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Molecular Taxonomy Of *Galactomyces* Spp. And *Dipodascus Capitatus* Associate With Dairy Based On Rdna Sequence Analysis In Iraq

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Abstract:
Galactomyces spp is a telomorph of Geotrichum candidum (anamorph name). is used as a culture for cheese making and in some traditional fermented milks, few studies have assessed the genetic diversity of Galactomyces spp that exist in traditional cheese making facilities. The aim of this study isolation and molecular taxonomically treated of Galactomyces spp and other Candida spp. correlated with dairy products. The results showed that most dairy samples companioned with two species of Galactomyces: G.candidum and G.geotrichum , D. capitatus and four species of Candida spp.: Candida krusei , C. kefyr, C. utilis and C.glabrata .A total of 23 fungal isolates were diagnosed based on universal primers ITS1 / ITS4 to amplify the Internal transcribed region spacer. Four doubtful G.candidum isolates showed unique sizes of products PCR ranged between 380-400 bp. other Candida PCR product ranged 420-780bp. Sequence analysis identified a doubtful G.candidum into G. candidum and G.geotrichum with 97% similarities and with D. capitatus showed 93% similarities, few intraspecific variation were observed at 312-380bp in the amplicons from the primer pair ITS1-ITS4 commonly are from 380 to 400 bp for G. candidum , G.geotrichum and D. capitatus and other variation in the ITS region of Candida spp under our interest .

Keywords: Taxonomy, Galactomyces, Dipodascus , dairy , rDNA sequence analysis

INTRODUCTION

Galactomyces is ascomycete fungus. Historically, many synonyms names designated for *Galactomyces* are :*Geotrichum candidum*, *Oidium lactis*, *Oospora lactis*, *Oidium nubilum*, *Oidium humi* (Guarro et al., 1999). *Galactomyces* geotrichum (abbreviate: *G.geotrichum*) is the teleomorphic of *Geotrichum* and other previous synonyms names.

The history of taxonomic treatment of *Geotrichum candidum* Lk.ex Pers was first described in 1809. Butler and Petersen (1972) was designated the *Endomyces geotrichum* as a telomorphic state of *Geotrichum candidum*.In 1977 Redhead & Malloch disengaged valid name *Galactomyces* geotrichum as telomorphic state of *G. candidum* instead of *E. geotrichum* and consider the *E. geotrichum* as synonym and the basionym of *G. geotrichum* based on de Hoogh and Smith (1986).

G. geotrichum is a ubiquitous filamentous yeast-like fungus commonly isolated from soil, air, water, milk, silage, plant tissues, digestive tract in humans and other mammals (. Eliskases-Lechner et al.,2011). This species is widely used as adjunct culture in the maturation of cheese(Dziuba et al.,, 2000). The form genus *Geotrichum* is composed of 18 -22 species (Pottier et al., 2008; Eliskases-Lechner et al.,2011). A recent taxonomic revision designated many telomorphic species out of genus *Geotrichum* such as *G. candidum* and *G. geotrichum*, *Dipodascus capitatus* (de Hoog & Smith (2004) . *Galactomyces* is morphologically very similar to *Dipodascus capitatus* .

G.geotrichum and *G. candidum* are the best-known species of the form genus *Geotrichum* , are an acid-tolerant yeast-like fungus, recognized as a yeast (de Hoog and Smith, 2004).

The development of *Galactomyces* spp is typical for many mold-ripened, smear-ripened, and acid-coagulated cheeses (Marcellino et al.,2001). *Galactomyces* spp *Galactomyces* spp contributes to the characteristic appearance, taste, and aroma of these cheeses((Prillinger et al., 1999;Marcellino et al., 2001). Proteolytic and lipolytic activities(Litthauer et al.,1996), as well as catabolism of amino acids and free fatty acids, and de acidification activity are of primary importance for their use in cheese making. the biochemical attributes of *G. candidum* impact the course of cheese ripening. Lipases and proteases of *G. candidum* release fatty acids and peptides that can be metabolized by ensuing

