J Ind Microbiol Biotechnol (2006) 33: 1019–1031
DOI 10.1007/s10295-006-0130-3

ORIGINAL PAPER

S. Gente · D. Sohier · E. Coton · C. Duhamel
M. Gueguen

Identification of *Geotrichum candidum* at the species and strain level: proposal for a standardized protocol

Received: 2 November 2005 / Accepted: 3 April 2006 / Published online: 20 July 2006

Abstract In this study, the M13 primer was used to distinguish *Geotrichum candidum* from the anamorphic and teleomorphic forms of other arthrospore-forming species (discriminatory power = 0.99). For intraspecific characterization, the GATA4 primer showed the highest level of discrimination for *G. candidum* among the 20 microsatellite primers tested. A molecular typing protocol (DNA concentration, hybridization temperature and type of PCR machine) was optimized through a series of intra- and interlaboratory trials. This protocol was validated using 75 strains of *G. candidum*, one strain of *G. capitatum* and one strain of *G. fragrans*, and exhibited a discrimination score of 0.87. This method could therefore be used in the agro-food industries to identify and to evaluate biodiversity and trace strains of *G. candidum*. The results show that the GATA4 primer might be used to differentiate strains according to their ecological niche.

Keywords Molecular typing · RAM-PCR · *Geotrichum candidum*

S. Gente (✉) · M. Gueguen
Laboratoire de Microbiologie Alimentaire,
Université de Caen Basse-Normandie,
Esplanade de la Paix, 14032 Caen cedex, France
E-mail: stephanie.gente@unicaen.fr
Tel.: +33-231-565621
Fax: +33-231-566179

D. Sohier
ADRIA Développement Créac'h Gwen,
29196 Quimper, France

E. Coton
ADRIA Normandie, Boulevard du 13 juin 1944,
14310 Villers-Bocage, France

C. Duhamel
Laboratoire de Microbiologie, CHU,
Avenue Côte de Nacre,
14033 Caen Cedex, France

Introduction

Geotrichum candidum, taxonomically located at the boundary between typical yeasts and molds, is an important microorganism in the agro-food industry as it is an integral part of the microflora of some products; it is also involved in biodegradation and depollution and may act as a contaminant.

This species is naturally present in raw milk [14, 15]. Because of its proteolytic and aromatic capacities as well as its covering properties, *G. candidum* is used in starter cultures for cheese production. It is a desirable component of the surface flora of pressed cheeses and soft mould or smear-ripened cheeses [8]. This species is also used during the malting process to inhibit of mycotoxin production and increase the enzymatic potential of the malt, which leads to an improvement in brewhouse performance [6, 20, 44]. Due to its biotechnological characteristics, it has therefore been the subject of numerous physiological and biochemical studies [4, 13, 18, 48, 61]. It may also hydrolyze native cellulose [43, 56, 62] and pectin [26], and degrade pesticide residues such as lindane [39]. It acts as a bleaching agent and eliminates phenolic components from the effluents of oil presses [1, 7, 11], and also bleaches molasses [33] and vinasse [21]. Biological treatment of the water used to rinse molasses or of wastewater from distilleries reduces COD (chemical oxygen demand) and BOD (biochemical oxygen demand) [19, 22, 50]. Furthermore, *G. candidum* can be used to produce highly digestible proteins (SCP or single cell proteins) and to extract flavonoids from orange peel [37, 58].

However, *G. candidum*, like other yeasts, is also a spoilage agent responsible for the deterioration of fresh cheeses, fruit juices and vegetables [10, 16, 53]. Growth has been observed in refrigerated cakes [42] and in frozen leaf vegetables [55] and attack on unripe fruits in orchards and ripe fruits in storage, causes a watery, thick or sticky rot [36]. *G. candidum* is also a commensal organism of human and animal digestive systems [27].

There is a clear link between the frequency of carriage in the digestive tract and the amount of *G. candidum* present in food, as the fungus survives in the digestive tract, which it colonizes transiently [5]. It is not unusual for this fungus to be isolated from sputum and skin samples from healthy individuals [25]. *G. candidum* is rarely an opportunistic pathogen of humans. About thirty cases of *G. candidum* fungemia have been reported in the last 35 years. Most of these cases concerned immunocompromised patients [31].

For many years, the methods used to identify yeasts were essentially based on morphological criteria [34, 38], physiological characteristics [2] or a combination of both [3, 35]. The standardized identification micromethods (API 32C Biomérieux, France; Auxacolor 2, Biorad, RapID Yeast System) [17], which were developed for the yeasts of clinical importance, are not suitable for yeasts encountered in the agro-food industries. Furthermore, it is not possible to distinguish species of the genus *Geotrichum* on the basis of phenotypic characteristics alone [49]. Recently, De Hoog and Smith [12] proposed a revision of the filamentous yeast-like fungi predominantly reproducing with arthric conidiogenesis. Results based on ITS rDNA sequences and nDNA/DNA reassociation data presented 32 taxa and a key (with nutritional tests) was provided.

Nevertheless, the high polymorphism and the variability of phenotypic characters of the *G. candidum* species, as well as the difficulties for growth test reading leads to the use of molecular techniques. However, the genetic characteristics of *G. candidum*—the anamorphic form of the ascomycete *Galactomyces candidus* (formerly *Gal. geotrichum*)—are still largely unknown, as are those of numerous filamentous fungi. Only the genes of the lipolytic system of this fungus have been studied [45, 46, 52]. A recent pulsed field gel electrophoresis (PFGE)-based study aiming to determine the number of chromosomes and the size of the genome of 13 strains of *G. candidum* revealed high levels of polymorphism related to the phenotypic diversity of the species [23].

