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Barcoding of Some Plant Species Using the *rbcL* Gene in the Mediterranean Oolitic Sand Dunes West of Alexandria, Egypt

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IN the Mediterranean Basin, oolitic sand dunes extend along the northeastern coast of Africa between the Gulf of Gabes (Tunisia) and the Nile Delta (Egypt), attenuating coastal vulnerability to storms and saltwater intrusion and protecting inland infrastructure. Psammophytes are typical and distinctive plants for dune habitats; they are the primary drivers for dune growth and stabilization by trapping sand particles in their shoot and root systems. The Anthropocene fingerprint may create an environment favorable for nonpsammophyte invasion and subsequent alteration in sand dune habitat structure and functions. The authentication of sand dune-inhabiting species should be a priority for conservation or restoration measures concerning sand dune habitats. This study is the first *rbcL*-based DNA barcoding documentation of 20 plant species inhabiting the vulnerable Egyptian oolitic sand dune habitats in Burg El Arab area, west of Alexandria. Results highlighted the applicability of the *rbcL* locus sequence for species authentication and recognition of the examined plant taxa sharing the same habitat. The phylogenetic analysis of *rbcL* barcode polymorphism revealed current taxonomic relationships among the assigned species. Results added valuable information to the authentication of sand dune plants, providing the first *rbcL* barcodes for species growing in oolitic sand dune habitats in Egypt, six of which were submitted for the first time to GenBank.

Keywords : Burg El-Arab, DNA-Barcoding, Egypt, *rbcL*, Ooliticsands dunes, Psammophytes.

Introduction

The growing erosion of coastal zones is a worldwide phenomenon under a changing climate and urbanization that is more pronounced in Asian and African developing countries (Grases et al., 2020). In Egypt, the northwestern coast is occupied by summer resorts, which is intended to host new settlements, for example, Al Alamein New City (Attia, 2019). However, approximately one-third of the world's coastal infrastructure and properties within 200 m from the seaboard may be lost in the next 50 years due to coastal erosion (UN-Habitat, 2009; Gracia et al., 2018). Coastal dunes have been characterized as one of the most efficient ecosystems for coastal protection (Temmerman et al., 2013; Van Slobbe et al., 2013). Avoiding the economic and environmental drawbacks of artificial wave breakers, coastal dunes act as natural barriers supporting resilient ecosystems and attenuating coastal vulnerability to storms and saltwater intrusion (Duran & Moore, 2013; Gracia et al., 2018; Maximiliano-Cordova et al., 2019).

Oolites are spherical marine sediments of 0.25 to 2.0 mm in diameter formed by the concentric lamellar precipitation of $CaCO_3$ around a nucleus; the latter is commonly a sand grain or fossil fragment (Yokoyama et al., 2006). These sediments are formed in shallow warm water supersaturated with $CaCO_3$ in the presence of insistent tidal agitation (Bardají et al., 2009). Oolitic sands accumulate in beach dunes in several

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localities worldwide, with the most famous example in The Bahamas. In the Mediterranean Basin, oolitic sand dunes are formed along the northeastern coast of Africa, extending between the Gulf of Gabes (Tunisia) and the Nile Delta (Egypt) (Lucas, 1955). The sampling area of this study (Burg El Arab area) in Egyptian territories is characterized by successive, interrupted, undulated calcareous sand dunes formed by loose oval oolitic grains (Zahran & Willis, 2008).

Dune formation is essentially driven by the availability of sediments and the carrying wind energy that enables sand accretion, which in turn determines the type and distribution of the plant cover. Then, the vegetation becomes the primary driver for the growth and stabilization of dunes by trapping sand particles in their shoot and root systems (Corbau et al., 2015; Ciccarelli et al., 2017).

Psammophytes are typical and distinctive plants inhabiting dunes, adapted for harsh conditions, including sand burial and mobile substrate, high winds, soil salinity, and salt spray (Liu et al., 2016). In addition to these severe conditions, populations of these dune-building species suffer from overuse and pollution related to human activities (Amer et al., 2020). Moreover, the Anthropocene fingerprint may create an overall environment favorable for nonpsammophyte invasion and subsequent alteration in dune habitat structure and functions (Valcheva et al., 2020). Thus, authentication of both types of sand dune-inhabiting plant taxa should be a priority for conservation or restoration measures concerning sand dune habitats.

