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Research Article

Discrimination of Two Biotypes of *Haplophyllum tuberculatum* (Forssk.) A. Juss. (Rutaceae) by Morphology, SDS-PAGE and RAPD

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Abstract

Background and Objective: *Haplophyllum tuberculatum* is a valuable medicinal species with wide distribution in Mediterranean coastal desert of Egypt. It was characterized by high level of intraspecific morphological and biochemical variability. The objective of study was to assess the species status diversity for its coordinated conservation. **Materials and Methods:** Twenty-seven Operation Taxonomic Units (OTU's) from nine populations were collected along the Mediterranean coastal desert of Egypt. Eighty seven macro and micro morphological characters, besides 114 molecular attributes from both seed storage proteins and RAPD were evaluated. The agglomerative cluster analysis conducted and the dissimilarity matrix analyzed through three sorting methods, average linkage UPGMA, single linkage and Ward's. **Results:** The OTU's from 19-21 of Abo-Tamr village were discriminated from the other populations due to a large distance between the inflorescence and the first leaf, united sepals and united stamens at base. The studied samples achieved 8 common bands and both El-Karma (OTU's 7-9) and El-Gophera villages were distinguished by the absence of both polymorphism and the band at 77 KDa. The discrete DNA products per primer ranged from 12-19 bands, the percentage of polymorphism from 24-31% and fingerprinting bands from 1-4. **Conclusion:** Two biotypes of *H. tuberculatum* can be distinguished, OTU's from 19-21 of Abo-Tamr and the other ecotypes. The determination of the rank of these biotypes needs the study of the morphologically similar *H. blanche* to confirm the presence of the two species.

Key words: Biotype, electrophenogram, dendrogram, genetic diversity, genomic DNA, macromorphology, micromorphology, seed storage proteins

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Haplophyllum (Forssk.) A. Juss. (Rutaceae) comprises about 70 species belonging to different floristic regions, Irano-Turanian, Mediterranean, Saharo-Arabian and Sudano-Zambezian and the Mediterranean region encloses 13% of its species diversity[1,2]. However, *H. tuberculatum* prolongs from Morocco to Western Pakistan[3]. In Egypt, Rutaceae is monogeneric which represented by only one species; *H. tuberculatum*[4]. However, Boulos[5,6] assigned two species, *H. tuberculatum* and *H. poorei* C.C. Towns and the later collected from Sinai. The medicinal values of *H. tuberculatum* was established, it used as a remedy for headaches, arthritis, asthma, nausea, fever, gastric pains, skin discoloration, malaria, gynecological disorders and as an antispasmodic[2,7,8]. While, the essential oil exhibited antimicrobial activity and potentially active against lung, prostate and liver carcinoma cell lines[9-13].

The most inclusive morphological description of *H. tuberculatum* was ascertained by Townsend[3]. This species was characterized by high level of intraspecific morphological variability[1,14]. Raissi *et al.*[2] claimed that these differences related to harvest time and local, climatic and seasonal factors or these samples belonged to different chemotypes.

The main goal of the study was to provide an evaluation of *H. tuberculatum* status diversity in Mediterranean coastal desert of Egypt derived from macro and micromorphological characters, seed storage proteins and RAPD for its coordinated conservation.

MATERIALS AND METHODS

Collection of specimens: Twenty-seven Operation Taxonomic Units (OTU's)-representing nine populations of *H. tuberculatum* were collected in 2009-2010 along the Mediterranean coastal desert of Egypt as follow: 1-9 from El-Karma village (155 km Alexandria-Matruh road), 10-12 from El-Gophera village (95 km Matruh-Alexandria road), 13-15 from Ras El-Hekma (64 km Matruh-Alexandria road), 16-21 from Abo-Tamr village (41 km Matruh-Alexandria road), 22-24 from 22 km Matruh-Alexandria road and 25-27 from 5 km Matruh-Salloum road. Voucher specimens were kept at the Alexandria University Herbarium, Egypt (ALEX).

Macro- and micromorphology: Fifty-eight stem and leaf macro- and micromorphological characters and 29 floral and seed characters were evaluated (Appendix 1). For stem, leaf and petiole anatomy the uppermost, medium and lowermost internodes of the longest branch and the longest petiole of each OTU were sectioned according to Beveridge *et al.*[15]. The prepared slides were photographed using REICHERT AUSTRIA N. 365 475 by the aid of a digital Camera OPTICA, Italy. For floral characters, three mature flowers and ripened fruits from each OTU were examined by using both stereomicroscope and 0.001 cm graduated scale.

