Studies in Fungi 2 (1): 199–207 (2017) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/sif/ 2/1/22

Reniforma rhynchophori sp. nov. (Basidiomycota, Microbotryales) from guts of red palm weevil

Moubasher AH[1,2*], Abdel-Sater MA[1,2] and Soliman Z[2]

[1] *Department of Botany and Microbiology, Faculty of Science, Assiut University, P.O. Box 71526, Egypt*
[2] *Assiut University Mycological Centre, Assiut University, P.O. Box 71526, Egypt*

Moubasher AH, Abdel-Sater MA, Soliman Z 2017 – *Reniforma rhynchophori sp. nov.* (Basidiomycota, Microbotryales) from guts of red palm weevil. Studies in Fungi 2(1), 199–207, Doi 10.5943/sif/2/1/22

Abstract

During a survey of mycobiota inhabiting guts of red palm weevil (*Rhynchophorus ferrugineus*) in Assiut area of Egypt, two interesting yeast isolates were obtained from 2 different gut samples (out of 11 gut samples investigated). Two isolates were identified phenotypically and genotypically with 87% ITS sequencing similarities with the type strain *Reniforma strues*. The name *Reniforma rhynchophori* was assigned for the new species. The two strains were deposited in Assiut University Mycological Centre Culture Collection and AUMC numbers 10263^{T} (as the type strain) and AUMC 10264 were given. The ITS gene sequences for AUMC 10263^{T} & AUMC 10264 were also deposited at the National Center for Biotechnological Information (NCBI) and accession numbers are given as KX011609 and KX015891 respectively. Full description and photos of the new species are presented. This new species was recorded as the second species of the genus *Reniforma*.

Key words – Assiut – intestinal tract –ITS sequencing – *Reniforma strues*

Introduction

The genus *Reniforma* was described by Pore & Sorenson (1990) to accommodate *Reniforma strues* Pore and Sorenson, budding yeast with reniform cells. *Reniforma strues* was isolated several times on *Prototheca* Isolation Medium (PIM) (Pore 1973) from the biological film of a primary wastewater treatment plant in Morgantown, West Virginia. *Reniforma strues* is a morphologically unique anamorphic yeast species, forming reniform cells and buds with a flat base circumscribed by a brim (Pore & Sorenson 1990, Pore & Fell 2011). Cells may be arranged in vertical stacks of two or more cells resembling stacks of books or bricks. Sexual reproduction was not detected. *Reniforma strues* did not ferment sugars and assimilation was limited to a small number of carbon compounds while nitrate was not assimilated. The cell wall, urease, and the diazonium blue B reactions were those of basidiomycetous yeasts (Pore & Sorenson 1990). Up-to-date, the genus is known only by the type species *R. strues*.

Materials and Methods

Collection of insect samples and isolation of yeast strains

Submitted 1 August 2017, Accepted 20 October 2017, Published 1 December 2017
Corresponding Author: Abdel-Aal H Moubasher – e-mail – ahamaumc@yahoo.com

A total of 11 samples of red palm weevils, RPW (*Rhynchophorus ferrugineus* Olivier, order: Coleoptera, family: Curculionidae) were kindly supplied by the Department of Plant Protection, Faculty of Agriculture, Assiut University in July 2013, February and March 2014. Insects obtained were put in separate clean containers (bottles or plastic bags) and kept for 3 days with slightly moistened filter paper so that surface debris is removed. Insects were then put each in a 250 ml. conical flask with 95% ethyl alcohol for 1-2 min and washed several times with sterile distilled water for surface sterilization. They were dissected using forceps and a cutter in a sterile Petri plate, and the gut was removed aseptically from the body. The gut is transferred into a sterile 1.5 ml Eppendorf tube containing 200 µl of 0.7% sterile saline solution and crushed with a pipette tip, and all the solution (including gut pieces) was spread over the surface of yeast extract-malt extract-peptone-glucose (YM) and acidified YM (pH = 3.7) (Wickerham 1951) agar. The inoculated plates were incubated at 25°C for 3-7 days (Suh & Blackwell 2004) and of the developing yeast colonies were picked up, and streaked three times by crossing the lines onto a YM agar plates to obtain single colony isolation.

Physiological characterization of yeast strains

Fermentation of sugars and oxidative utilization of carbon compounds were performed according to Barnett et al. (2000). Assimilation of nine nitrogen compounds, growth at high osmotic pressure, in the presence of cycloheximide and production of extracellular starch-like compounds were tested by the methods described by Suh et al. (2008).