Recently, DNA barcoding has been used as an accurate, rapid, cost-effective, and time-efficient tool for species identification utilizing short DNA sequences as internal species tags (Hebert & Gregory, 2005). It gives additional support for growing efforts aiming to preserve biodiversity on the planet (von Crautlein et al., 2011). Dissimilar from morphological identification, DNA barcoding is not marred by prejudices and does not impose taxonomic expertise (Bafeel et al., 2012). Considering sequence quality, recoverability, and levels of species discrimination, the Consortium for the Barcode of Life (CBOL) Plant Working Group recommended employing *rbcL* (ribulose bisphosphate carboxylase large) and/or *matK* (maturase K) in the barcoding of land plants (CBOL Plant Working Group, 2009). Compared to *matK*, *rbcL* has a high amplification success rate; it is the most characterized plastid coding region in GenBank (Kang et al., 2017). *rbcL* is routinely used to document endangered plant species (Alaklabi et al., 2014; Rajaram et al., 2019), endemic species (Amandita et al., 2019; Fouad et al., 2019; Shamso & Fouad, 2019), and species richness (Amandita et al., 2019; Worthy et al., 2019).

Sharing in the maintenance of oolitic sand dunes as natural barriers protecting inland developmental plans in the Egyptian northwestern coast against coastal erosion, this study presents the first *rbcL*-based DNA barcoding documentation for some plant species inhabiting this habitat in Burg El Arab, Egypt.

Materials and Methods

Study area and plant species

Twenty plant species were collected from the sampling area in Burg El Arab (lat. 30°54′N, long. 29°33′E), which belongs to the Mareotis sector of Mediterranean sand dunes coast west of Alexandria in Egypt (Fig. 1). It represents a unique limestone ridge close to the northwestern Mediterranean seashore in Egyptian territories (Bidak et al., 2015). The list and accession number of the sample species and their families and the geographic positioning system (GPS) coordinates in the study area are given in Table 1. Plant species were identified, and representative samples were deposited at the Cairo University Herbarium. Species identification was based on voucher herbarium specimens and the reference books on the flora of Egypt (Boulos, 1999, 2000, 2002, 2009); the species nomenclature is updated as in The Plant List (2013).

DNA extraction and rbcL barcoding procedures

Total genomic DNA was extracted from 20 mg fresh leaf tissue samples (5 samples per species) employing a Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The *rbcL* sequences were amplified according to the CBOL Plant Working Group (2009) in 50µL reaction mixture consisting of 25µL PCR Master Mix (Bioline), 50ng genomic DNA, and 1µL of each specific primer (5′-ATGTCACCACAAACAGAAAC-3′ and 5′-TCGCATGTACCTGCAGTAGC-3′). The amplification protocol was 94°C for 2min,

followed by 35 three-step cycles (1min at 94°C, 30s at 55°C, and 1min at 72°C) and a final extension step at 72°C for 7min. After a purification step with the aid of a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), amplicons were sequenced using BigDye Terminator Chemistry in 3130xl Genetic Analyzer (Life Technologies, South San Francisco, CA, USA) according to the standard manufacturer's protocol.

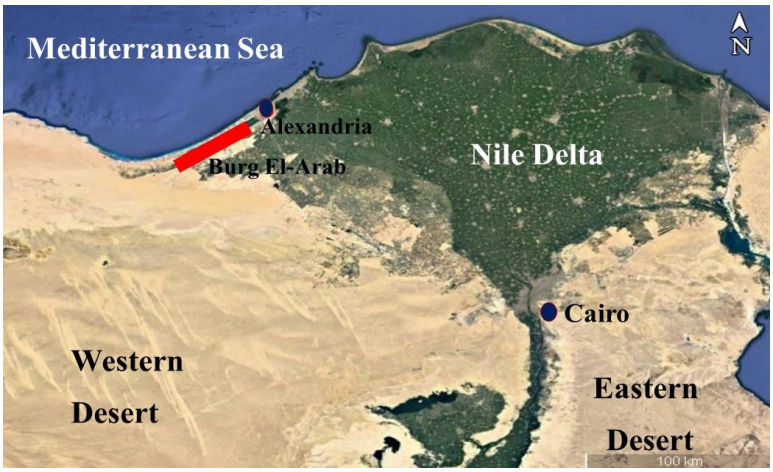

Fig. 1. Map of the Mediterranean coast of Egypt, west of Alexandria showing the area from which the sampled species (red rectangle) were collected

