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Molecular study of *Geotrichum* strains isolated from Armada cheese

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ABSTRACT

The aim of this work was the genetic characterization at the strain level of 39 presumed *Geotrichum candidum* isolates isolated throughout the artisanal manufacturing and ripening of Armada cheese and tentatively identified at genus and/or species level by phenotypic characteristics. The molecular identification of the strains included among others the amplification and sequencing of the D1/D2 domains of the 26S rRNA gene. A restriction fragment length polymorphism (RFLP) analysis with the ITS1-5.8S-ITS2 PCR amplicons and a randomly amplified polymorphic DNA (RAPD) analysis with five different primers were carried out. The bands pattern profile obtained through RFLP by enzymatic restriction with *Hin*fI was the same for all the strains studied, which confirmed the classification of the strains at species level. A RAPD-PCR analysis with three different primers was applied to assess the intraspecific diversity, in this way 16 band profiles were obtained for the 39 strains studied by the combined use of primers Ari1 and Omt1. This study contributes to know the occurrence and genotypic biodiversity of *G. candidum* in Armada cheese.

1. Introduction

Geotrichum candidum is naturally present in raw milk and usually found on the surface of smeared soft cheeses, mould-ripened and semi-hard cheeses (Guéguen, 1984; Eliskases-Lechner and Ginzinger, 1995), like it happens with Armada cheese. Armada cheese is a craft variety made in the North of Spain by traditional and artisanal methods from raw goats' milk without addition of starter cultures (Tornadijo et al., 1995). *G. candidum*, which grows very early during the ripening process and stimulates the development of the bacterial microbiota (Guéguen, 1984), is one of the main yeast species isolated during the manufacturing and ripening of this cheese, being the dominant fungal species (Tornadijo et al., 1998). In fact, almost one third (60 out of 188) of the identified yeast isolates from this artisanal cheese variety have been shown to belong to *G. candidum* (Tornadijo et al., 1998).

Little consideration has been given to the specific occurrence and significance of *G. candidum* in cheese (Boutrou and Guéguen, 2005), even though *G. candidum* has certain properties that are of particular interest to the dairy industry and has been added as a ripening agent for at least thirty years (Pottier et al., 2008).

The classification of *G. candidum* has been a matter of controversy. First classified as a mould, *G. candidum* has now been considered as a yeast for more than 20 years (Barnett et al., 1990). However, it is difficult nowadays to allocate this species between one group or another. As it shows both, yeast and mould characteristics, some authors consider it as a true yeast (Kurtzman and Fell, 1998; Barnett et al., 2000), while some other authors still include it among the moulds (Wouters et al., 2002). Anyway, it must be kept in mind that the terms "yeast" and "mould" do not have a formal taxonomic significance (Kirk et al., 2001). In fact, all *Geotrichum* species are considered as filamentous yeast-like fungi (De Hoog and Smith, 2004).

According to the taxonomy study of Kurtzman and Fell (1998) the so called *Galactomyces geotrichum* complex has been considered as the teleomorphic state of the anamorphic state *G. candidum* from 1977 to 2004. De Hoog and Smith (2004) proposed a revision of *Geotrichum* and its teleomorphs and it was concluded that the *G. geotrichum*/*G. candidum* complex contained four separate species. Therefore, *G. candidum* needs to be differentiated from the closely-related, newly-described species *G. geotrichum*/*Geotrichum* sp., *Galactomyces pseudocandidus*/*Geotrichum pseudocandidum* and *Geotrichum europaeum*.

Nowadays, *G. candidum* is the most common species of the genus *Geotrichum* and it has its teleomorphic state in *Galactomyces candidus* (De Hoog and Smith, 2004; Pimenta et al., 2005; Suh and

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Blackwell, 2006; Wuczkowski et al., 2006). Polyphasic phenotypic and genotypic approaches are usually used to identify *G. candidus*/*G. candidum* (Gente et al., 2006). Moreover, there is a recognized need to define precisely the type strain by studying more isolates from different sources and comparing their morphology, physiology and genome organization (Piegza et al., 2006).

According to Gente et al. (2006) the identification of *G. candidum* strains to include them in one group or another of the recent taxonomy proposed by De Hoog and Smith (2004) needs some confirmatory steps based on phenotypic tests before applying genetic assays. In addition, phenotypic data used in yeast identification are useful only in association with genotypic methods. An integrated approach based on DNA-fingerprinting techniques, such as the sequence analysis of the D1/D2 domain of 26S rDNA and phenotypic studies represents a reliable diagnostic system for food-borne yeasts. The sequences of the D1/D2 domain of 26S rDNA of all known yeasts are accessible from different sequence databases and often exhibit enough differences between yeasts that it can be used in the prediction of at least inter-species relationships (Kurtzman and Robnett, 1998; Kurtzman and Fell, 1998). These authors suggested that ascomycetous yeasts exhibiting less than 1% nucleotide differences in this region belong to the same species. In a recent study, multiple sequence alignments of the complete 18S, 5.8S, 26S rRNA genes and internal transcribed sequences from 18 *G. candidum* strains were made by Alper et al. (2011). They found 8 polymorphic sites (out of 588 bp) in the D1/D2 region concluding that conspecificity establishment through the analysis of this domain might not be applicable to *G. candidum*. In addition they clearly suggested that the analysis of ITS1-5.8-ITS2 was as effective at differentiating strains as an analysis of the entire 18S–5.8S–26S region.