Genotypic identification of yeast strains

The yeast strains were grown on YM plates and incubated at 25°C for 2 days. A small amount of yeast growth was scraped off and suspended in 100 µl of distilled water and boiled at 100°C for 15 mins following the manufacturer's protocol (SolGent Company, Daejeon, South Korea) and sent to Korea for DNA extraction and sequencing. Yeast DNA was extracted and isolated using SolGent purification beads at this company. Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA were amplified using ITS1 (5'- TCC GTA GGT GAA CCT GCG G -3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). Then amplification was performed using the polymerase chain reaction (PCR) (The GeneAmp® PCR System 9700 thermal cycler, Applied Biosystems, Foster City, California, USA). 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 (2.5 U) 0.25 µl, template 1.0 µl, DW up to 25 µl. Then the amplification was carried out using the following PCR reaction conditions: one round of amplification consisting of denaturation at 95°C for 15 mins 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 mins The PCR products were then purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. After that, the purified PCR products were reconfirmed (using size marker) by electrophoreses of the PCR products on 1% agarose gel. These bands were then eluted and sequenced. Each sample was sequenced in sense and antisense direction. Contigs were created from the sequence data using the CLC Genomics Workbench 8.0.1 program. The contig obtained from each isolate was further analysed using BLAST from the National Center of Biotechnology Information (NCBI) website. Sequences obtained together with those retrieved from the GenBank database were subjected to phylogenetic analysis using the neighbour-joining (NJ) method (Saitou & Nei 1987).

Results

Two interesting strains with kidney-shaped cells were isolated from 2 different gut samples that could be assigned to the genus *Reniforma* based on their phenotypic and genotypic characteristics (with 87% ITS similarity with the type strain of *Reniforma strues*). These strains were described as a new species in the genus *Reniforma*.

Reniforma rhynchophori Moubasher, Abdel-Sater & Zeinab Soliman, sp. nov. Figs 1–4

MycoBank: MB821288

Etymology – *rhynchophori*, named after the host genus of the insect, *Rhynchophorus ferrugineus* from which the fungus was isolated.

Cell morphology **–** when cultured on yeast extract malt extract agar (YM) for 3 d (Fig. 1), the cells kidney-shaped, observed as singles, pairs or small clusters (Fig. 2–4), with a flat base and tended to be arranged in stacks or layers (Fig. 3). The base of the cell was appended with a brim radiating from the basal margin of the cell. The brim 0.5–1.0 µm wide. Vegetative cell diameters 4.5–6.0 × 4.0–5.0 µm with an average of 5.0 × 4.5 µm. Budding enteroblastic, monopolar, bipolar and multipolar. Buds (2–) 3 (–4) µm diam, Pseudomycelium not produced (Fig. 2–4).

Growth on agar – growth on YM agar rapid at 25°C especially when few drops of water are added to the agar surface. Colonies cream to off-white.

Growth in broth – after 3–15 days of incubation, it forms a pellicle plus sediment when grown to test its ability to assimilation carbon compounds (e. g. on D-glucose, α, α-trehalose, glycerol, xylitol, D-glucitol, D-mannitol, glucono-d-lactone, D-gluconate, D-galacturonate, and ethanol.

Fermentation – ability of the 2 strains to ferment sugars was lacking.

Assimilation of carbon compounds – D-glucose, α, α-trehalose, inulin, glycerol, D-mannitol, 2-keto-D-gluconate, citrate, methanol and ethanol were assimilated by the 2 strains. However, the two strains gave different growth responses towards other carbon compounds (Table 1).