Seed protein electrophoresis: Fifty mg of seeds bulked OTU's from each population macerated with hexane, then centrifuged at 10.000 rpm for 10 min and stored at -20°C. The total seed proteins extracted through Laemmli[16] method. An aliquot of 15 µL of the extracted seed storage proteins loaded onto 12.5% resolving gel, using Hoffer scientific LKB 2001 vertical electrophoresis unit with LKB 2301 MACRODRIVE 1 power supply, at 15 mA/gel through the stacking gel, then completed at 25 mA/gel for about 5-6 h. The resultant gels were stained in Coomasei Briliant Blue R_{250} solution.

The bands produced by each sample were scored as binary characters and the percentage of polymorphism was determined from:

$$\text{Polymorphism (\%)} = \frac{\sum \text{Bands for each sample-} \sum \text{Common bands for all sample}}{\sum \text{Bands for all samples}} \times 100$$

Random amplified polymorphic DNA analysis (RAPD): Isolation of DNA from one gram fresh leaves pooled OTU's from each population carried out using cetyl trimethyl ammonium bromide (CTAB) method[17]. The analysis of RAPD achieved through six primers OPB05 (TGCGCCCTT), LA13

Appendix 1: List of characters and the mode of assessment of character states

Stem macromorphological characters
Length of stem (cm)
Length of lowest internode (cm)
Length of medium internode (cm)
Length of upper-most internode (cm)
Hair density
Sparsely hairy
Moderately hairy
Densely hair
Stem colour
Yellowish green
Light green
Green
Stem micromorphological characters
Uppermost internode
Epidermis thickness (mm)
Cortex thickness (mm)

Appendix 1: Continue

- Phloem thickness (mm)
- Xylem thickness (mm)
- Cortex/vascular bundle
- Pith thickness (mm)
- Pith/cortex
- Cortex+vascular bundle/pith
- Number of glands

Medium internode

- Epidermis thickness (mm)
- Cortex thickness (mm)
- Phloem thickness (mm)
- Xylem thickness (mm)
- Cortex/vascular bundle
- Pith thickness (mm)
- Pith/cortex
- Cortex+vascular bundle/pith
- Number of gland

Lowermost internode

- Epidermis thickness (mm)
- Cortex thickness (mm)
- Phloem thickness (mm)
- Xylem thickness (mm)
- Cortex/vascular bundle
- Pith thickness (mm)
- Pith/cortex
- Cortex+vascular bundle/pith
- Number of cortical glands

Leaf macromorphological characters

Leaf length (cm)
Leaf width (cm)
Leaf length/Leaf width
Leaf shape

- Linear lanceolate
- Linear

Leaf hair

- Sparsely hairy
- Moderately hairy
- Densely hairy

Leaf colour

- Light green
- Green

Petiole length (cm)
Petiole length/leaf length

Leaf micromorphological characters

Thickness of leaf upper epidermis (mm)
Thickness of leaf lower epidermis (mm)
Thickness of leaf upper palisade tissue (mm)
Thickness of leaf lower palisade tissue (mm)
Thickness of leaf spongy tissue (mm)
Thickness of leaf upper collenchyma (mm)
Thickness of leaf lower collenchyma (mm)
Thickness of leaf xylem (mm)
Thickness of leaf phloem (mm)
Number of lysogenous ducts on leaf
Thickness of petiole epidermis (mm)
Presence of petiole collenchyma

- Absent
- Present

Presence of petiole chlorenchyma

- Absent
- Present

Presence of petiole phloem fibers

Appendix 1: Continue

- Absent
- Present

Thickness of petiole phloem (mm)
Thickness of petiole xylem (mm)
Number of lysogenous ducts on petiole

Floral and seed morphological characters

Flowering period

- Two months
- Three months
- Four months

Colour of flower

- Yellow
- Dark yellow

Pedicel of flower

- Absent
- Present

State of sepals

- Free
- United

Distance between inflorescence and first leaf (cm)
Calyx length (cm)
Calyx width (cm)
Calyx length/calyx width
Shape of sepals