TABLE 1. List and accession number of the sample species and their families and the GPS coordinates in the study area

Ser. No.	Species	AC. No.	Family	Coordinates	
				Lat.	Long.
1	*Cakile maritima* Scop.	MT645206.1	Brassicaceae	30.943446	29.505183
2	*Carrichtera annua* (L.) DC.	MT645213.1	Brassicaceae	30.943059	29.503907
3	*Reseda decursiva* Forssk.	MT645214.1	Resedaceae	30.947283	29.502405
4	*Thymelaea hirsuta* (L.) Endl.	MT645212.1	Thymelaeaceae	30.947283	29.502405
5	*Euphorbia paralias* L.	MT645205.1	Euphorbiaceae	30.947714	29.502269
6	*Tetraena alba* (L.f.) Beier & Thulin.	MT645202.1	Zygophyllaceae	30.944449	29.502179
7	*Fagonia cretica* L.	MT645207.1	Zygophyllaceae	30.944449	29.502179
8	*Emex spinosa* (L.) Campd.	MT645216.1	Polygonaceae	30.943713	29.505891
9	*Crucianella maritima* L.	MT645210.1	Rubiaceae	30.944872	29.502823
10	*Echinops spinosissimus* Turra.	MT645211.1	Asteraceae	30.947283	29.502405
11	*Limbarda crithmoides* (L.) Dumort.	MT645203.1	Asteraceae	30.943446	29.505183
12	*Senecio glaucus* subsp. *coronopifolius* (Maire) C. Alexander.	MT645215.1	Asteraceae	30.943059	29.503907
13	*Otanthus maritimus* (L.) Hoffmanns. & Link.	MT645201.1	Asteraceae	30.944449	29.502179
14	*Picris asplenioides* L.	MT645204.1	Asteraceae	30.943059	29.503907
15	*Bassia indica* (Wight) A. J. Scott.	MT645208.1	Amaranthaceae	30.943446	29.505183
16	*Gymnocarpos decandrus* Forssk.	MT645209.1	Caryophyllaceae	30.944872	29.502823
17	*Mesembryanthemum crystallinum* L.	MT645218.1	Aizoaceae	30.943713	29.505891
18	*Avena sterilis* L.	MT645217.1	Poaceae	30.943059	29.503907
19	*Aegilops bicornis* (Forssk.) Jaub. & Spach.	MT645219.1	Poaceae	30.943059	29.503907
20	*Bromus catharticus* Vahl.	MT645220.1	Poaceae	30.943059	29.503907

Forward and reverse sequences repeated identically in at least three amplicons for each species were assembled employing Codon Code Aligner version 7.1.2. Contig sequences were submitted to GenBank and used to establish a phylogenetic tree and calculate pairwise distance utilizing Neighbor-Joining method based on the Tamura 3-parameter model (Tamura, 1992) with the aid of MEGA 6 (Tamura et al., 2013).

Results

The sample species belonged to 12 families, of which Astraceae was the most represented family by five species, followed by Poaceae represented with three species. Among the remaining families, only Zygophyllaceae and Brassicaceae were exemplified with two species each, whereas others were represented by one species each. Sequences of the *rbcL* loci of the 20 collected species were deposited in GenBank and acquired accession numbers MT645201 to MT645220 (Table 1). The obtained sequences were the first *rbcL* barcodes obtained from sample species growing in Egyptian oolitic sand dune habitats in the Mediterranean coast of Egypt.

Moreover, this study reported the first records of *rbcL* barcodes for *Otanthus maritimus*, *Picris asplenioides*, *Gymnocarpos decandrus*, *Crucianella maritima*, *Echinops spinosissimus*, and *Bassia indica* to GenBank. BLASTN of the generated sequences reflected matching with reference sequences at the family level for the first record sequences and at the species level for the remaining sequences.

To assess the potential taxonomic insights of the newly recorded *rbcL* sequences for the sample species, evolutionary distances between the *rbcL* sequences for the 20 sample species were estimated based on the Tamura 3-parameter model (Tamura, 1992) (Table 2). The phylogenetic tree was constructed using the maximum likelihood method (Fig. 2). All sequences could be cleared up to particular species, and collected taxa showed excellent grouping patterns at the family level manifested in a monophyletic clade for each family represented by more than one species, viz., Brassicaceae, Zygophyllaceae, Asteraceae, and Poaceae.