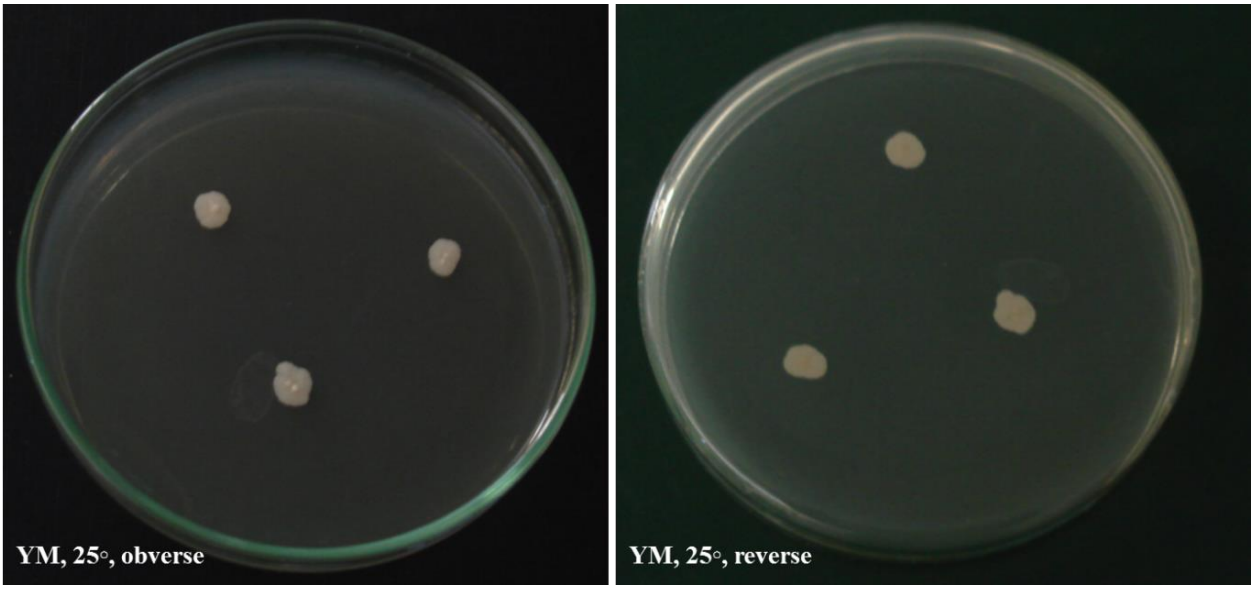

Fig. 1 – *Reniforma rhynchophori* AUMC 10263, growth on YM plates.

Assimilation of nitrogen compounds – ethylamine, L-lysine, D-glucoseamine and D-tryptophane were assimilated. Nitrate, nitrite, creatine, creatinine and imidazole were not assimilated by the 2 strains.

Other miscellaneous tests – growth was not detected on 0.01% and 0.1% cycloheximide, 50% D-glucose, 60% D-glucose, 10% NaCl and 16% NaCl. Hydrolysis of urea was positive, reaction to diazonium blue B was positive and starch-like compound was not produced (Table 1).

Genotypic characterization – genetic characteristics of the new species revealed 87% ITS similarity with the type strain of *Reniforma strues* (Table 2). Phylogenetic placement of *Reniforma rhynchophori* sp. nov. together with *R. strues* and other closely related species based on sequence of rDNA ITS region is presented in Fig. 5.

Habitat – the new species was isolated from the guts of red palm weevil, *Rhynchophorus ferrugineus* obtained from Department of Plant Protection, Faculty of Agriculture, Assiut University, Assiut, Egypt.

Holotype – the type strain has been deposited in the culture collection of Assiut University Mycological Centre as AUMC 10263^{T} (collection date 15-Feb-2014, collected by Zeinab Soliman from the gut of red palm weevil) and its ITS sequence was deposited at the National Center for Biotechnological Information (NCBI) and accession number is given as KX011609 and the MycoBank number is MB821288. The other strain (AUMC 10264 with ITS accession number KX015891, collection date 19-Mar-2014; by Zeinab Soliman) was also deposited.

