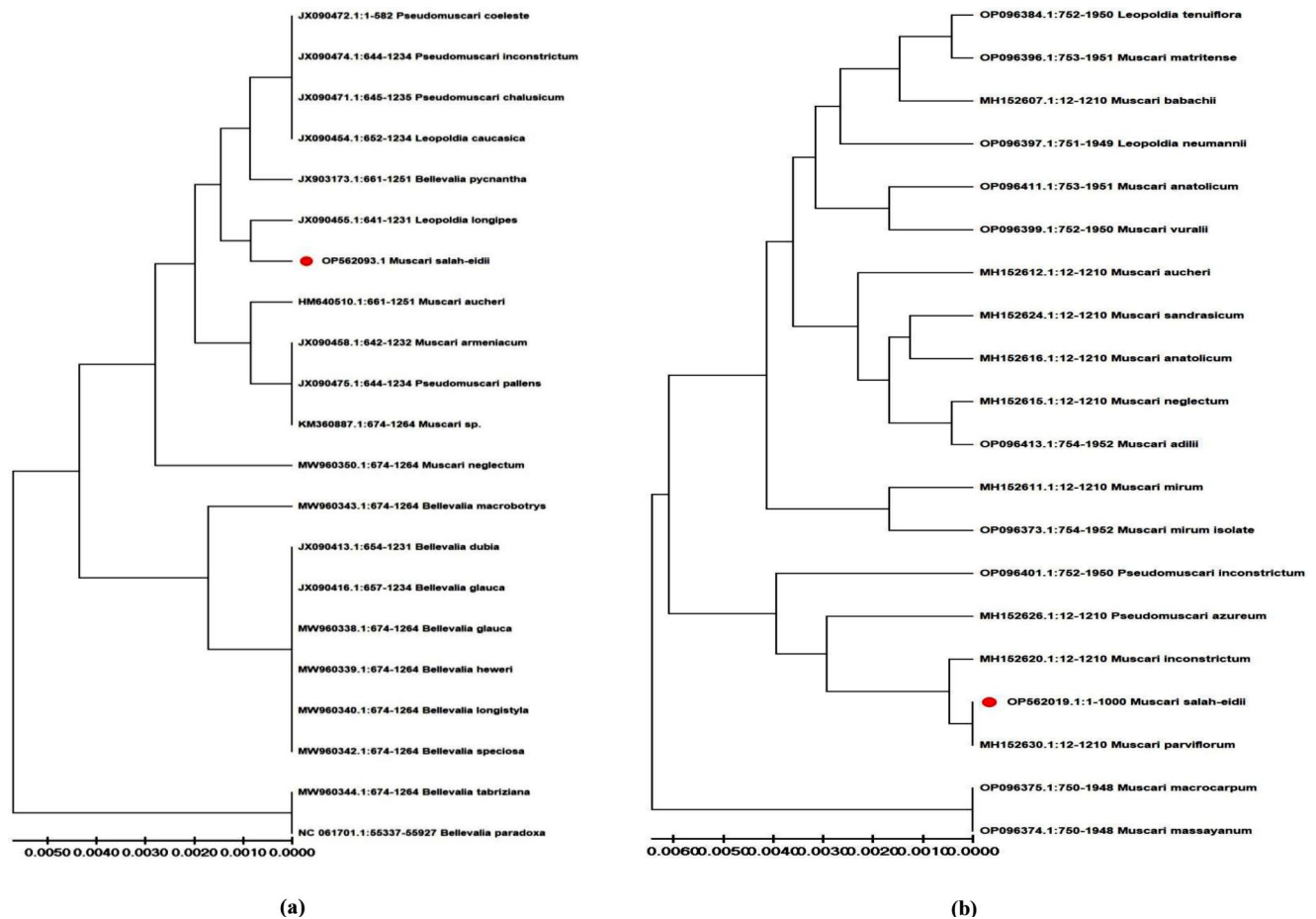


Fig. 7 Phylogenetic tree using UPGMA method based on P-distance model for *rbcL* and *matK* sequences of *Muscari salah-eidii*

that is critically endangered and steno-endemic to Egypt, was successfully identified at the genus and species levels using molecular identification based on the sequences of multiple markers, including *ITS*, *matK*, *rbcL*, and *trnL-F*, individually or in combination (El-Banhawy et al. 2020b). The ability of the obtained sequences to correlate endemic specimens to the species or genus level demonstrates their success. On the species level, it was reported that the identification of species is considered successful when the similarity percentage scores more than 95% and includes a single species. However, on the generic level, DNA barcoding is considered successful when all BLAST searches score similarity percentage of more than 95% and include a single genus (De Groot et al. 2011).

According to NCBI, the present study has priority for *rbcL* and *matK* barcoding for three taxa (*A. mareoticum*, *B. aegyptiacus* and *M. salah-eidii*), that weren't previously recorded in NCBI databases. In the current study, the highest identity percentages between the examined taxa and the other similar species were arranged between 98.2 and 100% for *rbcL* and *matK* genes. In our study, *P. arabicum* was identified at both species and genus levels for *rbcL* marker

and at the genus level for *matK* marker. The most closely related species are *P. arabicum*, *P. maritimum*, and *P. tenuifolium*. *P. arabicum* and *P. maritimum* exhibit morphologically and ecologically related characteristics considering *P. arabicum* as a variant of *P. maritimum* (Täckholm 1974). El-Hadidy et al. (2011) documented that both taxa appeared to be closely related to one another and to occupy the same biological niche. El-Hadidy et al. (2012) resolved this problem using RAPD data, but the created dendrogram demonstrated the strong resemblance between *P. maritimum* and *P. arabicum*. De Castro et al. (2012) indicated that *P. maritimum* complex included notable species like *P. arabicum* and *P. linosae* based on four plastid DNA markers: *ndhF* and *rbcL* genes, *trnL(UAA)–trnF(GAA)* intergenic spacer and *trnL(UAA)* intron.