- Broadly ovate
- Orbicular
- Elliptic

Calyx apex

- Rounded
- Acute

Petal length (cm)
Petal width (cm)
Petal length/petal width
Shape of petal

- Elliptic
- Oblong
- Lanceolate

Petal apex

- Rounded
- Acute

Hairs on petals

- Absent
- Present

State of stamens

- Free
- United

Filament length (cm)
Anther length (cm)
Filament length/Anther length
Density of hairs on filaments

- Sparsely hairy
- Moderately hairy
- Densely hairy

Ovary length (cm)
Style length (cm)
Ovary length/style length
Ovary width (cm)
Ovary length/ovary width
Ovary shape

- Broadly globose
- Elliptic

Length of seed (cm)
Width of seed (cm)

(CACCACGCCT), OPU16 (CTGCGCTGG), OPB10 (CTGCTGGGAC), A7 (GAAACGGGTG), M13 (GAGGGTGGCGGTTCT) procured from Pharmacia Biotech (Amersham Pharmacia Biotech UK Limited, England HP79NA). DNA amplifications carried in a Gene Amp Polymerase Chain Reaction (PCR) System Cycler. The reaction consisted of 40 cycles, each cycle of denaturation at 94°C for 30 sec followed by annealing at 30°C for 30 sec and extension at 72°C for 30 sec. There was an initial delay for 15 min at 95°C at the beginning of the first cycle and 10 min delay at 72°C at the end of the last cycle as a post extension step[18] then the product stored at -20°C. Amplification products analyzed in 1.5% agarose, supplemented with ethidium bromide (0.5 µg mL^{-1}) and compared with standard DNA (mixture of λ Hind III and ΦX 179 DNA/Hae III). The gels photographed with polaroid film type Photo Doc-It™ Imaging Systems (photo Doc Uvb England) and the sharp discrete bands scored as binary characters. The percentage of polymorphic bands calculated as:

$$\text{Polymorphic bands}(\%) = \frac{\text{Polymorphic bands per primer}}{\text{Total bands per primer}} \times 100$$

Data analysis and computer programs: All types of data-morphological, anatomical, seed protein electrophoresis and RAPD were subjected to cluster analyses through PAST program[19]. The agglomerative cluster analysis conducted with both Euclidean and Jaccard coefficients for mixed data set[20]. The dissimilarity matrix analyzed through three sorting methods, average linkage UPGMA, single linkage and Ward's.

RESULTS

The generated dendrogram via different methods of sorting and coefficients resulted to congruent forms (Fig. 1). The average taxonomic distance of the studied OTU's was 0.91, where the OTU's from Abo-Tamr village (19-21) were isolated in group I. These OTU's were characterized by a large distance between the inflorescence and the first leaf, united sepals and united stamens at base. At 0.953 similarity level, four clusters were discriminated, clusters 1 and 2 represented the OTU's from both El-Karma (7-9) and Abo-Tamr (16-18) villages, respectively. Cluster 3 segregated OTU's from El-Karma village (1-6), Ras El-Hekma (13-15) and 22 km Matruh-Alexandria road (22-24). Cluster 4 assembled the OTU's from El-Gophera village (10-12) and 5 km Matruh-Salloum road (25-27).

The electrophenogram produced by SDS-PAGE (SDS-Polyacrylamide gel electrophoresis) of seed proteins of studied samples were presented in Fig. 2. The samples