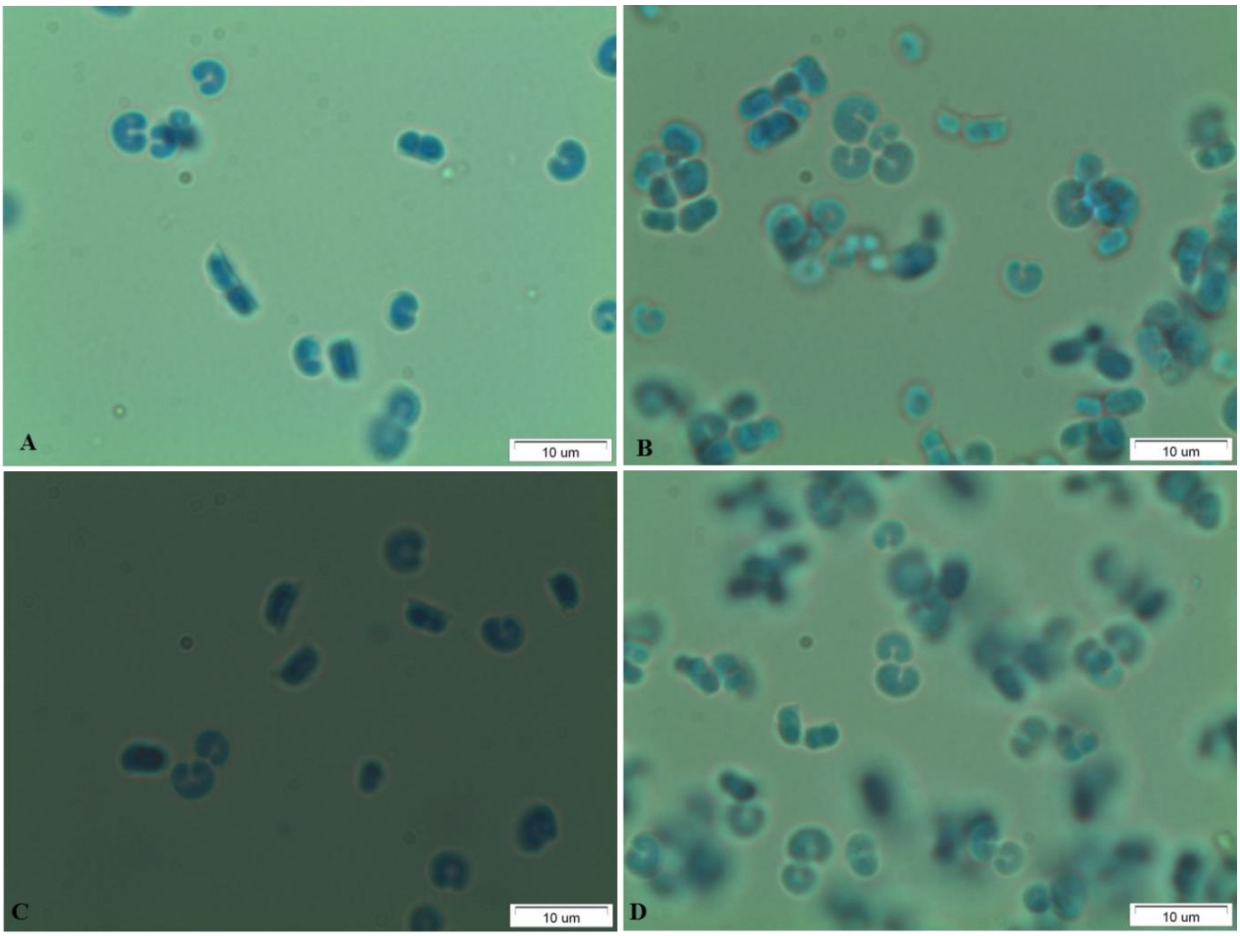

Fig. 2 – *Reniforma rhynchophori* AUMC 10263, A-D: reniform-shaped vegetative and budding cells.

Discussion

Basidiomycetous yeasts usually produce a positive Diazonium Blue B (DBB) test (van der Walt & Hopsu-Havu 1976, Hagler & Ahearn 1981) and a positive urease test (Weijman *et al.* 1988), whereas ascomycetous yeasts are typically DBB negative and urease negative. The new *Reniforma* species (*R. rhynchophori***)** and the type species (*R. strues*) are DBB and urease positive. These characteristics confirm basidiomycetous affinities for both species. The reniform-shaped vegetative and budding cells are a unique character in *Reniforma*. However, a number of ascomycetous yeasts produce reniform ascospores (some species of *Kluyveromyces*) or hat-shaped ascospores (*Pichia* species), but *Kluyveromyces* reniform ascospores lack the brim observed in *R. strues* and *R. rhynchophori* cells and the *Pichia* hat-shaped ascospores with a brim. In addition, vegetative and budding cells of *Kluyveromyces* and *Pichia* species are spherical or ellipsoidal and they ferment glucose and occasionally other sugars as well (van der Walt & Yarrow 1984), whereas