With the development of molecular analysis techniques it has become possible to demonstrate inter- and intraspecific DNA polymorphisms in fungi [60]. Microsatellites or simple repeat sequences are sequences consisting of a few repeated bases located randomly throughout the genome [32]. These loci present a high level of polymorphism and have been used for identification purposes and to study the diversity of various eukaryotic organisms [9, 28] including *G. candidum* [24]. Another tool for identification (species level and intraspecific diversity) derived from the core sequence of the M13 phage, specific for a minisatellite, is widely used in yeasts [41].

The aims of this study were to develop an intraspecific identification and discrimination method and to evaluate the discriminatory power and reproducibility of the method to allow for its recommendation as a standardized method.

Materials and methods

Strains used and culture conditions

Twenty-six representatives of species belonging to the genus *Geotrichum* and their teleomorphs *Galactomyces, Dipodascus* and *Trichosporon*, all reproducing with arthric conidiogenesis, were used to confirm the strains that belonged to the species *G. candidum* (Table 1). Seventy-five strains of *G. candidum*, plus one strain of *G. capitatum* and one strain of *G. fragrans*, were selected, according to the biotope they were isolated from and their morphotype, and used for molecular typing (Table 2). Ten of these strains (indicated by an asterisk in Table 2) were used to test the different primers. The optimization, intralaboratory repeatability and interlaboratory reproducibility tests were carried out with six strains (in bold in Table 2).

For culture, cellophane films (boiled for 5 min in water containing SDS, rinsed and sterilized for15 min at 120°C in distilled water) were placed in Petri dishes containing PDA (potato dextrose agar). Plates were inoculated by spreading and incubated them at 25°C for 72 h. The fungi were collected by removing the cellophane and were stored at −70°C until DNA extraction.

Extraction of genomic DNA from Geotrichum and related genera

The collected mycelia were frozen at −70°C and ground in a MM200 Mixer Mill (Retsch BmbH) in liquid nitrogen (3 min, 30 oscillations/s) to form a fine powder. DNA was then extracted by the Raeder & Broda method [51] for interspecific experiments or using the DNeasy Plant Mini kit (Qiagen) for typing experiments. To do so, the powder (about 100 mg) was dissolved in 400 µl of lysis buffer. The manufacturer's protocol was then followed. Total DNA was eluted from silicon columns in two lots of 100 µl of preheated (65°C) elution buffer. Finally, the extracted DNA was quantified using a fluorometer and the Picogreen® reagent (Molecular Probes)

PCR fingerprinting

Each reaction tube contained: 25 ng DNA, 1 × PCR buffer (2.5 mM $MgCl_2$), 0.2 mM dNTP, 100 pmol primer (Table 3), 0.2 µl Taq polymerase (Qbiogene, 15 U/µl) in a final volume of 50 µl. The PCRs were run in several different PCR machines: PTC 200 (MJ Research), I-Cycler (Biorad) (with different purchase dates) and Master Gradient (Eppendorf).

To amplify the intersimple sequence repeats (ISSR), the following program was used: initial denaturation at 94°C for 4 min, then 35 cycles of 1 min at 94°C, 1 min at the hybridization temperature (Table 4) and 2 min at

Table 1 Strains investigated for species differentiation

Strain	Genus/species	Original substrate	Geographical area of sampling
ATCC 204307	*Geotrichum candidum (Galactomyces candidus)*	Pont l'évêque	Normandy, France
CBS 110.12	*Geotrichum candidum Galactomyces candidus)*	Milk	France
CBS 193.34	*Geotrichum candidum (Galactomyces candidus*	Human sputum	USA
CBS 560.97	*Geotrichum candidum (Galactomyces candidus*	Human stools	Denmark
CBS 108.12	*Geotrichum magnusii (Magnusiomyces magnusii)*	Unknown	Unknown
CBS 152.25	*Geotrichum fragrans (Saprochaete suaveolens)*	Water brewery	Unknown
CBS 175.53	*Dipodascus aggregatus*	Pupal gallery of *Ips acuminatus* in *Pinus sylvestris*	Germany
CBS 175.89	*Geotrichum citri-aurantii*	Soil from orange orchard,	Salisbury, Zimbabwe
CBS 179.30	*Geotrichum klebahnii*	Brown slime flux in *Ulmus* sp.	Germany
CBS 179.60	*Galactomyces reessii*	Cold water retting of *Hibiscus cannabinus*	Java, Indonesia
CBS 184.80	*Dipodascus geniculatus*	Pulp of *Psidium guajava*,	Pune, Maharashtra, India
CBS 192.55	*Dipodascus ovetensis (Magnusiomyces ovetensis)*	Tannin concentrate	Spain
CBS 244.85	*Dipodascus spicifer (Magnusiomyces spicifer)*	Cactus rot	Arizona, USA
CBS 259.82	*Dipodascus macrosporus*	*Badhamia utricularis* (myxomycete) slime trail	Bristol, UK
CBS 425.71	*Geotrichum clavatum (Saprochaete clavata)*	Human lung	USA
CBS 439.83	*Geotrichum fermentans*	Wood pulp	Sweden
CBS 517.90	*Dipodascus ingens (Saprochaete ingens)*	Wine cellar	South Africa
CBS 571.82	*Geotrichum capitatum (Magnusiomyces capitatus)*	Wood pulp factory	Stockholm, Sweden
CBS 625.74	*Dipodascus australiensis*	Decaying cladodes of *Opuntia inermis* (cactus)	Australia
CBS 626.83	*Geotrichum pseudocandidum (Galactomyces pseudocandidus)*	Stomach of elk	France
CBS 749.85	*Dipodascus ambrosiae (Magnusiomyces ovetensis)*	Insect gallery	Yosemite, California, USA
CBS 765.70	*Dipodascus tetrasperma (Magnusiomyces tetrasperma)*	Wet conveyor at a prune dehydratation plant	Davis, California, USA
CBS 772.71	*Galactomyces geotrichum*	Soil	Puerto Rico
CBS 817.71	*Dipodascus armillariae*	*Gills of mushroom (Armillariae* sp.)	Netherlands
CBS 866.68	*Geotrichum europaeum*	Wheat field soil	Germany
CBS 8189	*Trichosporon gracile*	Sour milk	Germany

Names of species indicated in brackets correspond to the new nomenclature proposed by de Hoog and Smith [12]
ATCC American Type Culture Collection, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands

72°C, followed by a final extension at 72°C for 5 min. The PCR products were kept at 4°C.