To confirm the results of phenotypic studies and sequence analysis, other techniques must be used. Simple molecular techniques, such as RFLP-PCR rDNA (Restriction Fragment Length Polymorphism of rDNA) and microsatellite RAPD (Randomly Amplified Polymorphic DNA) developed for some other yeasts (Barszczewski and Robak, 2004; Prillinger et al., 1999; Walczak et al., 2007), may be useful for the study of the *G. candidum* genome. In fact, a RAPD technique using the primer M13 can discriminate the species belonging to the genus *Geotrichum* and its teleomorphs (Gente et al., 2006; Pottier et al., 2008). In addition, strains can be differentiated using a molecular typing method proposed by Flórez et al. (2007) based on RAPD using specific primers.

This paper reports on the molecular identification and typing of 39 presumed *G. candidum* isolates isolated from Armada cheese by using genetic methods such as rDNA sequencing, and RFLP-PCR and RAPD-PCR fingerprinting. The successive application of all these techniques allowed the identification of the yeast strains, as well as the characterization of their intraspecific genetic diversity.

2. Materials and methods

2.1. G. candidum strains and culture conditions

Thirty nine strains of *G. candidum* had been isolated from four batches of Armada cheese elaborated by experienced cheese makers according to traditional methods. Milk, curd (2 days after coagulation and before salting), and 1-, 2-, 4-, 8- and 16- weeks- old cheese samples were taken from each batch at different times of the year (Tornadijo et al., 1995). The isolates obtained were purified and maintained in Sabouraud dextrose agar (Oxoid, Unipath Ltd, Basingstoke, UK) tubes under refrigeration, covered with sterile paraffin oil in order to reduce their metabolic activity and to avoid agar dehydration. Strains had been identified previously by morphological and physiological tests (Tornadijo et al., 1998) and their technological properties characterized (Sacristán et al., 2012).

2.2. Isolation and purification of total DNA

The extraction and purification of DNA from the *G. candidum* strains was carried out according to Flórez et al. (2007). In short, an aliquot (4 mL) of the microbial cultures grown on TSB supplemented with 0.6% Yeast Extract (Oxoid, Unipath Ltd., Basingstoke, UK) at 25 °C for 72 h with shaking was centrifuged at 14,000 *g* for 5 min. The pellet was collected, washed with TE (10 mmol/L Tris–hydrochloride, 1 mmol/L EDTA, pH 8.0) and frozen overnight. The cell lysis was accomplished by vortexing the pellet in 500 μL of a lysis buffer (200 mmol/L Tris–hydrochloride, 25 mmol/L EDTA, 250 mmol/L NaCl and 0.5% SDS pH 7.55) with chloride acetate (300 μL) and glass beads (100 μL of 150–22 μm diameter) during 10 min to ensure cell lysis. The mixture was incubated with proteinase K (Sigma; Sigma–Aldrich Química SA., Madrid, Spain) for 30 min at 50 °C. After centrifugation to eliminate the cell debris, the DNA was purified by phenol: chloroform: isoamyl alcohol (24:24:1) extractions and precipitated with one volume of isopropyl alcohol. The pellet was washed twice with ethanol at 70%, vacuum dried, suspended in 100 μL of TE and stored until use at −20 °C.

2.3. Molecular identification of the strains at species level

2.3.1. Sequencing of D1/D2 domains of the 26S rRNA encoding gene

Primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used to amplify a fragment of approximately 600 bp of the D1/D2 variable domains of the 26S ribosomal DNA (rDNA) gene (Lopandic et al., 2006; Kurtzman and Robnett, 1998). PCR was performed in a final volume of 50 μL containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 mM of each primer, 1.25 UI *Taq*-polymerase (Promega) and 2 μL of the extracted DNA. Amplifications were carried out according to Cocolin et al. (2002) as follows: initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 60 s, 52 °C for 45 s and 72 °C for 60 s, and a final extension at 72 °C for 7 min.

Five microliters of the amplified mixture were analyzed in a 1% 0.5× TBE agarose gel electrophoresis at 100 V during 2 h and visualized after staining with 0.5 μg/mL ethidium bromide. The results were photographed under UV illumination. The 600 bp fragment was sequenced and used in a similarity search by means of the BLASTN program from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.2. Identification of the G. candidum/G. candidus species

A RAPD-PCR analysis using the primer M13 (5′-GAGGGTGGCGGTTCT-3′) was carried out. The PCR conditions were: initial denaturation at 94 °C for 4 min, then 35 cycles of 30 s at 94 °C, 1 min at 50 °C and 30 s at 72 °C, followed by a final extension at 72 °C for 6 min, according to Gente et al. (2006).

2.3.3. Restriction fragment length polymorphism (RFLP) of the ITS1-5.8S-ITS2 region and sequencing and comparison of the amplicons obtained

Amplification of the ITS1-5.8S-ITS2 region as well as the digestion and sequencing of amplicons obtained were carried out according to Álvarez-Martín et al. (2007). Primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′GGAAGTAAAAGTCGTAACAAGG-3′) (White et al., 1990) were used to amplify a segment of the DNA coding for the rRNA operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers ITS1 and ITS2. The PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 °C for 60 s and 72 °C for 2 min, and a final extension