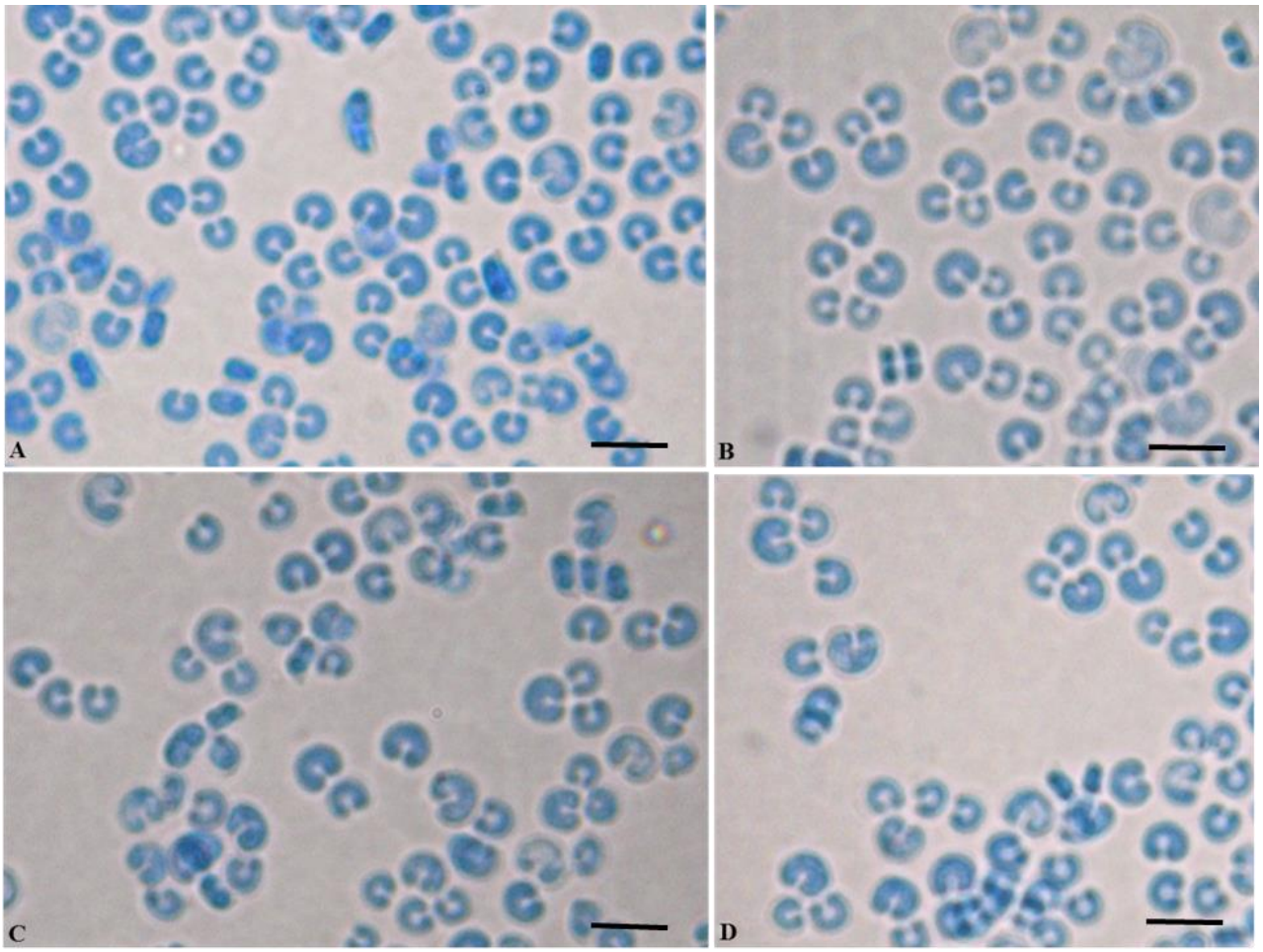

Fig. 3 – *Reniforma rhynchophori* AUMC 10263, A–D reniform–shaped vegetative and budding cells, B–D vegetative cells solitary or arranged in stacks of 2-5 (mounted in lactophenol cotton blue), bar = 10 µm.

Table 1 – Physiological comparison of the tested strains of the new yeast species *Reniforma rhynchophori* isolated from insect guts versus the type strain of the previously described species *R. strues*.