For minisatellite (M13) amplification, the following program was used: 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. The PCR products were kept at 4°C.

Analysis of PCR products

The PCR products (size range = 200 bp to 2.5 kb) were separated on 1% agarose gels in 1 × TBE. A molecular weight marker was included in each run to make it possible to determine the sizes of fragments (Marker XIV, Roche). After electrophoresis (120 V for 3 h), the gel was stained in 0.2 mg/ml ethidium bromide (EtBr). The bands were then visualized under UV light. The stained gel was scanned and analyzed with Bionumerics software version 2.0 (Applied Maths, Belgium). The intensity and positions of bands in the profiles obtained were translated into densitometric curves by calculating Pearson's similarity coefficient [54]. The profiles were grouped and used to build dendrograms by the UPMGA method (unweighted pair group method using arithmetic averages) [59]. We evaluated the extent to which microsatellite amplifications distinguished between isolates using Simpson's diversity index (D) [29].

$$D = 1 - 1/N(N-1)\sum x_j(x_j - 1);$$

Table 2 Strains investigated for intraspecies differentiation

Strain	*Geotrichum* sp.	Substrate	Geographical area of sampling
CBS 240.62*	*candidum*	Germinated grain of *H. vulgare*	Netherlands
GEO A*	*candidum*	Starter (Danisco)	Unknown
IFBM 93.132*	*candidum*	Malting environment	France
LCP 51.590*	*candidum*	Sandy soil	Burgos, Spain
UCMA 292*	*candidum*	Corn silage	Normandy, France
UCMA 294*	*candidum*	Teat	Normandy, France
UCMA 297*	*candidum*	Udder	Normandy, France
UCMA 299*	*candidum*	Teat	Normandy, France
UCMA 300*	*candidum*	Teat	Normandy, France
UCMA 382*	*candidum*	Raw cream	Normandy, France
UCMA 91 = ATCC 204307	***candidum***	**Pont l'évêque cheese**	**Normandy, France**
CBS 110.12	***candidum***	**Milk**	**France**
UCMA 291	***candidum***	**Grass**	**Caen, France**
UCMA 293	***candidum***	**Corn silage**	**Normandy, France**
UCMA 302	***candidum***	**Cows' milk**	**Normandy, France**
UCMA 937	***candidum***	**Human stools**	**CHU, Caen, France**
CBS 181.33	*candidum*	Nail of man	Netherlands
CBS 184.56	*candidum*	Child tongue	Germany
CBS 185.56	*candidum*	Human sputum	Netherlands
CBS 193.34	*candidum*	Human sputum	USA
CBS 558.97	*candidum*	Human stools	Unknown
CBS 559.97	*candidum*	Penis	Unknown
CBS 560.97	*candidum*	Human stools	Denmark
GEO B	*candidum*	Starter (Danisco)	France
IFBM 93.201	*candidum*	Malting environment	France
IHEM 1178	*candidum*	Human	Arlon, Belgium
IHEM 1484	*candidum*	Leg ulcer	Brussels, Belgium
IHEM 3001	*candidum*	Human stools	Brussels, Belgium
IHEM 3136	*candidum*	Human sputum	Belgium
IHEM 4830	*candidum*	Human sputum	Visé, Belgium
IHEM 6815	*candidum*	Human stools	Brussells, Belgium
IP 1447.83	*candidum*	Stools	Paris, France
IP 285.54	*candidum*	Human	Unknown
IP 651.61	*candidum*	Human	Unknown
LMSA 1	*candidum*	Corn fodder	Brittany, France
LMSA 2	*candidum*	Tomato	Brittany, France
WISBY 1	*candidum*	Starter (Wisby)	Unknown
UCMA 290	*candidum*	Grass	Caen, France
UCMA 541	*candidum*	Sows'faeces	Brittany, France
UCMA 542	*candidum*	Sows'faeces	Brittany, France
UCMA 939	*candidum*	Stools (same patient as UCMA 942)	CHU, Caen, France
UCMA 940	*candidum*	Human stools	CHU, Caen, France
UCMA 941	*candidum*	Human stools	CHU, Caen, France
UCMA 942	*candidum*	Throat (same patient as UCMA 939)	CHU, Caen, France
UCMA 943	*candidum*	Human stools	CHU, Caen, France
UCMA 944	*candidum*	Human stools	CHU, Caen, France
UCMA 945	*candidum*	Nail	CHU, Caen, France
UCMA 946	*candidum*	Human stools	CHU, Caen, France
UCMA 947	*candidum*	Human stools	CHU, Caen, France
UCMA 949	*candidum*	Human stools	CHU, Caen, France
UCMA 951	*candidum*	Human stools	CHU, Caen, France
UCMA 952	*candidum*	Human stools	CHU, Caen, France
UCMA 953	*candidum*	Gluteal fold	CHU, Caen, France
UCMA 954	*candidum*	Human stools	CHU, Caen, France
UCMA 955	*candidum*	Human stools	CHU, Caen, France
UCMA 956	*candidum*	Human stools	CHU, Caen, France
UCMA 957	*candidum*	Human stools	CHU, Caen, France
UCMA 959	*candidum*	Human stools	CHU, Caen, France
UCMA 960	*candidum*	Human stools	CHU, Caen, France
UCMA 961	*candidum*	Human stools	CHU, Caen, France
UCMA 963	*candidum*	Human stools	CHU, Caen, France
UCMA 964	*candidum*	Human stools	CHU, Caen, France
UCMA 965	*candidum*	Human stools	CHU, Caen, France
UCMA 966	*candidum*	Human stools	CHU, Caen, France
UCMA 967	*candidum*	Human stools	CHU, Caen, France
UCMA 968	*candidum*	Human stools	CHU, Caen, France
UCMA 970	*candidum*	Human tongue	CHU, Caen, France
UCMA 972	*candidum*	Human stools	CHU, Caen, France

