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Research Article

Neurospora tetraspora D. Garcia, Stchigel & Guarro (= *Gelasinospora tetrasperma* Dowding) as a first record to Egypt

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ABSTRACT

An interesting isolate of homothallic, ascosporic filamentous fungus having 4-spored asci, was recovered once from a non-rhizosphere soil sample collected from a grapevine plantation in the village of El-Khawaled, Sahel-Saleem city, Assiut. It was isolated on DYM agar plate at 25°C in June 2008. The isolate was identified phenotypically and genotypically as *Neurospora tetraspora* (= *Gelasinospora tetrasperma*) and was deposited in the culture collection of Assiut University Mycological Centre as AUMC no. 6784 and ITS gene sequence of the strain was deposited at the National Center for Biotechnology Information (NCBI) and assigned a GenBank accession number JQ425383. *N. tetraspora* is being recorded in the current work for the first time in Egypt. By this addition, the genus is now known in Egypt by four species. A key is provided for the four species.

Keywords: Assiut; Grapevine; Genotypic and phenotypic characterization; ITS; Soil.

1. INTRODUCTION

During the course of a survey of mycobiota of grapevine plantations in Assiut Governorate, Egypt in 2008/2009, an interesting ascosporic species was isolated from the soil by dilution-plate method [1] on dichloran yeast extract malt extract agar plate, DYM [2]. The macro- and micro-morphological characteristics of the isolate proved to be sufficiently similar to those of the genera *Neurospora*/*Gelasinospora* [3-6].

The generic name *Neurospora* was introduced by Shear & Dodge [3] for four species characterized by dark ascospores, with a grooved surface with longitudinal ribs. Later, numerous species were added [7-14] and so far 12 species have been accepted. In 1933, the genus *Gelasinospora* was erected by Dowding [15] to accommodate two species with ascospores similar to those of *Neurospora*, but with a pitted wall. Further species were added by others [4, 16-25].

The phylogenetic relationships between members of *Gelasinospora* and *Neurospora* were investigated by Dettman et al. [26] analysing four genes: ITS/5.8S rRNA, glyceraldehyde 3-phosphate dehydrogenase (*gpd*), mating-type *A-1*, and mating-type *a-1*. Although only five species of *Gelasino-*

spora were included in that study, they concluded that pitted vs. ribbed ascospores is not a useful criterion for the separation of these genera. Also, partial sequences of the 28S rDNA gene from 27 species of both genera were analysed by Garcia et al. [6] to infer their phylogenetic relationships. Species of the two genera were interspersed in the different clades and confirmed that they are genetically very similar. The name *Neurospora* has priority under the Code, and *Gelasinospora* was treated as a synonym of *Neurospora*, whose generic diagnosis is amended, and a dichotomous key to 49 species of *Neurospora* recognized in the genus was presented [6].

The internal transcribed spacer (ITS) region, located between the 18S and 28S rRNA genes, is an area of particular importance in discriminating between closely related species or at intraspecific level, because it has areas both of high conservation and high variability. ITS sequencing has been used to identify *Neurospora* species [6, 27].

In Egypt, of this genus only the anamorphic stage of *Neurospora crassa* (*Chrysonilia crassa*), the 4-spored ascosporic species (*Neurospora tetrasperma*) and the 8-spored ascosporic species *Neurospora hippopotama* (= *Gelasinospora hippopotama*) were reported. *N. crassa* has been reported from animal and bird pens materials [28], soybean meal [29], combine harvestor sorghum dust [30], caraway and cumin seeds [31], the air of Bahariya Oasis, Western Desert [32], grapevine soil, Assiut [33]. *N. hippopotama* (J.C. Krug, R.S. Khan & Jeng) D. Garcia, Stchigel & Guarro (= *Gelasinospora hippopotama* Krug, Khan & Jeng) was first isolated and described from sandy soil, Dakhleh Oasis, Western Desert in 1994 [24]. Also, *N. tetrasperma* was recently recorded from the air of citrus plantations in Sahel-Saleem city, Assiut, Egypt [34].

2. MATERIALS AND METHODS

2.1. Strain examined

The strain examined was isolated from a soil sample collected from a grapevine plantation in El-Khawaled village, Sahel-Saleem city at approximately 25 km south-east of Assiut town, Egypt (about 6 km to the east border of the river Nile). The strain was isolated in June 2008, on dichloran yeast exract malt extract agar, DYM [2] at 25°C, by Zeinab Soliman in a laboratory of Assiut University Mycological Centre (AUMC), Assiut, Egypt. It was misidentified as *Gelasinospora bonaerensis* [33] based on its ITS sequence similarity with the type strain (99%). However, the strain produces 4-spored asci and that of *G. bonaerensis* 8-spored asci.