		R. rhynchophori		***R. strues***
Test /Species strain number	**Code**	**AUMC 10263^T**	**AUMC 10264**	**CBS 8263^T**
Fermentation (D-glucose)	F1	-	-	-
Assimilation of carbon compounds				
D-glucose	C1	+*	+*	+
D-galactose	C2	-/+	+/-	-
L-sorbose	C3	-/w	+	-
D-glucosamine	C4	-/w	-	-
D-ribose	C5	-/+	-	-
D-xylose	C6	-/+	+/-	-
L-arabinose	C7	-/+	-	-
L-rhamnose	C9	-/+	+/-	-
Sucrose	C10	-	+/-	-
Maltose	C11	-/w	+/w	-
α, α-trehalose	C12	+*	+*	-
Methyl-α-D-glucoside	C13	-/+	+	-
Cellobiose	C14	-/w	-/+	-

Table 1 – Continued.

Test /Species strain number	Code	*R. rhynchophori* AUMC 10263^T	*R. rhynchophori* AUMC 10264	*R. strues* CBS 8263^T
Salicin	C15	-	w/+	-
Arbutin	C16	-/w	w/+	d
Lactose	C18	-/+	w	-
Raffinose	C19	-/w	-/w	-
Melezitose	C20	-	+	-
Inulin	C21	+	+	-
Soluble starch	C22	-	+	-
Glycerol	C23	+*	+*	-
Meso-erythritol	C24	-/w	-/w	-
Ribitol	C25	-/w	-/w	-
Xylitol	C26	-/w	-/+*	-
D-glucitol	C28	-/w	w/+*	-
D-mannitol	C29	+*	+*	+
Galactitol	C30	-/+	-/+	-
Myo-inositol	C31	-/+	+	-
Glucono-d-lactone	C32	w/-	w/+*	+
2-keto-D-gluconate	C33	+	+	-
D-gluconate	C35	+/-	+*	-
D-glucuronate	C36	-	-/+	-
D-galacturonate	C37	-	-/+*	-
DL-lactate	C38	-/+	+	-
Succinate	C39	-	+	-
Citrate	C40	+/w	w/+	+
Methanol	C41	+	w/+	-
Ethanol	C42	+*	+*	-
Propane 1,2 diol	C43	-/-	-/+	-
Butane 2,3 diol	C44	w/-	-/+	-
Quinic acid	C45	-	+/-	-
Nitrogen compounds				
Nitrate	N1	-	-	-
Nitrite	N2	-	-	-
Ethylamine	N3	+	+	+
L-lysine	N4	+	+	w-
Creatine	N6	-	-	-
Creatinine	N7	-	-	-
D-glucosamine	N8	+	+	-
Imidazole	N9	-	-	-
D-tryptophane	N10	+	+	+
Miscellaneous				
0.01% cycloheximide	O1	-	-	-
0.1 % cycloheximide	O2	-	-	-
50% D-glucose	O4	-	-	-
60% D-glucose	O5	-	-	-
10% NaCl	O6	-	-	-
16% NaCl	O7	-	-	-
Starch formation	M1	-	-	-
Urease	M3	+	+	+
Diazonium Blue B	M4	+	+	+

+: growth; w: weak growth; d, delayed; –: no growth; * means surface growth

Data of *R. strues* CBS 8263^T are derived from Pore & Sorenson (1990), Barnett et al. (2000) and Wang et al. (2016).

Table 2 – Assiut University Mycological Centre accession number (AUMC) of the new yeast (*Reniforma rhynchophori*) isolated from red palm weevil guts with accession GenBank numbers given 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 (%)	Species
10263	KX011609	583	CBS8263^T=NR_073314	518/594(87.21)	*Reniforma strues*
			CBS9038^T=KY101703	167/178(94%)	*Bannoa ogasawarensis*
			JCM10336^T=NR_121198	167/178(94%)	*Bannoa hahajimensis*
			CBS 9041^T=AB035721	167/178(94%)	*Bannoa bischofiae*
			CBS9040^T= KY101704	166/178(93%)	*Bannoa syzygii*
10264	KX015891	592	CBS 8263^T=NR_073314	525/601(87.35)	*Reniforma strues*
			CBS9038^T=KY101703	167/178(94%)	*Bannoa ogasawarensis*
			JCM10336^T=NR_121198	167/178(94%)	*Bannoa hahajimensis*
			CBS 9041^T=AB035721	167/178(94%)	*Bannoa bischofiae*
			CBS9040^T= KY101704	166/178(93%)	*Bannoa syzygii*