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microbial populations and that contribute to the development of distinctive flavors and other qualities (Bertolini et al.,1995, Holmquist,1998,) However, *Galactomyces* spp, like other yeasts, is also frequently recorded as a spoilage organism. An overgrowth on the surface of mold-ripened soft cheeses leads to slippery-rind defect. Its presence leads to product spoilage of fermented milks and fresh cheeses, *Galactomyces* spp is a fungus that colonizes nearly all fungal surface-ripened cheeses during the early stages of ripening (Berger et al.,1999). On some cheeses, it is responsible for the appearance of the cheese, imparting a uniform, white, velvety coat to the surface (Guéguen et al.,1992).

Although *G. candidum* strains are readily isolated from dairy products, few studies have assessed the genetic diversity of strains that exist in traditional cheese making facilities. This issue is important since the industry trend toward standardization of ripening conditions may lead to the loss of empirically derived biodiversity. Prillinger et al.(1999) have used random amplification of polymorphic DNA (RAPD)-PCR to classify isolates of the genus *Geotrichum* at the species level . Phenotypic tests such as morphology, carbon source utilization, and salt tolerance, chosen for their relevance to cheese technology, were also done.Unfortunately rare ITS typing studies were performed on *Geotrichum*, thus making it necessary to consider a range of ITS sequence variants, Nakamura and Iwai (2010). de Hoogh and Smith (2004) based on data concerning ribosomal RNA genes, have separated the genus of *Galactomyces* into six species. One of these species is described as *G. geotrichum* without a described anamorphic form, and another one as *G. candidus* with *Geotrichum candidum* as anamorphic state. Most of the anamorphic species of *G. candidum*, *Oidium lactis*, *Oospora lactis* belong to the last group.

The aim of this study review the taxonomy of the *Galactomyces* spp based on morphology and physiology are reviewed by performing simple PCR, and emphasizing the identification of genotype variations for *Galactomyces* spp and some species of *Candida* by using the sequencing and supporting software and constructed phylogeny tree.

Material and Methods

1-Fungal species isolation

A total of 356 dairy product samples of diary collected from the markets and houses of cows and sheep and included 205 samples of cheese and 151 yogurt. were collected during the study conducted in 2014-2015 in the laboratory of bio-technology / All Women Science college / University of Babylon. To isolate fungi, approximately 1 g of cheese and yogurt was added to 5.0 ml of Sterile Distil Water and mixed gently until the formation of a suspension. Loop full each cheese and yogurt suspension were streaked on Sabouraud Dextrose Agar (SDA) plates and incubated at 25-28°C for 48 h. Colonies whose morphology resembled that of *G. candidum* were re-streaked to obtain a pure culture. Others *Candida* colonies were streaked on CHROMagar and incubated under same conditions and preliminary identified based on Nadeem et al.,(2010) .

2-DNA extraction:

The DNA template extraction was performed according to Imran and Al

Rubaiy 2015 .The culture media for each of the *G.geotrichum* isolates were frozen for 1 h and tiny portions of the mycelia mat were harvested into 1.5 ml tube. The harvested mats were suspended in 400 µl of lysis extraction buffer (Promega Co. USA), then vortexed for 5 min and added to 10 µL protinase K. Tubes were incubated in 65°C water bath overnight. A mixture of phenol: chloroform: Isoamyl alcohol (25:24:1) was added to the tubes. Tubes were centrifuged at 5000 rpm for 10 min. The aqueous supernatant was transferred to a new tube. An equal volume of cool isopropanol was added and agitated many times; it was centrifuged at 1000 rpm for 10 min. The supernatant was poured out. The pellet containing DNA was rinsed with 70% ethanol. It was air dried; pellets were re-suspended with 100 µL TE and placed in 70°C water bath. 6 µL of RNase A was added, and incubated at 37°C for 30 min. The tubes were centrifuged at 5000 rpm for 2 min. The supernatant was transferred to a new tube and frozen at -20°C until use (Imran and Al Rubaiy , 2015). . while the genomic DNA of *Candida* spp was extracted based on Imran and Al Asadi , 2014).