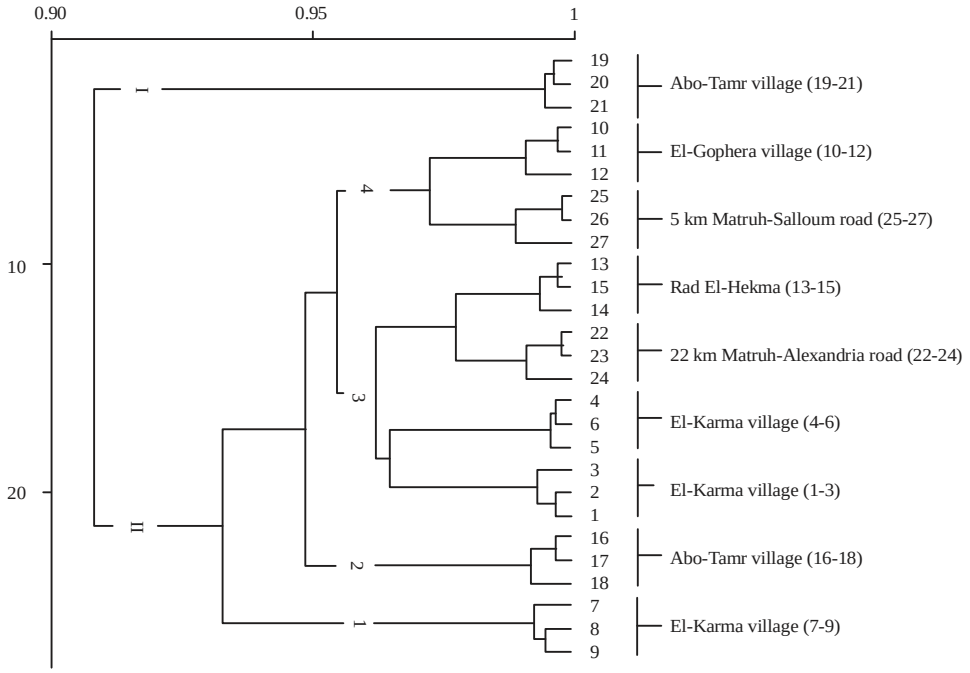

Fig. 1: Dendrogram resulting from UPGMA method of sorting of 27 OTU's of *H. tuberculatum*

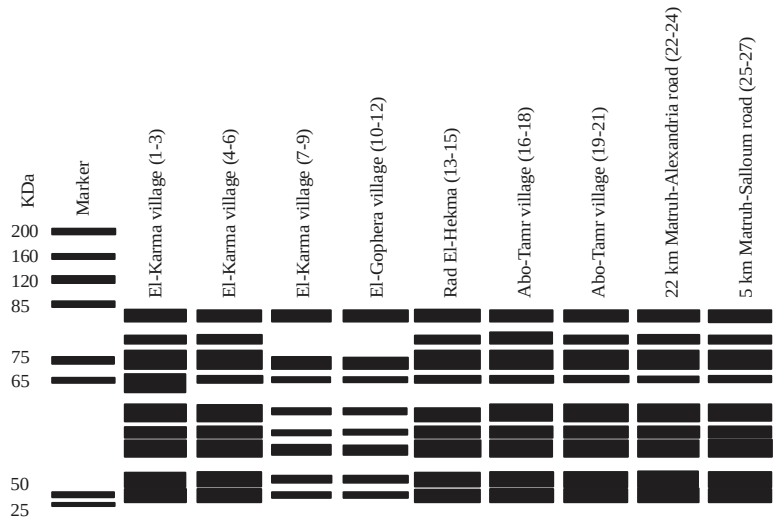

Fig. 2: Electrophenogram resulting from seed protein electrophoresis of the nine populations of *H. tuberculatum*

achieved 8 common bands at 50, 52, 54, 57, 61, 65, 75 and 80 KDa without any specific bands. The OTU's of both El-Karma (7-9) and El-Gophera villages were distinctive by the absence of both polymorphism and the band at 77 Kda.

The six primers effectively primed the amplification of genomic DNA samples of *H. tuberculatum* and generated 95 bands, of which 27 were common among studied populations (Fig. 3). The discrete DNA products per primer ranged from 12 bands generated from primer A7 to 19 bands generated from primers LA13 and OPB10. The percentage of polymorphism ranged from 24-31% in the samples from 5 km Matruh-Salloum road and Abo-Tamr village (16-18), respectively. Each sample achieved fingerprinting bands which varied from one band in the samples from El-Karma village (1-3), El-Gophera village and Ras El-Hekma to 4 bands in the sample from Abo-Tamr village (19-21) (Table 1).

DISCUSSION

The protection of biodiversity is central to conservation biology and the loss of it, particularly in arid and semi-arid regions, is considered as a global challenge. The destruction or alteration of natural habitat by humans is the major proximate threat to biodiversity worldwide[21,22]. Many of the less cuddly, less spectacular organisms are more important to future than most publicized endangered species. Genetic diversity is one of the three forms of biodiversity recognized by the World Conservation Union (IUCN) as deserving conservation and it is required for populations to evolve in response to environmental changes[23,24]. The loss of genetically distinct populations within species is an important problem as the loss of entire species. Level of genetic variation is of concern because of the potentially deleterious effects of inbreeding on the ability of a population to adapt to novel and changing environments[25].