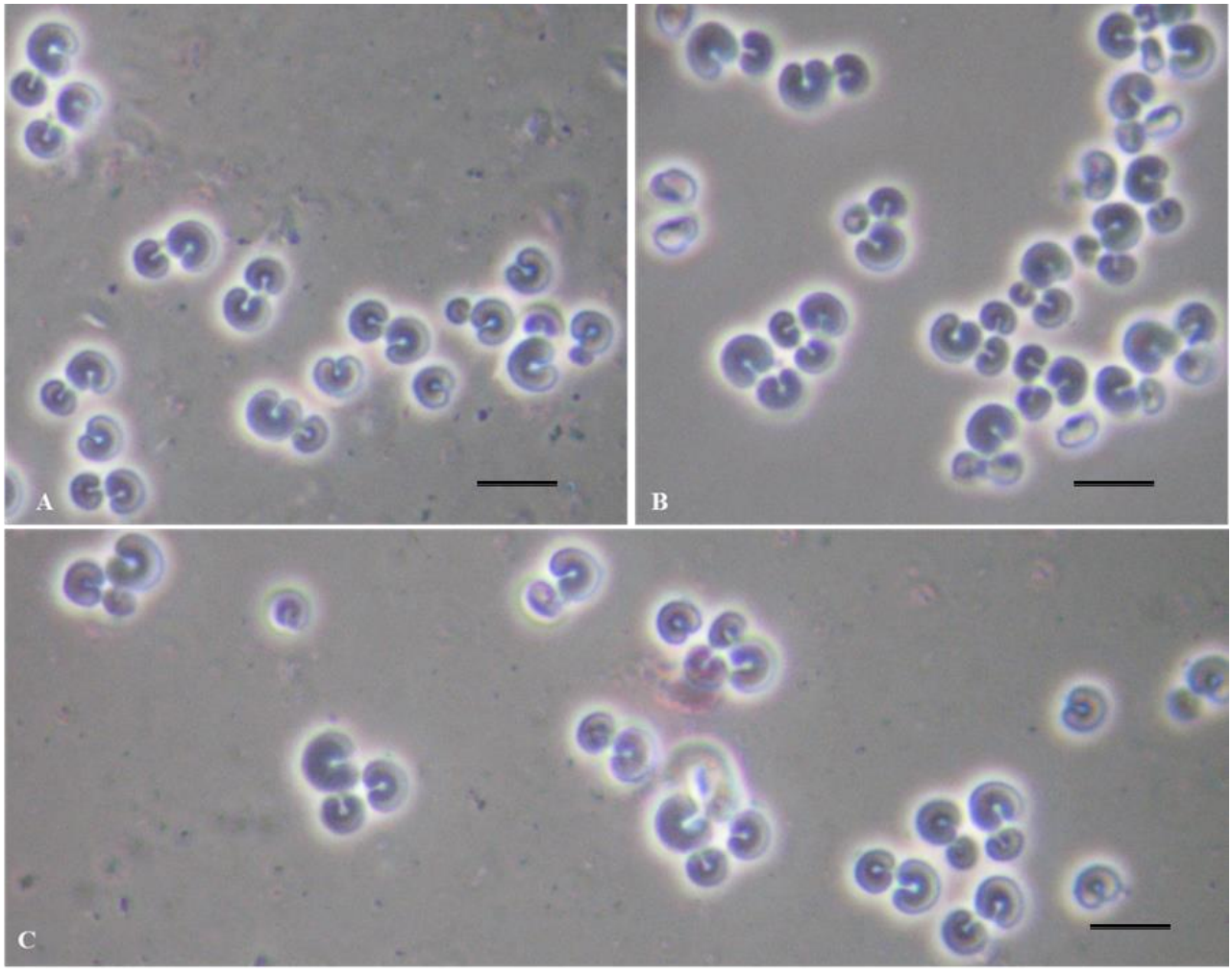

Fig. 4 – *Reniforma rhynchophori* AUMC 10263, A–C: reniform–shaped vegetative and budding cells (phase contrast), bar = 10 µm.

the two *Reniforma* species do not ferment sugars. The remarkable reniform vegetative cells which produce miniature reniform buds distinguish the two *Reniforma* species from all known yeasts.

Reniforma rhynchophori could be distinguished from *R. strues* by its ability to: 1) assimilate the carbon compounds α, α-trehalose, inulin, glycerol, 2-keto-D-gluconate, methanol and ethanol; 2) assimilate the nitrogen compounds L-lysine and D-glucosamine.