Table 2 (Contd.)

Strain	*Geotrichum* sp.	Substrate	Geographical area of sampling
UCMA 4573	*candidum*	Mouth	CHU, Besançon, France
UCMA 4575	*candidum*	Human stools	CHU, Besançon, France
UCMA 4585	*candidum*	Sigmoïd colon abscess	CHU, Besançon, France
UCMA 4587	*candidum*	Human tongue, HIV + patient	CHU, Besançon, France
UCMA 4590	*candidum*	Human tongue, HIV + patient	CHU, Besançon, France
UCMA 4591	*candidum*	Ascitic fluid	CHU, Besançon, France
UCMA 4577	sp.	Human sputum	CHU, Besançon, France
CBS 571.82	*candidum*	Wood pulp factory	Sweden
CBS 152.25	*fragrans*	Water brewery	Unknown

Strains indicated by an asterisk were used for probe selection. Strains indicated in bold were used for optimization tests
ATCC American Type Culture Collection; *CBS* Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; *IFBM* Institut Français de Brasserie Malterie, Vandoeuvre les Nancy, France; *IHEM* Biomedical Fungi and Yeasts Collection, Brussells, Belgium; *IP* Collection Institut Pasteur; *LCP* Museum National d'Histoire Naturelle, Laboratoire de Cryptogamie Paris, France; *LMSA* Laboratoire de Microbiologie et Sécurité Alimentaire, ESMISAB (Brest, France); *NCPF* National Collection of Pathogenic Fungi, Bristol, UK; *UCMA* Université de Caen laboratoire de Microbiologie Alimentaire (Caen, France)

N: number of isolates
X_j: number of isolates per group.

Results

Identification of the species *G. candidum*

Twenty-six strains (Table 1) belonging to genera or species closely related to both the anamorphic and teleomorphic stages of *G. candidum* were chosen. Table 1 presents previous and revised nomenclature of the *Galactomyces* complex. The objective of the molecular identification technique was to distinguish rapidly and readily between *G. candidum* and other fungi with similar macro- and microscopic features (production of arthrospores). The M13 primer allowed for distinction between the species tested with a discriminatory power of 99% (Fig. 1).

The four strains of *G. candidum* (CBS 193.34, CBS 560.97, CBS 110.12 and ATCC 204307) displayed a strong band, characteristic of the species, with a molecular weight of 860 bp. This band was not found in the type strain of *Galactomyces geotrichum* (CBS 772.71), which had no bands in common with the four strains of *G. candidum*. To confirm the allocation to the species *G. candidum* of the 75 strains used in intraspecific study, they were tested with the M13 primer (data not shown). All of them presented the *G. candidum* pattern. The slight intraspecies variability observed for the four species representative strains (Fig. 1) was confirmed.

Traceability of *G. candidum*

The microsatellite primers tested had a simple structure: a core sequence repeated × times and a degenerate sequence at the 5′ end encoding many possible sequences other than the core sequence, such that each

Table 3 Primers tested for identification and tracing of *Geotrichum candidum* (D = A or T or G, B = T or C or G, H = A or T or C, V = A or C or G)

Primer	Sequence (5′-3′)	Annealing temperature (°C)
$(ACA)_5$	BDBACAACAACAACAACA	50
$(AAT)_5$	BBVAATAATAATAATAAT	48
$(CA)_8$	DBDBCACACACACACACACA	50
$(CAC)_5$	DBDBCACCACCACCACCAC	50
$(CAT)_4$	DBVCATCATCATCAT	48
$(CCA)_5$	DBDBCCACCACCACCACCA	50
$(CGA)_5$	DHBCGACGACGACGACGA	48
$(CGAT)_4$	DHBVCGATCGATCGATCGAT	50
$(CT)_8$	DVDVCTCTCTCTCTCTCTCT	50
$(GAA)_5$	HBBGAAGAAGAAGAAGAA	50
$(GACA)_4$	HBDBGACAGACAGACAGACA	50
$(GACC)_4$	HBDBGACCGACCGACCGACC	50
$(GAG)_5$	HBHGAGGAGGAGGAGGAG	48
$(GATA)_4$	HBVBGATAGATAGATAGATA	50/51[a]
$(GGAT)_4$	HHBVGGATGGATGGATGGAT	50
$(GGTG)_5$	HHVHGGTGGGTGGGTGGGTGGGTG	50
$(GTG)_5$	HVHGTGGTGGTGGTGGTG	48
$(TG)_8$	VHVVHTGTGTGTGTGTGTG	50
$(T3G3)_3$	VHHGTTTGGGTTTGGGTTTGG	50
1283	GCGATCCCA	48
M13	GAGGGTGGCGGTTCT	50

[a]Annealing temperature was optimized, depending on the thermocycler used

primer annealed to the end of the microsatellite, resulting in the amplification of the sequence between the microsatellites.