PCR procedure were as previously described, except that the ITS1 and ITS4 primers were used for amplification of DNA extracted from yeasts cell suspension , one colony for each was suspended in 200 ml sterile distilled water in a sterile tube. DNA was obtained by lysing the cells at 95 C for 5 min followed by immersing in ice. PCR was performed according to the previously described method by using the primers ITS1/ITS4 (Imran and Al.Shukry ,2014).

3-PCR and sequencing assays

PCR assay was performed the following condition : The primer pair that targeted the sequences site of the ITS1-5.8S-ITS2 gene was ITS1-ITS4 the isolates were used. The PCR mixture (25 µl) consisted of 12.5 µl of 20x Master Mix (Promega), 2 µl (10 pemole) of each primer and 1 µl template DNA, made up to 25 µl with molecular-grade water. The PCR mixture was amplified by the thermal cycler PCR System (Labnet, USA) .initial denaturation temperature 95ºC for 5 min, , 30 cycles, 95ºC for 30 sec , annealing temperature of 58ºC for 1.5 min , extension temperature of

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72ºC for 1 min ,final extension temperature 72ºC for 10 min ,cool step by 4 ºC.

four PCR products for both *Geotrichum* and Candida species were sent to Macrogen Laboratory in UAS and the received the sequences data for different fungal species . Prior to the sequencing reaction. The sequencing results were subjected for alignment .The sequence alignment were performed by BioEdit software.

4-Phylogeny tree

The phylogeny tree of eight yeasts species was instructed based on Mega6 software.

Results and Discussion

Galoctomyces spp showed fast growing colony that reached 5–6 cm diameter at 5 days on Sabouraud-glucose agar colony color of *G. geotrichum* and *G.candidum* milky color while *D. capitatum* has brown color on SDA (Figure 1).

Figure 1:Colony texture of *Galactomyces candidum* and *Dipodascus capitatums* on Sabouraud Dextrose Agar 48h at 28ºC incubation condition.

Microscopically, the two species *G.candidum* and *D. capitatum* were characterized by the special conidial shapes ; *G.candidum* showed rectangular shape while *D. capitatum* showed cylindrical conidial shape (Figure 2).these characters coincidence with the description of de Hoogh and Smith (1986).

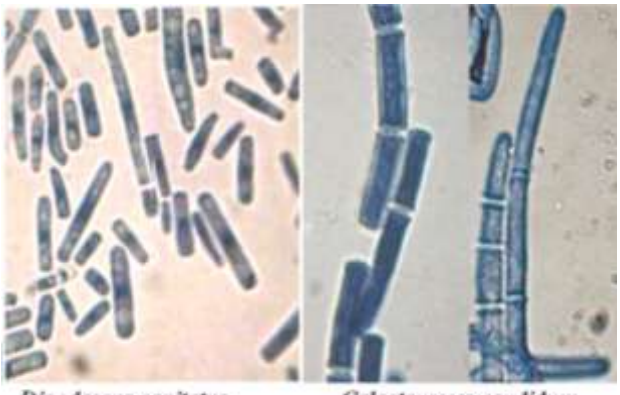
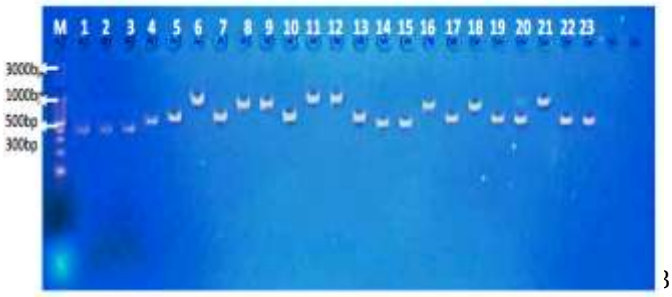


Figure 2-Microscopicaic characters of *Galactomyces candidum* and *D. capitatus* on Sabouraud Dextrose Agar 48h at 28ºC incubation condition. Magnification power X40.