Conclusion

Pollen grains and seeds of four Mediterranean endemics in Egypt were analyzed using both a light microscope (LM) and scanning electron microscopes (SEM). Documentation

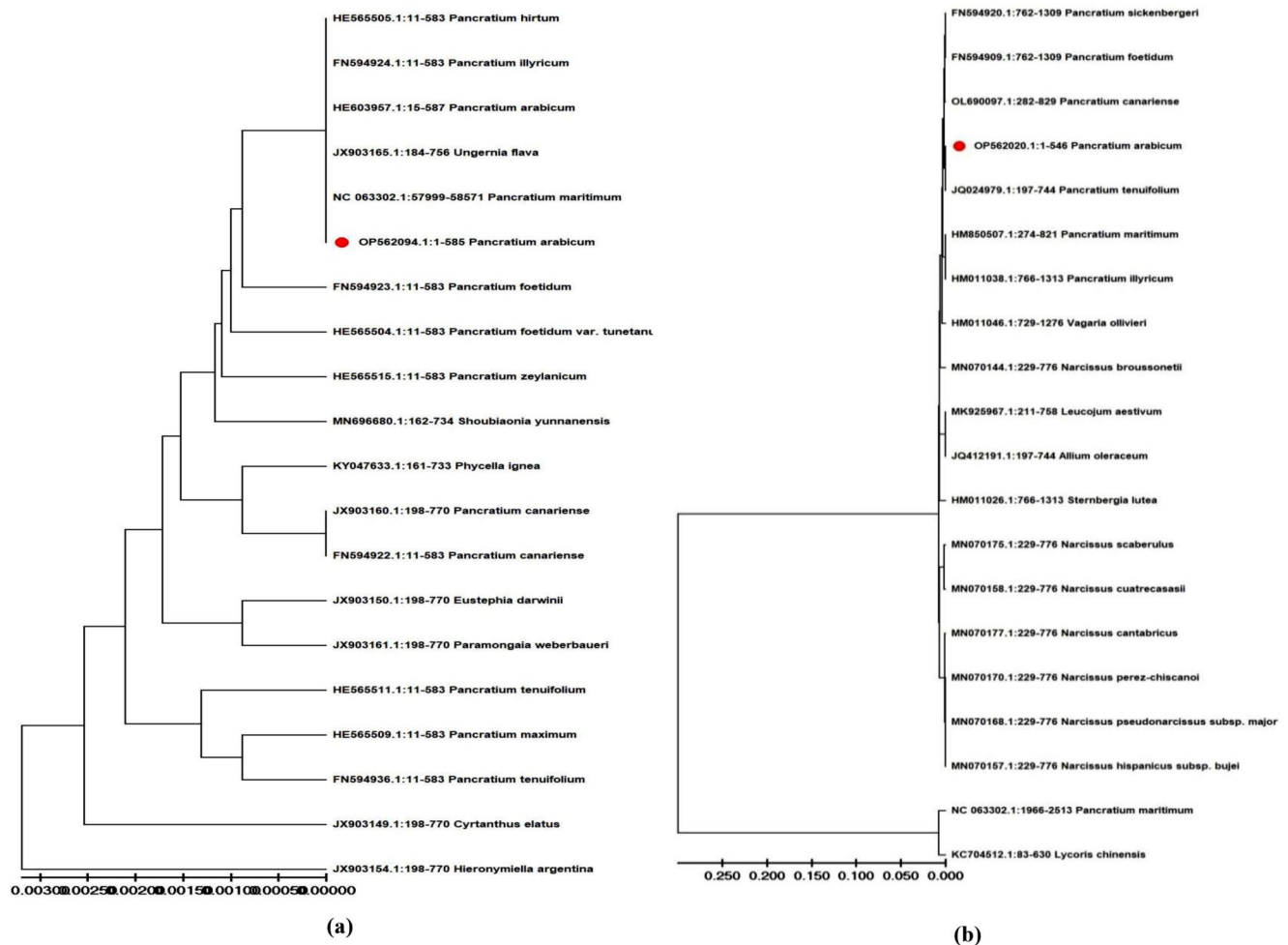


Fig. 8 Phylogenetic tree using UPGMA method based on P-distance model for *rbcL* and *matK* sequences of *Pancratium arabicum*

of the four species involved DNA barcoding. Rapid amplification and sequencing of the *matK* and *rbcL* genes have been employed. BLAST matching and phylogenetic tree analysis of the examined species using the two markers as barcodes revealed more than 95% similarity with the highly similar species. The two genes were successful in identifying *Allium mareoticum*, *Bromus aegyptiacus*, and *Muscari salah-eidii* at the genus level. *Pancratium arabicum* was identified at both species and genus levels for *rbcL* marker and the genus level for *matK* marker. This study has priority in *rbcL* and *matK* barcoding for 3 taxa (*Allium mareoticum*, *Bromus aegyptiacus* and *Muscari salah-eidii*), as these taxa weren't previously recorded in NCBI databases. The current study demonstrates the potential use of DNA barcode analysis in documenting endemic endangered species and assigning them to the correct taxonomic position. Furthermore, it is recommended to examine other markers, such as nuclear gene markers to entirely identify the plant more accurately.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were

performed by Mohamed Mahmoud El-Khalafy, Aziza S. El-Khoily and Mohamed A. Salem. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Not applicable.

Declarations

Consent to participate None.

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