We tested 20 primers (Table 3) on 10 strains of *G. candidum* (indicated by asterisks in Table 2), to identify the most discriminatory primer. The Simpson's diversity index (*D*) [29], calculated from the dendrograms obtained with the various primers, was used to assess the capacity of primers amplifying the sequences between microsatellites to distinguish between isolates. The cut-off threshold was set at 85%. This percentage corresponds to the reproducibility threshold before optimization of the method.

Most of the primers (16/20) were only useful for interspecific discrimination. Only four primers allowed intraspecific differentiation: CGA5, GACA4, GGAT4 and GATA4.

GATA4 had the highest discriminatory power (0.94) of any of the 20 primers tested. The genetic profiles of the strains differed in terms of the number of fragments (mean = 7), their position and their intensity. All bands varied according to the strain studied.

Table 4 Final protocol for RAM-PCR fingerprinting of *G. candidum* strain

Growth conditions					
PDA / cellophane 72 h - 25°C					
DNA extraction					
Mycelium ground in liquid nitrogen (mixer mill) DNeasy plant kit (Qiagen) or equivalent					
DNA quantification					
by fluorimetry with Picogreen® reagent (Molecular Probes) or equivalent					
PCR : Mix composition (50µl)					
Primer : $(GATA)_4$ Make aliquots			5' HBVBGATAGATAGATAGATA 3'		
Primer (initial concentration = 100pmol/µl)			1 µl (=100 pmol in the mix)		
dNTP mix (5mM)			2µl		
Incubation mix 10X with $MgCl_2$ (2.5mM)			5µl		
Taq DNA polymerase (15U/µl) (Qbiogen)			0.2 µl		
DNA			25 ng in 6µl of AE buffer (Dneasy Plant kit)		
distilled H_2O (in vial)			Qsp 50µl		
PCR : Parameters (I-CYCLER/Biorad or equivalent)					
Cycle	**objectif**	**Step**	**Temperature (°C)**	**Length mn : sec**	**repetition**
1	denaturation	1	94	04 :00	1
2	amplification	1	94	01 :00	35
		2	51	01 :00	
		3	72	02 :00	
3	final extension	1	72	05 :00	1
4	conservation	1	4	∞	1
Analytical electrophoresis : migration					
Agarose type and concentration			Standard Agarose (Quantum AGAR 0050 or equivalent) 1%		
Type of gel			Maxi size (200 ml)		
Volume of PCR products to load			35 µl +5 µl of loading buffer		
voltage and run length			120 V - 3 h		
weight marker			XIV (Roche) or equivalent		
Number of well			20		
Q C strain					
Geotrichum candidum			ATCC204307, CBS 110.12, UCMA 291, UCMA 293, UCMA 302, UCMA 937.		

Intralaboratory repeatability of profiles

We compared profiles within a long-standing databank and assessed the intralaboratory repeatability of the molecular typing profiles, by evaluating several parameters within a single laboratory, laboratory A:

- The amount of DNA present in the amplification mixture, with a view to its standardization. The DNA for a given assay was obtained from independent cultures of the same strain.
- Annealing temperature and PCR machine used (I-Cycler, BioRad). The two machines were both of the same model, produced by the same manufacturer but were of different ages. This made it possible to simulate the aging of a machine within a laboratory.

The various steps (culture, DNA extraction, constitution of the amplification mixture, amplification and electrophoresis) were carried out totally independently. DNA was systematically extracted from pure cultures on silicon columns to facilitate subsequent analysis, to guarantee the speed of manipulations and to minimize the effects of extraction buffers made within the laboratory. The stability of the profiles generated for six strains (shown in bold in Table 2) was studied (Fig. 2).

Standardization of the amount of DNA in amplification mixtures Despite standardized culture conditions, the amount of DNA extracted from independent cultures of the six tested strains varied considerably (from 700 ng to 8 µg of DNA per extraction). This variability probably results, at least in part, from the hand grinding of the samples. In studies aiming to establish links between strains and to create homogeneous groups, it is essential to standardize the amount of DNA in amplification mixtures. The DNA extracts were thus systematically quantified by fluorimetry and normalized before using for amplification. Different quantities of DNA were tested: 25 ± 5, 50 ± 5 and 75 ± 5 ng. The profiles generated were compared (data not shown).

The highest percentage of similarity was obtained with 25 ± 5 ng of DNA, in 6 µl of elution buffer. Figure 2 presents the amplification patterns obtained with three different DNA quantity (20, 24 and 28 ng) by random amplified microsatellite (RAM)-PCR.