In Egypt, diversity of natural communities has never been more highly valued than they are now, as they became increasingly threatened by the environmental crisis. Intensive urbanization and industrialization are among the most serious threats that contribute to declines in biological diversity and rapid fragmentation of habitats[26]. It is conceivable that since the Western Mediterranean region of Egypt has been subjected to human manipulations and the conservation activities should encompass all habitats and species. These anthropogenic disturbance leads to the diminishing of the germplasm reservoir which give the priority to study populations rather than whole species or higher taxa[27,28].

The current study declares low level of genetic diversity among *H. tuberculatum* populations and denotes that the species must be listed as a threatened one[29], which is confirmed through the uniformity in seed storage proteins. Ladizinsky and Hymowitz[30] clarified that despite the morphological differences the taxonomic categories below the species level still possess basically the same seed protein profiles. The study recommends primer LA13 for the population discernment because of its high percentage of polymorphism (32%) and high number of fingerprinting bands (5 bands).

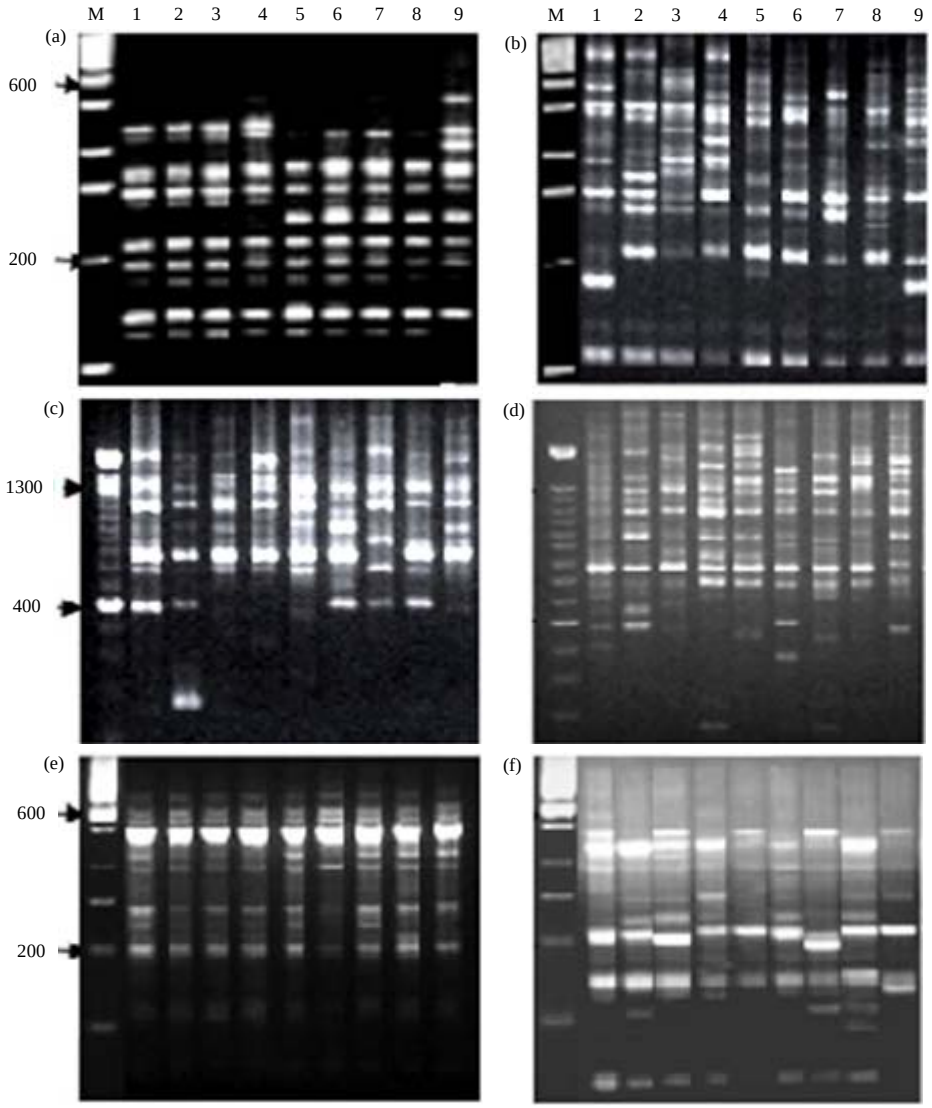

Fig. 3(a-f): RAPD pattern obtained from genomic DNA of the examined nine populations of *H. tuberculatum*, (a) OPB05, (b) LA13, (c) OPU16, (d) OPB10, (e) A7 and (f) M13