This effective and rapid testing was performed for the diagnosis of the yeasts after inoculated and incubated the results showed different colors compared with the standard Nadeem et al.,(2010) key ,The results of this test showed set of different colonies in the colors on the CHROMagar medium.The colony was pink color (pink to cream) for *C. krusei.*

A-PCR Assay :

A total of 23 fungal isolates were diagnosed based on universal primers ITS1 / ITS4 to amplify the Internal transcribed region spacer. Four doubtful *Geotrichum candidum* isolates showed unique sizes of products PCR ranged between 380-400 bp. other *Candida* PCR product ranged 420-780bp. (Figure 3).Our results coincidence with molecular weight key based on Fujita et al.,(2001).



fungal isolates were diagnosed based on ITS1 / ITS4. Well 1-4 =*G.geotrichum* (380-400bp),wells:5,7,10,13,17,19-20,22-23=*C,utilis*(554bp),14-15= *C.regusa* (420bp),wells 8-9,16,18,= *Kluyveromyces marxianus* (720bp),wells 6,11-12, 21, *C.glabrata*(780bp).Molecular marker 100bp for each step.

Results showed relay recording three new species of analysis of Iraq, a *G.geotrichum* and *G.candidum* and *Dipodascus capitatus*. Unfortunately no previous molecular studies were performed on *Galactomyces* spp. in Iraq .The phylogeny tree constructed based on UPGMA software convergence isolates species *G. geotrichum and G. candidum* and distancing itself from the species *D. capitatus* which confirms the diagnosis and degree of speciation for three species.

B-rDNA Sequence analysis

The universal fungal primers ITS1 / ITS4 amplified the ITS1, 5.8S, and ITS2 regions for all 23 isolates and in each case, satisfactory sequencing results were obtained. ITS analysis are shown in (Figure 4).High sequence similarities were observed in the ITS region between *G. geotrichum*

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and *G. candidum* (97% similarities) and distancing itself from the species *D. capitatus* (93% similarities) the sequence of *D. capitatus* was compared with reference strain of *D. capitatus* accession number:HQ014712 , few intraspecific variation were observed at 312-380bp in the amplicons length from the primer pair ITS1-ITS4 commonly are from 380 to 400 bp for *G. candidum* , *G.geotrichum* and *D. capitatus* and other variation in the ITS region of *Candida* spp under our interest .

: High similarity from 10 to 310 nucleotides (Nts) and high variation occur from 312 to 380Nts were labeled by red rectangle in sequence between *Galactomyces* spp (Figure 5).