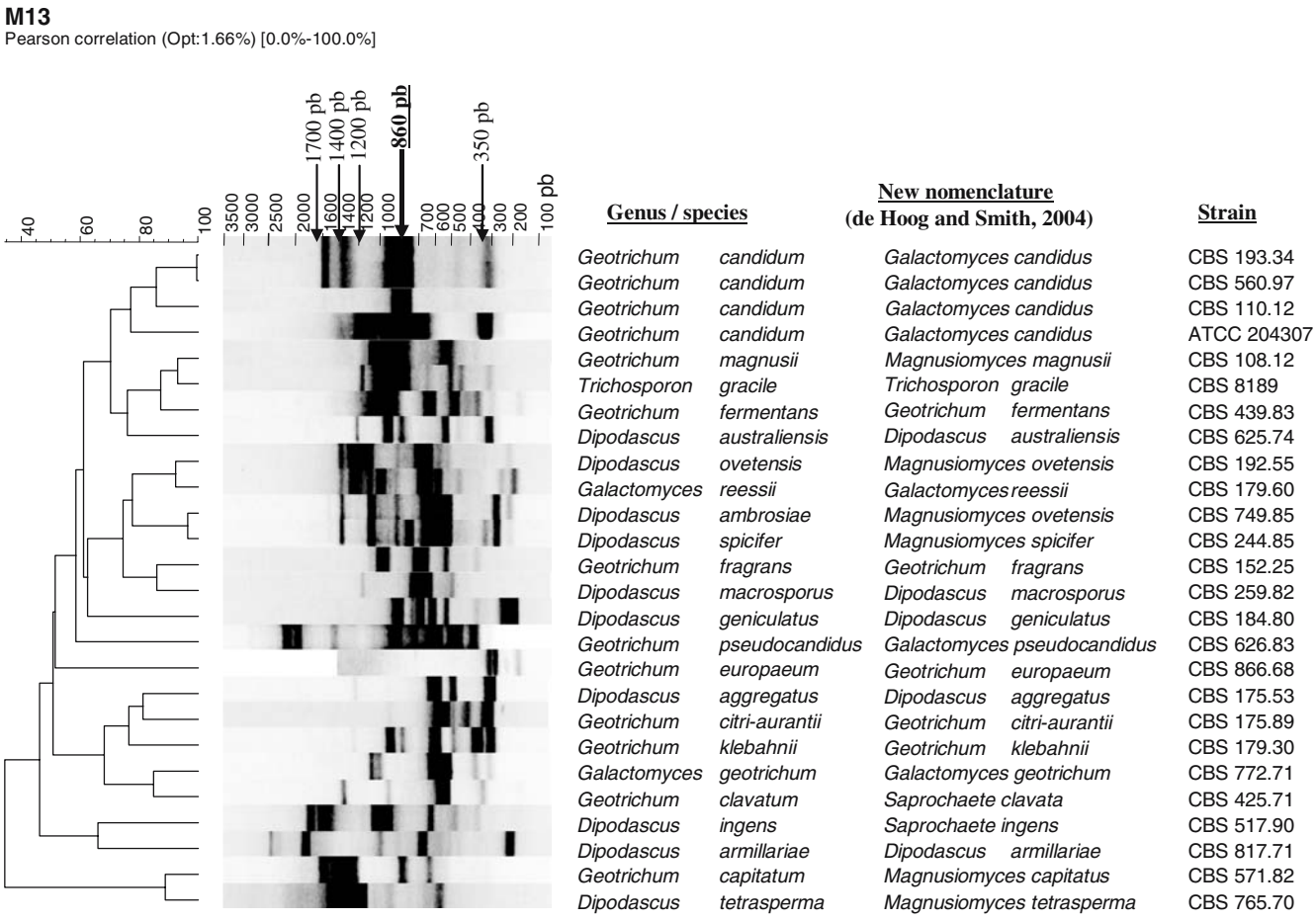

Fig. 1 UPGMA dendrogram derived from patterns generated by M13 primer on strains belonging to genera or species closely related to both the anamorphic and teleomorphic stages of *G. candidum*. *Arrows* label bands of the *G. candidum* pattern. Band sizes are indicated