The 20 species representing 12 families were grouped into two unequal clades: a minor clade representing the monocots represented by the three species of Poaceae and a major clade for the eudicots 11 families represented by 17 species. The major clade is delimited into three major groups, Rosids, Astrides, and Caryophyllids, whereas Zygophyllaceae and Euphorbiaceae were grouped into Fabids. Brassicaceae, Resedaceae, and Thymelaeaceae were assembled into Malvids. Both Fabids and Malvids were clustered into a monophyletic clade for Rosids.

Similarly, Campanulids and Lamiids were represented as Asteraceae and Rubiaceae, respectively, clustered to form the Astrides clade. In contrast to the monophyly exhibited by Rosids and Astrides, a paraphyly appeared in the clustering of Caryophyllids representative families. In contrast, Caryophyllaceae, represented by Aizoaceae and Amaranthaceae, clustered into a single clade. Polygonaceae was nested in a distinct clade from Caryophyllids, as it connects the Rosids and Asterids clades (Fig. 2).

Discussion

This investigation indicated a complete matching between *rbcL* and the identification based on morphological characteristics to the species level for the previously *rbcL*-barcoded taxa. This study also reported the first record of *rbcL* barcode for *Otanthus maritimus*, *Picris asplenioides*, *Gymnocarpos decandrus*, *Crucianella maritima*, *Echinops spinosissimus*, and *Bassia indica* to GenBank. BLASTN of the generated sequences agreed with reference sequences at the family level for the first record sequences and at the species level for other sequences. The discriminatory potentials for the *rbcL* sequences for DNA barcoding in land plants were recommended by Newmaster et al. (2006). The discriminatory power of the *rbcL* barcode locus was also proven useful for the authentication of some United Arab Emirates native plants by Maloukh et al. (2017), endangered plants in genus *Aquilaria* by Thitikornpong et al. (2018), and selected *Acacia* species by Ismail et al. (2020). It was also utilized in the barcoding analysis and phylogenetic relationships of mangroves in Guangdong Province in China by Wu et al. (2019).

TABLE 2. Estimates of evolutionary distances between the *rbcL* sequences for 20 plant species growing in Egyptian oolitic sand dune habitats based on the Tamura 3-parameter model (Tamura, 1992)

	Cakile maritima	***Carrichtera annua***	***Reseda decursiva***	***Thymelaea hirsuta***	***Euphorbia paralias***	***Tetraena alba***	***Fagonia cretica***	***Emex spinosus***	***Crucianella maritima***	***Echinops spinosissimus***	***Limbarda crithmoides***	***Senecio glaucus***	***Otanthus maritimus***	***Picris asplenioides***	***Bassia indica***	***Gymnocarpos decander***	***Mesembryanthemum crystallinum***	***Avena sterilis***	***Aegilops bicornis***
Carrichtera annua	0.006																		
Reseda decursiva	0.040	0.042																	
Thymelaea hirsuta	0.080	0.083	0.064																
Euphorbia paralias	0.094	0.093	0.078	0.082															
Tetraena alba	0.121	0.120	0.097	0.104	0.100														
Fagonia cretica	0.125	0.129	0.109	0.108	0.103	0.033													
Emex spinosus	0.076	0.074	0.072	0.089	0.088	0.095	0.115												
Crucianella maritima	0.126	0.122	0.112	0.098	0.121	0.122	0.133	0.100											
Echinops spinosissimus	0.105	0.100	0.089	0.070	0.093	0.106	0.107	0.075	0.082										
Limbarda crithmoides	0.111	0.109	0.114	0.086	0.100	0.120	0.119	0.088	0.094	0.011									
Senecio glaucus	0.110	0.105	0.100	0.084	0.089	0.101	0.108	0.075	0.099	0.016	0.026								
Otanthus maritimus	0.107	0.105	0.095	0.088	0.109	0.102	0.103	0.098	0.092	0.023	0.026	0.028							
Picris asplenioides	0.128	0.124	0.118	0.094	0.095	0.115	0.111	0.079	0.084	0.010	0.023	0.017	0.029						
Bassia indica	0.121	0.116	0.108	0.112	0.120	0.125	0.143	0.091	0.121	0.108	0.117	0.104	0.114	0.115					
Gymnocarpos decander	0.116	0.112	0.103	0.089	0.092	0.110	0.120	0.078	0.096	0.092	0.104	0.093	0.100	0.100	0.071				
Mesembryanthemum crystallinum	0.120	0.116	0.122	0.088	0.129	0.113	0.113	0.101	0.110	0.097	0.111	0.107	0.109	0.142	0.073	0.063			
Avena sterilis	0.135	0.137	0.129	0.124	0.119	0.133	0.132	0.136	0.149	0.136	0.128	0.116	0.121	0.128	0.125	0.121	0.164		
Aegilops bicornis	0.150	0.148	0.133	0.141	0.145	0.144	0.154	0.137	0.148	0.142	0.149	0.132	0.136	0.147	0.125	0.128	0.148	0.019	
Bromus catharticus	0.166	0.166	0.151	0.154	0.142	0.159	0.149	0.151	0.164	0.146	0.138	0.124	0.134	0.139	0.143	0.148	0.176	0.034	0.025