2.2. Morphology

For macromorphological observations, the strain was grown in the dark on Czapek yeast autolysate agar (CYA) and potato sucrose agar (PSA) at 25ºC for 7 days (for medium formulations see Samson et al. [35]. For micromorphological observations, microscopic mounts were made in lactophenol cotton blue from PSA plates after 10-15 day-old culture.

2.3. Growth of the fungus and DNA extraction and sequencing

The fungus was grown on CYA plates and incubated at 25°C for 7 days. A small amount of fungal mycelia was scraped and suspended in 100 µl of distilled water and boiled at 100°C for 15 minutes and stored at -70°C. Samples were sent to SolGent Company (Daejeon, South Korea) to carry out the whole procedure from DNA extraction till the final step of DNA sequencing. Fungal DNA was extracted and isolated using SolGent purification bead. Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA were amplified using universal primers: ITS 1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS 4 (5' - TCC TCC GCT TAT TGA TAT GC - 3'). Then amplification was performed using the polymerase chain reaction (PCR) (ABI, 9700). The PCR reaction mixtures were prepared using Solgent EF-Taq as follows: 10X EF-Taq buffer 2.5 µl, 10 mM dNTP (T) 0.5 µl, primer (F-10p) 1.0 µl, primer (R-10p) 1.0 µl, EF-Taq (2.5U) 0.25 µl, template 1.0 µl, DW to 25 µl. Then the amplification was carried out using the following PCR reaction conditions: one round of amplification was performed consisting of denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min,

with a final extension step of 72°C for 5 min.

Then the PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. Then the purified PCR products were reconfirmed (using size marker) by electrophoreses of the PCR products on 1% agarose gel. Then these bands were eluted and sequenced. Each sample was sequenced in the sense and antisense direction. Contigs were created from the sequence data using the CLCBio Main Workbench program. The sequence obtained was further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Sequence obtained together with those retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov) were subjected to the Clustal W analysis using MegAlign software version 5.05 (DNASTAR Inc., Madison, Wisconsin, USA) for the phylogenetic analysis [36].

3. RESULTS AND DISCUSSION

Neurospora tetraspora D. Garcia, Stchigel & Guarro 2004 = *Gelasinospora tetrasperma* Dowding, Can J Res 1933; 9(3): 294 = *Gelasinospora calospora f. tetrasperma* (Dowding) C. Moreau & M. Moreau 1951

3.1. Culture morphology

AUMC strain 6784 on PSA and CYA at 25ºC after 7 days: colonies filling the agar plates (9 cm diam.), producing a rapid and dense growth of mycelium which is white at first, becoming gray or slightly pinkish (Fig. 1).