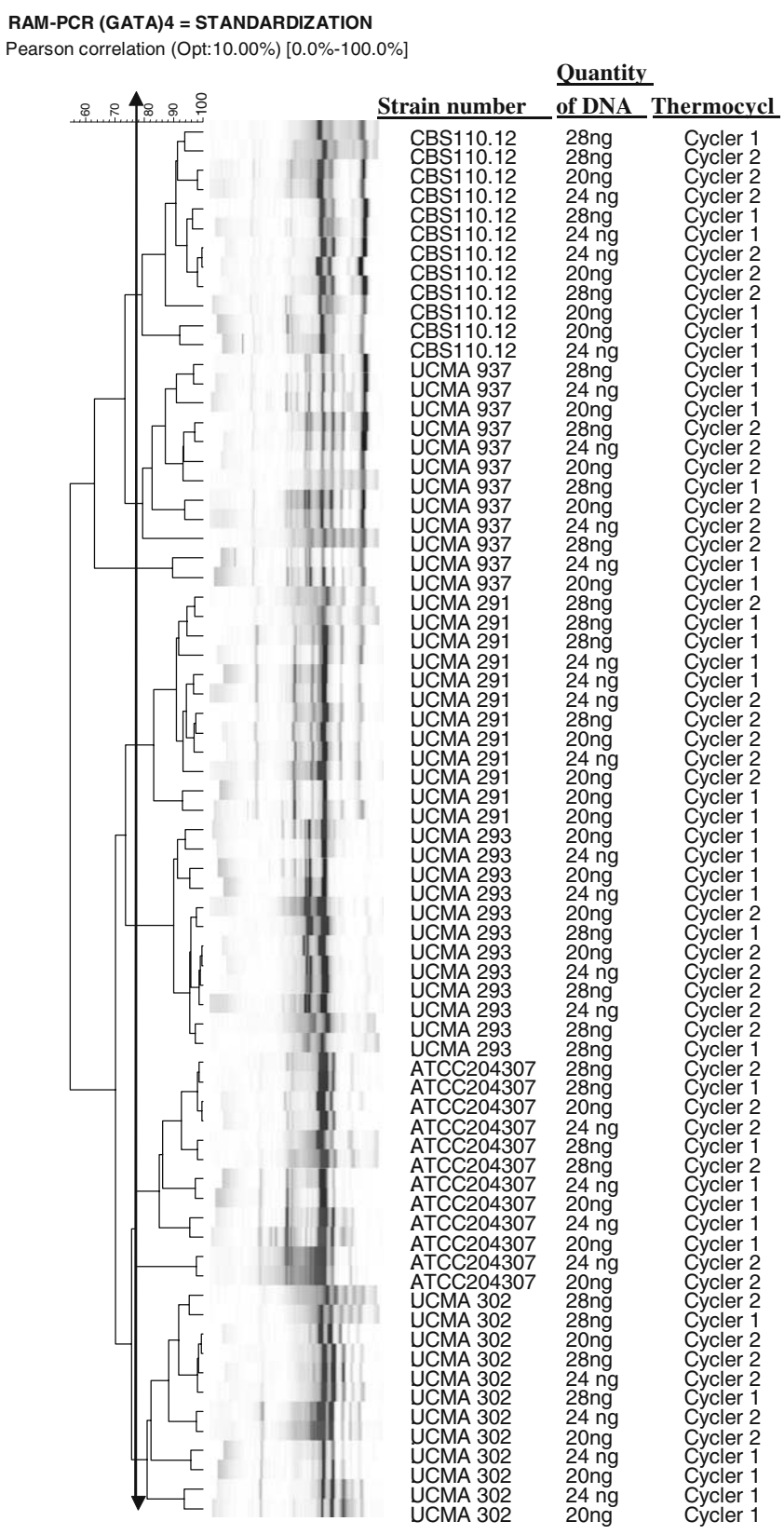

Fig. 2 Stability of the profiles generated for 6 strains of *G. candidum* by the GATA4 probe. Three DNA quantities tested: 20, 24 and 28 ng (corresponding to 25 ± 5 ng) with annealing temperature of 51°C. Two thermocyclers tested: cycler 1 and cycler 2

Effect of annealing temperature and age of PCR machine The temperature at which primers hybridize to the DNA matrix as well as the age and model of PCR machine play an essential role. Amplification conditions were optimized by testing various hybridization temperatures (data not shown). We tested two identical PCR machines of different ages (Fig. 2). The profiles obtained with an annealing temperature of 51°C presented satisfactory discriminatory power and repeatability within laboratory A. For a given strain, profiles obtained in a totally independent manner were 75–85% similar.

In conclusion, the profiles were clearly repeatable within a given laboratory:

- for quantities of DNA of between 20 and 28 ng,
- regardless of the age of the machine,
- using DNA extracted independently from different cultures and
- if the primer annealing temperature was 51°C.

Interlaboratory comparison of genetic profiles We then determined the conditions allowing interlaboratory (i.e. different consumables and different PCR machines) comparisons. Two independent laboratories (A and B) analyzed the same set of six strains (Fig. 3).

As different PCR machines were used, it was necessary to adjust the annealing temperature of the primers in each laboratory. Both laboratories classified the strains similarly, although slight differences in profile were observed.

The six strains tested will be referred to hereafter as the quality control (QC) strains for random amplified microsatellite-PCR (RAM-PCR). Laboratories using RAM-PCR for the molecular typing of strains of *G.blank;candidum* should always include the six QC strains tested and should adjust the hybridization temperature according to the PCR machine used so that the obtained dendrogram is in accordance with the ones obtained in this study.

The conditions required for intralaboratory repeatability and for interlaboratory comparisons are summarized in Table 3.

Validation

The standardized protocol was applied (in the laboratory C) on 77 strains (Table 2) to evaluate *G. candidum* biodiversity.

The discriminatory power of the GATA4 primer was lower (D = 0.87) than previously reported, probably because most of the strains tested were of human origin (Fig. 4a). This primer revealed a high degree of diversity, illustrated by variations of the number and length of the observed bands. Four types of profile were observed based on the presence or absence of a 600-bp band and an 800-bp band (group I to IV, Fig. 4b), which were 56–80% identical. All 28 strains in the first group displayed a 600-bp band. All 11 strains in the second group displayed an 800-bp band. The third group (29 strains) exhibited both the 600- and the 800-bp bands. Finally, the nine strains in the fourth group contained neither of these two bands.

Discussion

As previously indicated, the taxonomy of the genus *Geotrichum* has been recently revised [12], based on ribosomal sequence comparisons (18S, 26S, ITS),

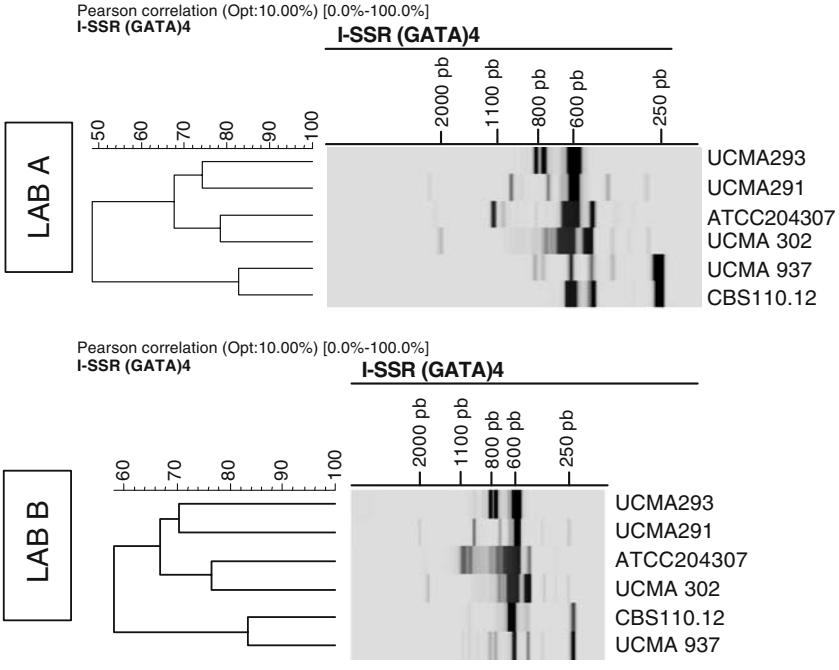

Fig. 3 Interlaboratory comparison of genetic profiles of 6 strains of *G. candidum* generated by the random amplified microsatellite (RAM)-PCR technique (using the GATA4 oligonucleotide)