Lanes: M: Marker, 1: El-Karma village (1-3), 2: El-Karma village (4-6), 3: El-Karma village (7-9), 4: El-Gophera village (10-12), 5: Ras El-Hekma (13-15), 6: Abo-Tamr village (16-18), 7: Abo-Tamr village (19-21), 8: 22 km Matruh-Alexandria road (22-24), 9: 5 km Matruh-Salloum road (25-27)

The constructed dendrogram acquires from 77 of both morphological and anatomical attributes and 114 molecular characters segregates the OTU's from 19-21 of Abo-Tamr village at 0.91 similarity level mainly due to the large distance between inflorescence and the first leaf (7 cm and more), united sepals and united filaments at base. Notwithstanding, Boulos[5,6] defined the Egyptian *H. tuberculatum* with the fused filaments. This study suggests the presence of two morphs within the species in Egypt, the first with free filaments (OTU's from 19-21 of Abo-Tamr) and the second specifies with united filaments. These OTU's of Abo-Tamr are also specified with 4 specific bands, two from primer OPU16, one from primer OPB10 and one from primer M13. Salvo *et al*.[1] pointed out that *H. tuberculatum*, *H. blanche* and *H. buxbaumii* with high level of intraspecific morphological variability. It was also asserted that the first two species were morphologically similar and it is difficult to separate between them on the basis of morphology and the main distinguishing feature being the distinctly fused filaments of *H. blanche*[3].

Table 1: DNA banding patterns obtained from 6 Operon primers for the nine populations of *H. tuberculatum*

	El–Karma village (1-3)			El–Karma village (4-6)			El–Karma village (7-9)			El–Gophera village (10-12)			Ras El–Hekma (13-15)			Abo-Tamr village (16-18)			Abo-Tamr village (19-21)			22 km Matruh-Alexandria road (22-24)			5 km Matruh-Salloum road (25-27)		
Primers	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)	TNB	F. #	P (%)
OPB05	11	0	31	11	0	31	10	0	25	13	1	44	9	0	18	11	0	31	12	0	38	11	0	31	12	1	38
LA13	11	1	42	9	0	31	9	2	31	11	0	42	8	1	26	9	0	31	6	0	17	10	1	37	9	0	31
OPU16	7	0	26	7	1	26	6	0	20	7	0	26	8	0	33	9	2	40	8	2	33	5	0	13	7	0	20
OPB10	7	0	10	13	1	42	9	0	21	12	0	37	13	0	42	11	1	32	10	1	26	9	0	21	11	0	32
A7	9	0	20	7	0	10	8	0	10	8	0	10	9	0	20	8	0	10	9	0	20	10	0	30	9	0	20
M13	8	0	31	9	1	38	10	0	44	7	0	25	5	0	13	10	0	44	8	1	31	8	1	31	6	1	19
Mean P (%)			26.6			28			25			30.6			25			31			27.5			27			24
∑TNB	53			56			52			58			52			58			53			53			54		

TNB: Total number of bands, P (%): Percentage of polymorphic bands, # F: Number of fingerprinting bands

CONCLUSION

The study concluded that *H. tuberculatum* can be discriminated into two biotypes or higher taxonomic rank, OTU's from 19-21 of Abo-Tamr and the other ecotypes and to determine this rank need to extend the study to *H. blanche* to confirm the presence of the two species of Egypt.

SIGNIFICANCE STATEMENT

This study discriminates two biotypes of *H. tuberculatum* and declares the low level of genetic diversity among its populations in the Mediterranean coastal desert of Egypt and claims that the species must be listed as a threatened one. This study help for the determination of the germplasm collection for coordinated conservation of the species which subjected to severe anthropogenic disturbance.

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