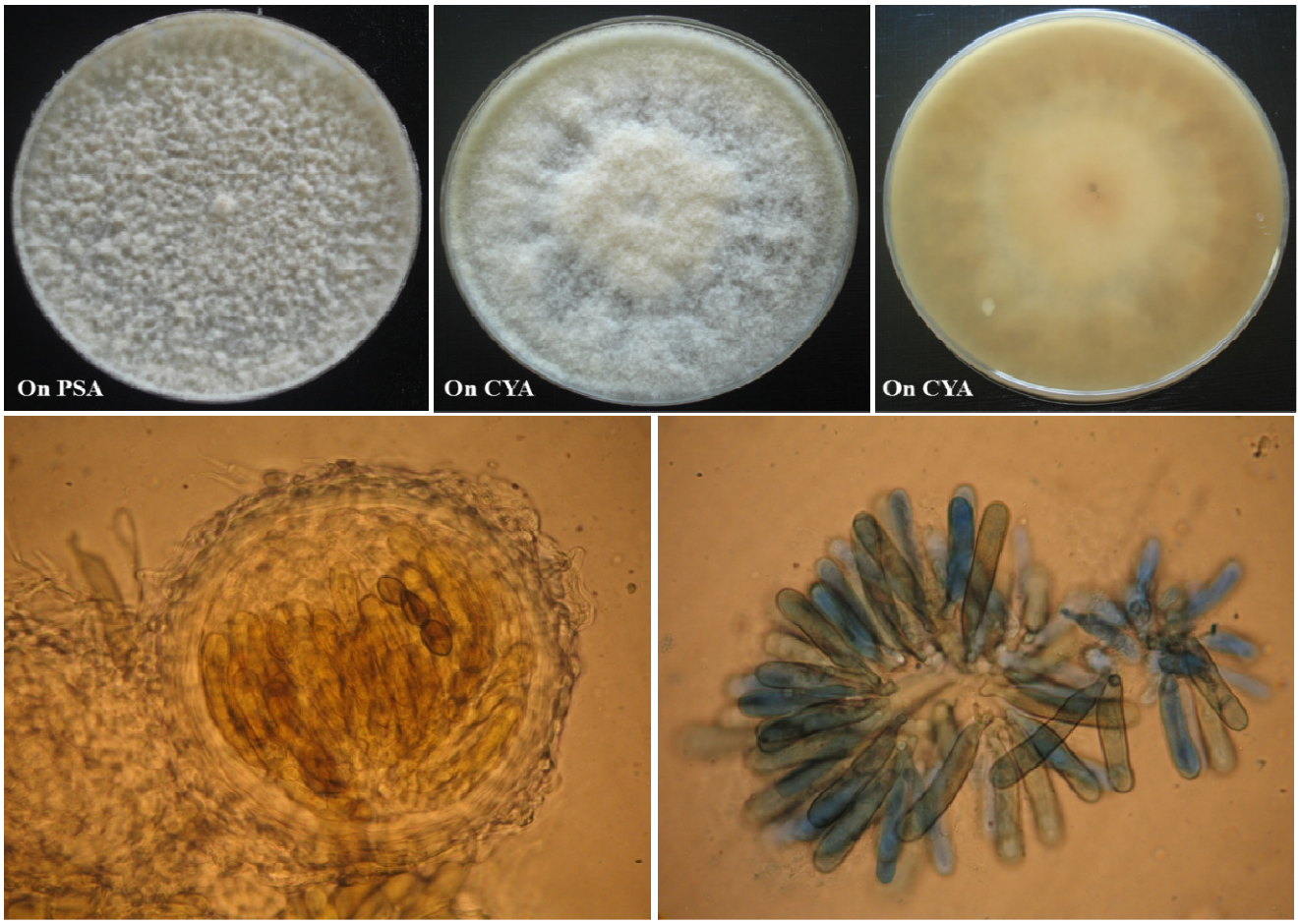

Figure 1. *Neurospora tetraspora* (*Gelasinospora tetrasperma*) AUMC 6784, 7-day-old colony obverse on PSA (upper left) and on CYA (upper middle) and reverse on CYA (upper right), ascoma (lower left) and asci (lower right).