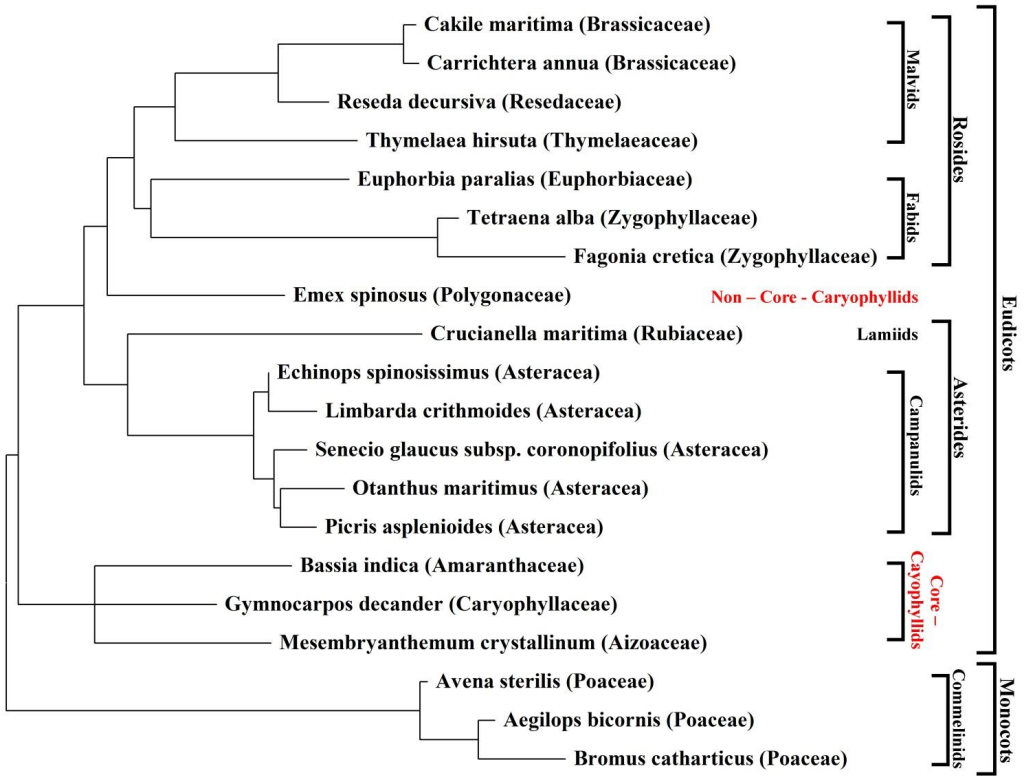

Fig. 2. Molecular phylogenetic analysis based on *rbcL* sequence for the 20 sampled species using maximum-likelihood method [The evolutionary history was implied based on the Tamura 3-parameter model (Tamura, 1992). Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 1447 positions in the final data set]

Phylogenetic analysis (Fig. 2) and evolutionary distances among species (Table 2) showed the competence of the obtained *rbcL* sequences to confirm the taxonomic relationships among the studied plant species. The tree reflected a clear resolution between monocots and eudicots into two separate clades. Moreover, the eudicot clade clearly manifests three large groups, viz., Astroids, Rosides, and Caryophyllids, as different groups, which agreed with Judd & Olmstead (2004). Additional evidence supporting the taxonomic utility of *rbcL* in this investigation is that Astroids split into Campanulids and Lamiids, whereas Rosides bifurcated into Fabids and Malvids, coinciding with the classification of the Angiosperm Phylogeny Group (Chase et al., 2016). In contrast, the evolutionary distances calculated among the species provided a species discriminatory tool for their corresponding taxa. In addition, the phylogenetic tree showed a grouping of species assigned to the same families supporting the taxonomic competence of *rbcL* at the family level, as reported by Maloukh et al. (2017) and Shinwar et al. (2018).