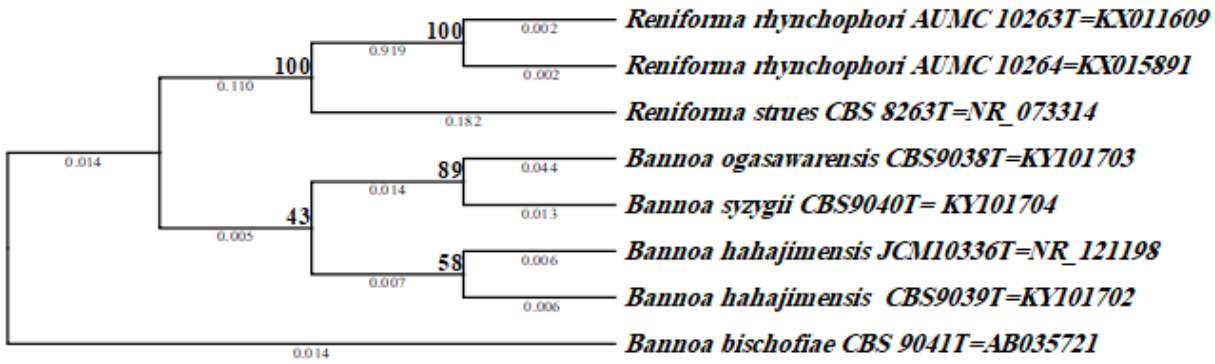

Fig. 5 – Phylogenetic placement of *Reniforma rhynchophori* sp. nov. among the closely related species based on sequence of ribosomal DNA internal transcribed spacer region. Bold numbers in the tree are bootstrap values (in percent) for the internal nodes, and non-bold numbers are the branch lengths, which connect nodes to the parent nodes.

Acknowledgement

Assiut University Mycological Centre, Assiut University, Egypt is acknowledged for the financial support.

References

Barnett JA, Payne RW, Yarrow D. 2000 – Yeasts: characteristics and identification. 3rd edition, Cambridge University Press, London, UK.

Hagler AN, Ahearn DG. 1981 – A rapid DBB test to detect basidiomycetous affinity of yeasts. International Journal of Systematic Bacteriology 31, 204–208.

Pore RS. 1973 – Selective medium for the isolation of *Prototheca*. Applied and Environmental Microbiology 26, 648–649.

Pore RS, Fell JW. 2011 – *Reniforma* Pore & Sorenson (1990). In: The yeasts, a taxonomic study (Kurtzman CP, Fell JW, Boekhout T, eds.), 5th edition. Elsevier, Amsterdam: 1869 –1872.

Pore RS, Sorenson WG. 1990 – *Reniforma strues*, a new yeast from waste-water. Mycologia 82, 549–553.

Saitou N, Nei M. 1987 – The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425.

Suh S-O, Blackwell M. 2004 – Three new beetle-associated yeasts in the *Pichia guilliermondii* clade. FEMS Yeast Research 5: 87–95. http://dx.doi:10.1016/j.femsyr.2004.06.001

Suh SO, Zhang N, Nguyen N, Gross S, Blackwell M. 2008 – Lab Manual for yeast study. Mycology Lab., Louisiana State University, PP. 38.

Wang Q-M, Yurkov AM, Göker M, Lumbsch HT. 2016 – Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. Studies in Mycology 81, 149–189. http://dx .doi.org/ 10.1016/j. simyco.20 15.12.002

Walt JP van der, Hopsu-Havu VK. 1976 – A colour reaction for the differentiation of ascomycetous and hemibasidiomycetous yeasts. Antonie van Leeuwenhoek 42, 157–163.

Walt JP van der, Yarrow D. 1984 – Methods for the isolation, maintenance, classification and identification of yeasts. Pp. 45–104. In: The Yeasts: A taxonomic study. 3–rd edition., Elsevier Science Publishers, Amsterdam.

Weijman ACM, Rodriguez de Miranda L, van der Walt JP. 1988 – Redefinition of *Candida* Berkhout and the consequent emendation of *Cryptococcus* Kutzing and *Rhodotorula* Harrison. Antonie van Leeuwenhoek 54, 545–553.
Wickerham LJ. 1951 – Taxonomy of yeasts. Technical Bulletin 1029, US Deparment of Agriculture, Washington D.C.