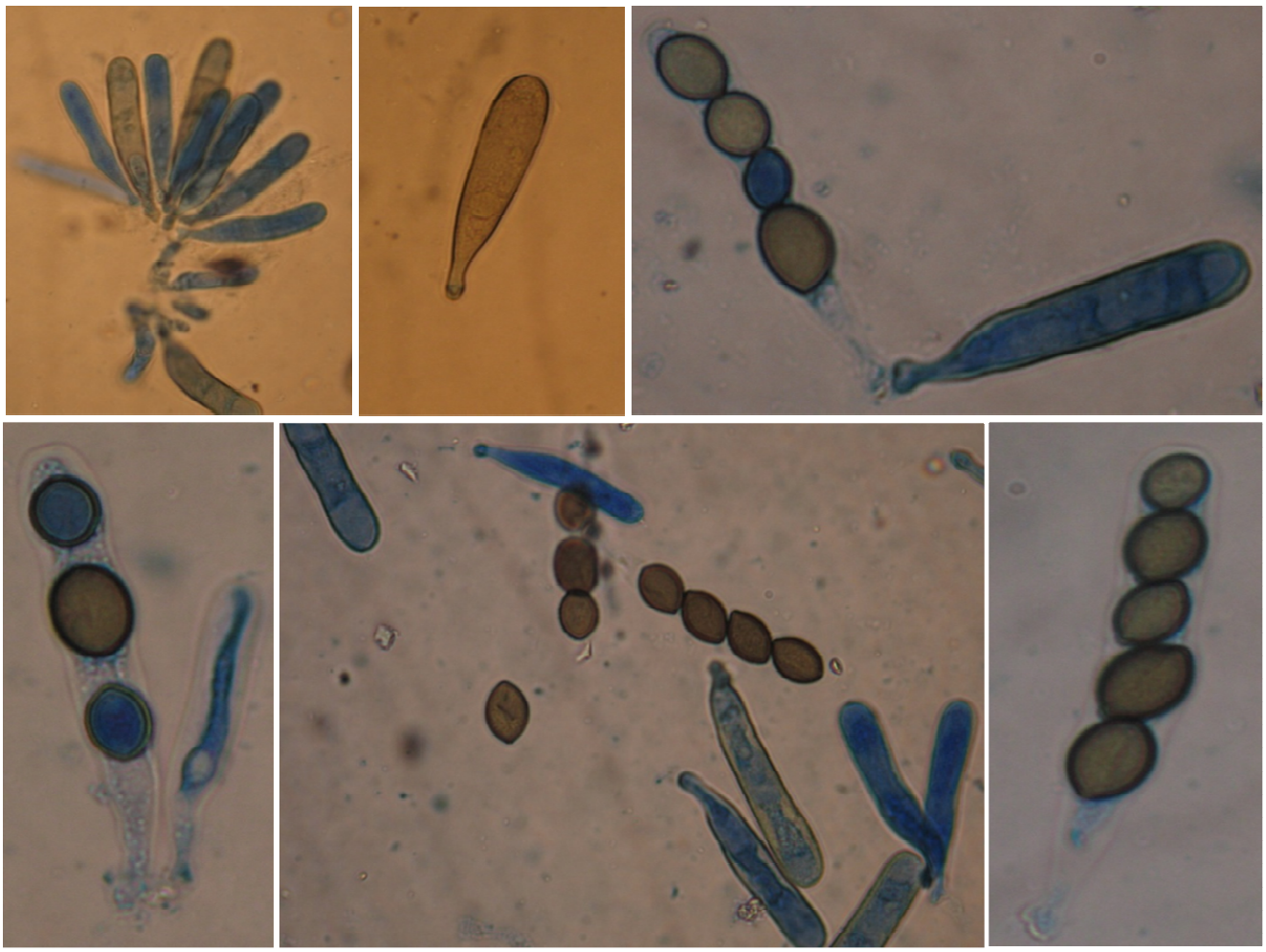

Figure 2. *Gelasinospora tetrasperma* AUMC 6784: asci and ascospores.

3.2. Micromorphology

Ascomata superficial or slightly immersed, black, pyriform, ostiolate, 450-600 x 270-450 µm. Asci cylindrical, 4-spored, but 3- and 5-spored asci were seen in the AUMC strain. Ascospores uniseriate, ellipsoidal or elongate, 22-33 x 12-20 µm, broadly rounded at the ends, dark brown, becoming black and opaque, walls with slightly irregular pits, one germ pore at each end (Figs. 1 & 2). Asexual stage (conidial state) not observed.

3.3. Genotypic characterization

Comparison of ITS sequence of AUMC strain 6784 with eight *Neurospora/Gelasinospora* species showed close similarity ranging from 97.91%-98.44%, with the highest ITS similarity to the type strains of *G. tetrasperma* (98.44%, with no gaps) and *G. udagawae* (98.44%, with 2 gaps) followed by *N. dictyophora* (98.43%, with 1 gap) and *N. pannonica* (98.38%, with 2 gaps). The previous species along with those presented in Table 1 produce 8-spored asci, but only *G. tetrasperma* has 4-spored asci. Thus, morphological characteristics along with the highest ITS sequences similarity and no gaps with the type species confirmed its identity as *G. tetrasperma* (= *Neurospora tetraspora*) (Table 1, Fig. 3). From this finding, both molecular and phenotypic features are helpful in such identification.

3.4. Distribution

N. tetraspora (= *G. tetrasperma*) was found on dung of ptarmigan, horse, rabbit and cow from Ontario, Quebec, Manitoba, Venezuela, England and Germany and was also found on seeds of *Beta vulgaris* L. and *Festuca rubra* L. from Quebec [4] and from *Vaccinium* sp. in Quebec [37]. It was

also reported in Canada, England, Finland, Norway, Russia, Spain, Sweden, and USA, from dung and soil [6]. Also, *N. tetraspora* was one of the most frequent isolated ascomycete microfungi from rotting wood in Norhern Albert forests, Canada suggesting that these fungi are significant component of wood decaying fungal communities [38].

In the current study, it was reported from a non-rhizosphere soil sample collected from a grapevine plantation in El-Khawaled village, Assiut, Egypt. The presence of this fungus together with other fungal communities in grapevine soil [33] may help in decaying the woody and non-woody debris of vine.

Living culture of the fungus is deposited at the culture collection of the Assiut University Mycological Centre (AUMC 6784), Assiut, Egypt. The strain is registered with a GenBank accession number JQ425383 (http://www.ncbi.nlm.nih.gov.). It is worthy to mention that only 2 strains registered in the GenBank and these are CBS178.33^{T}= NR_077163 [27], GEN002=GQ922543 [39], in addition to ours.