The constructed evolutionary relationships (Fig. 2) also showed two subclades comprising Caryophllids (Brockington et al., 2009) as core Caryophyllales represented by Caryophyllaceae, Aizoaceae, and Amaranthaceae, whereas the second was termed as noncore Caryophyllales that included Polygonaceae. The splitting of Caryophllids into two taxonomic groups is supported with morphological and molecular clues. The absence of sufficient common morphological criteria as conducted by Dahlgren (1983) considered these groups as distinct superorders, viz., Caryophyllales and Polygonales, placed close but not necessarily related to each other. The comparative sequencing of the *rbcL* gene provided early molecular support for this conclusion; it showed Polygonales and Caryophyllales as unallied groups (Giannasi et al., 1992). The molecular evidence for splitting Caryophllids was cumulated and finally crowned by Brockington et al. (2009) based on a combination of two nuclear genes, nine plastid genes, and the plastid inverted repeat.

The paraphyly of Caryophllids in this study did not deviate from *rbcL* as a taxonomic tool and can be attributed to the utilization of *Echinops spinosa* (Family Polygonaceae) as a representative for Polygonales. Based on the *rbcL* sequences, phylogenetic relationships in Polygonaceae reflected *Emex* as a paraphyletic genus (Frye & Kron, 2003). To resolve the phyletic origin of Caryophyllids, future studies need to address more species from different Polygonales families and different genetic loci.

Conclusion

This study reported the *rbcL* sequences for the authentication and discrimination of 20 plant species in a vulnerable sand dune habitat west of Alexandria. The results are the first authentication of plants in a vulnerable sand dune habitat in Egypt. However, the phylogenetic analysis of the *rbcL* barcode polymorphism revealed current taxonomic relationships between the assigned species.

Conflict of interest: The authors declare no conflict of interest.

Author contributions: Wafaa Amer and Azza Hamed collected and identified the plant specimens. Ahmed Fouad and Rehab Hafez Conducted the remaining practical work. All authors shared in original draft preparation, writing—review and editing.

Ethical approval: Not applicable

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باركود بإستخدام جين *rbcL* لبعض الأنواع النباتية بالكثبان الرملية الجيرية على شاطئ البحر المتوسط غرب مدينة الأسكندرية بمصر

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تمتد الكثبان الرملية الجيرية فى حوض البحر المتوسط عبر الساحل الشمالى الأفريقى بين خليج قابس بتونس ودلتا النيل بمصر موفرة الحماية للمنشآت الساحلية ضد العواصف وتخلل المياة المالحة. وتعد النباتات الرملية العامل الأساسى لتكوين والحفاظ على تلك الكثبان بما لها من قدرة على إصطياد الرمال والإحتفاظ بها ملتصقة بإجزائها المختلفة. تتسبب الأنشطة البشرية فى تغيرات بيئية مشجعة لغزو الأنواع النباتية الأخرى بما يترتب على ذلك من تغيرات فى الكثبان وقدرتها على القيام بوظيفتها فى حماية الشواطئ. لذلك يجب أن يكون توثيق الانواع النباتية المختلفة الموجودة بالكثبان الرملية الجيرية على رأس أولويات الحفاظ على تلك الكثبان. تهدف الدراسة الحالية إلى توثيق 20 نوع نبات من تلك النامية ببيئة الكثبان الرملية الجيرية بمنطقة برج العرب غرب مدينة الأسكندرية بمصر بإستخدام تقنية باركود الحامض النووى بالإعتماد على جين *rbcL*. أوضحت النتائج قدرة التقنية المستخدمة على تعريف وتوثيق الأنواع النباتية موضوع الدراسة ، كما أظهرت الشجرة التطورية نفس العلاقات التصنيفية المعروفة بين تلك الأنواع. وتعد نتائج هذه الدراسة إضافة ذات قيمة لتوثيق النباتات النامية بالكثبان الرملية الجيرية حيت قدمت أول توثيق من نوعه لتلك النباتات بمصر ومن بينهم 6 أنواع تم نشرهم ببنك الجينات للمرة الأولى على الإطلاق.