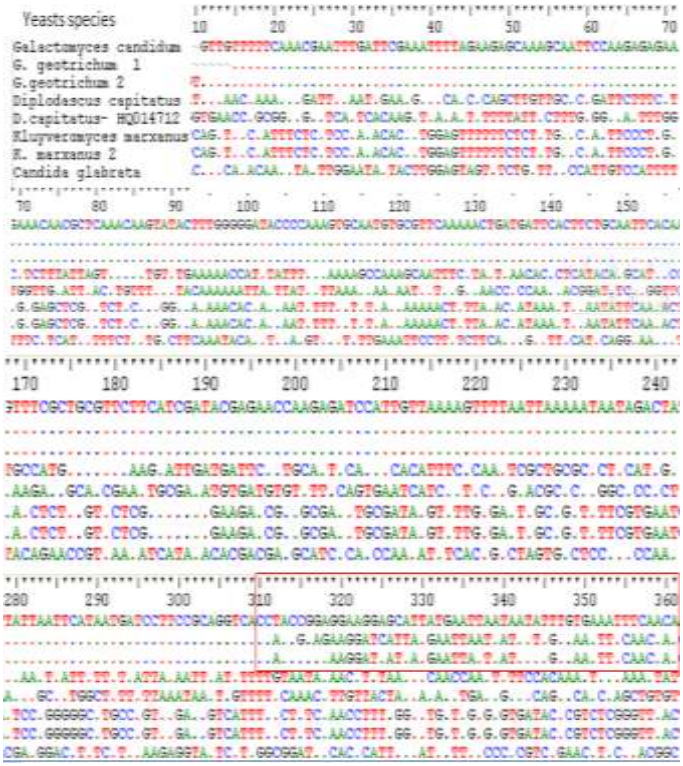

Figure (5):Selected sequences alignment in the ITS regions for sequences of 8 species of *Galactomyces* spp. and *Candida* spp were aligned by using the BioEdit software. *Galactomyces candidum* was considers as leading sequence. High homologous(10-310Nts) and high variation occur from 312 to 380Nts were labeled by red rectangle sequence between *Galactomyces* spp .

C-Phylogeny tree:

UPGMA dendrogram tree of *Galactomyces* spp., *Dipodascus capitatus* and other *Candida* spp on the basis of their ITS sequences was constructed with data for standard strains of gene bank: The sequence of *D. capitatus* and the reference strain of *D. capitatus* accession number:HQ014712 were showed in cluster 1 , *Galactomyces* spp cluster 2 while two isolates of *Kluyveromyces marxianus* showed in cluater 3 while the *C.glabrata* recognized in cluster 4 (Figure 6) . A high degree of homology was observed between the sequences described in the literature and those determined in the present study. Table 1 shows the distance between isolates of yeasts species under interest according to the absolute number of different bases (absolute distances) and the percentage of divergent bases in relation to the total number of bases sequenced (mean distances).

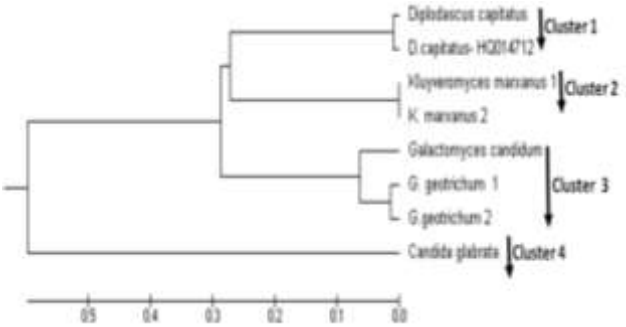

Figure 6: UPGMA dendrogram showing relationships among 8 fungal isolates of *D. capitatus* and reference strain of *D. capitatus* accession number:HQ014712. Custer 2 of *K. marxianus*. *Galactomycese* spp. in cluster 3 and *C. glabrata* in cluster 4.

Table 1**.** Distance matrix between the ITS rDNA sequences of 18 isolates of dairy fungal. Below the diagonal are the absolute distances corresponding to the number of divergent bases. Above the diagonal are the percentages of different bases in relation to the total number of bases sequenced.

	1	2	3	4	5	6	7	8
1. Galactomyces candidum								
2. G. geotrichum 1	0.1							
3. G.geotrichum 2	0.1	0.0						
4. Diplodascus capitatus	0.6	0.6	0.6					
5. D.capitatus- HQ014712	0.6	0.6	0.6	0.0				
6. Kluyveromyces marxanus 1	0.5	0.6	0.6	0.6	0.5			
7. K. marxanus 2	0.5	0.6	0.6	0.6	0.5	0.0		
8. Candida glabrata	1.1	1.1	1.2	1.2	1.2	1.2	1.2	

The conclusion of this study referred that dairy products complain and contaminated with many yeasts ,some of this yeasts may be pathogenic or opportunistic pathogens ,others contribution in the repine of cheese .we still in need survey and identification of dairy yeasts periodically and determine their role in milk fermentation .Uses of molecular markers that be considered as useful tools for proper identification of the yeasts species. These results agreed with finding

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reported molecular markers have proved to be powerful tools in molecular identification for provided to speed the process of identifying fungal species.

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