3.5. Key to the four species of *Neurospora* so far recorded in Egypt

1. Homothallic ……..... 2
1. Heterothallic, asci 8-spored; ascospores with longitudinal striations, 27-36 x14-16 µm; anamorph present, colonies orange, conidia 6-8 µm diam ..... *N. crassa*
2. Asci 8-spored, ascospores pitted, 35-45 x 25-32 µm …..... *N. hippopotama* (= *G. hippopotama*)
2. Asci 4-spored ……..…….…….....…… 3
3. Ascospores with longitudinal striations, (24-)31-36(-40) x 15-19(-22) µm, anamorph present .... *N. tetrasperma*
3. Ascospores pitted, 22-33 x 12-20 µm; anamorph absent .....….....…..... *N. tetraspora* (= *G. tetrasperma*)

Table 1. The Assiut University Mycological Centre accession number, AUMC 6784 of *N. tetraspora* isolated from grapevine soil with its accession GenBank number together with the closest match in the GenBank database and sequence similarity in percent to the match as inferred from Blastn searches of ITS sequences.

AUMC number	Accession GenBank number	Length (bp)	Closest GenBank match # ITS	Sequencing similarity (%)	Gaps	Species	References
6784	JQ425383	590	CBS178.33^{T}=NR_077163	569/578 (98.44%)	0/578 (0%)	*G.tetrasperma*	[27]
			CBS 298.63^{T}=AY681176	575/587 (97.96%)	1/587 (0%)	*N. terricola*	[27]
			FGSC1889^{T}= NR_077131	544/554 (98.19%)	1/554 (0%)	*N. terricola*	[26]
			CBS 435.74^{T}=AY681184	569/579 (98.27%)	1/579 (0%)	*G. saitoi*	[27]
			CBS309.91^{T}=NR_103582	567/576 (98.44%)	2/576 (0%)	*G. udagawae*	[27]
			CBS 529.95^{T}=AY681181	565/574 (98.43%)	1/574 (0%)	*N. dictyophora*	[27]
			CBS 571.69^{T}=GQ922531	562/572 (98.25%)	2/572 (0%)	*N. africana*	[39]
			FGSC 1740^{T}=NR_077130	550/560 (98.21%)	2/560 (0%)	*N. africana*	[26]
			CBS561.94^{T}=NR_137146	562/574 (97.91%)	1/574 (0%)	*G. hippopotama*	[27]
			CBS 270.91^{T}=GQ922532	548/557 (98.38%)	2/557 (0%)	*N. pannonica*	[39]
			CBS315.74^{T}=NR_144834	508/596 (85.23%)	31/596 (5%)	*C. madrasense*	[40]

N = Neurospora, G = Gelasinospora, C = Chaetomium.

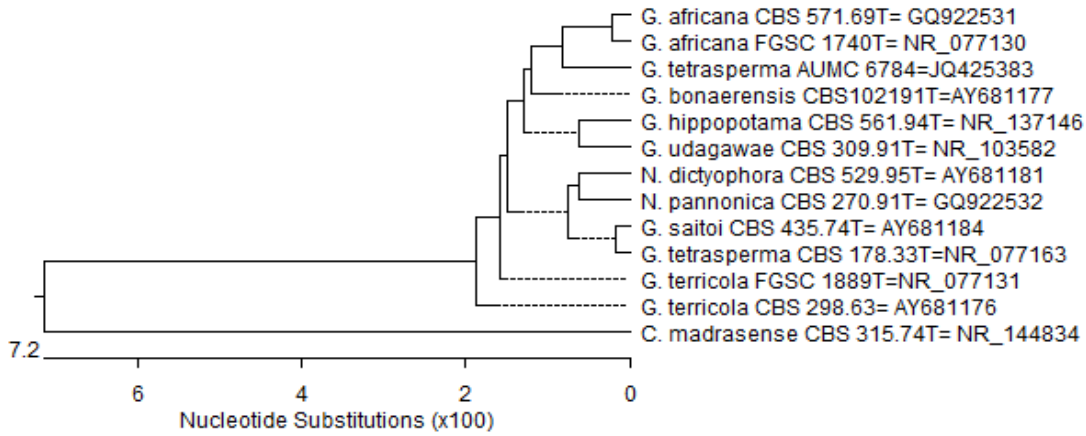

Figure 3. Phylogenetic tree based on DNA sequence data of ITS region of *N. tetrasperma* AUMC 6784 (= *G. tetraspora*), compared with 11 reference strains in Genbank of closely related *Neurospora/Gelasinospora* species and *Chaetomium madrasense* as an outgroup.

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AUTHOR'S CONTRIBUTION

All authors shared in the experimental designed and assisted in the work, formatting the tables, interpretation of data and in preparation and editing of the manuscript. The final manuscript has been approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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