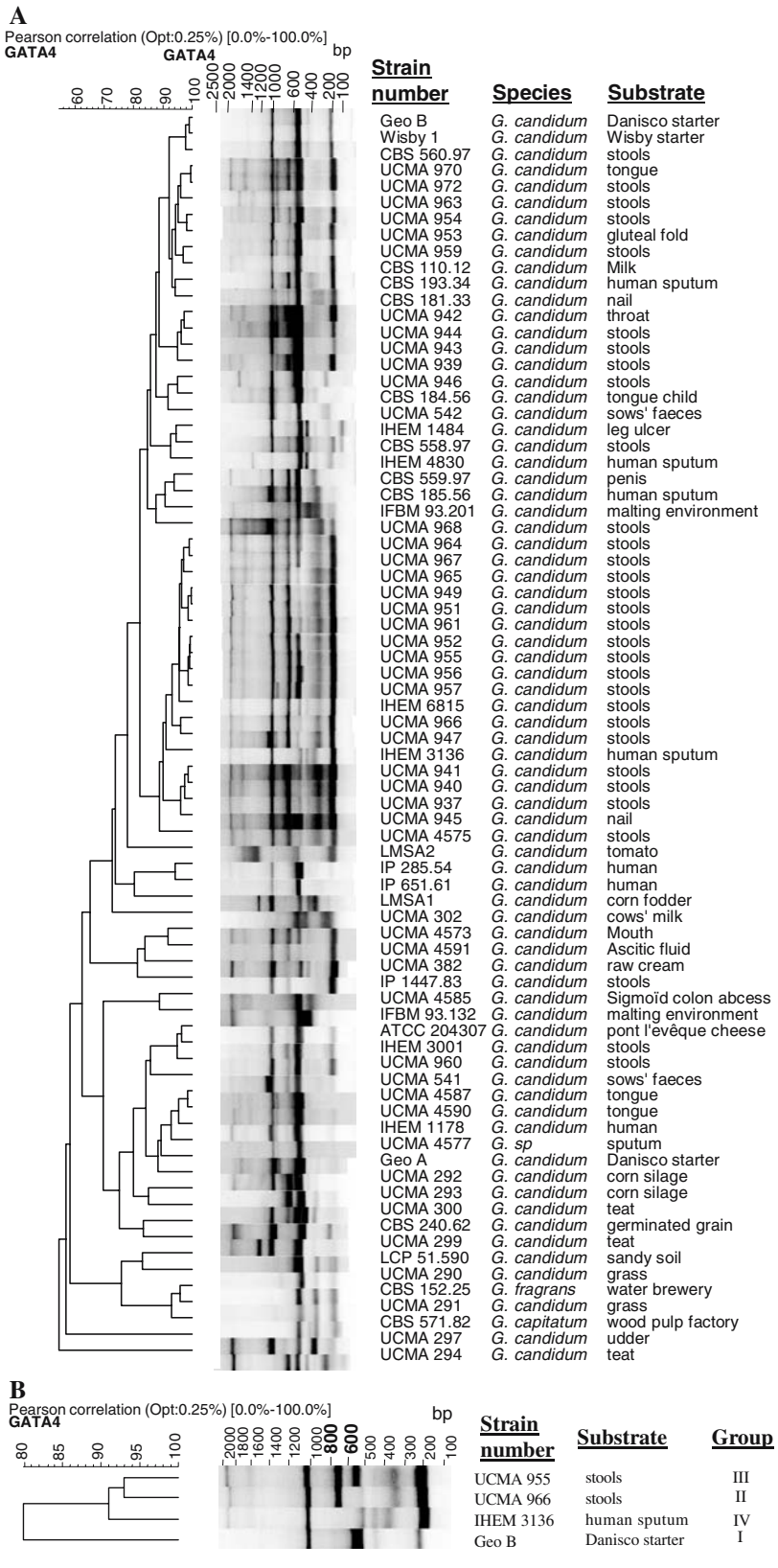

Fig. 4 **a** Validation step. UPGMA dendrogram derived from pattern generated by GATA4 primer on 75 *G. candidum* strains, 1 *G. capitatum* strain and 1 *G. fragrans* strain. **b** Example of strains corresponding to the four types of profile encountered after RAM-PCR amplification with the GATA4 primer in the *G. candidum* species. Group I: 600 bp band, Group II: 800 bp band, Group III: 600 + 800 bp bands, Group IV: neither of these two bands

resulting in proposed modifications of the teleomorph/anamorph genera. *Galactomyces* and *Dipodascus* were linked with *Geotrichum* anamorphs and *Magnusiomyces* with *Saprochaete* anamorphs. Moreover, the *G. geotrichum*/*G. candidum* complex which contained four separate species: *Gal. geotrichum* sensu stricto, and A, B and

C are now classified, respectively, as *Gal. geotrichum*, a new teleomorph named *G. candidus* has been designated for *G. candidum* (ex species A), *Gal. pseudocandidus* (ex species B) and a new species was created named *Geo. europaeum* (ex species C).

In the first part of this work a rapid and reliable method for the identification of the *G. candidum*/*Gal. candidus* species was developed. As a prerequisite to the molecular method proposed (M13-PCR), it is necessary to observe the strain under a microscope to ensure that it is indeed a fungus with holothallic spore production (hyphae splitting at the septum to form individual cells called arthrospores). The M13 primer discriminated very efficiently the tested species, *G. candidum* species was characterized by the presence of a common three bands profile, among which a major band at 860 pb was observed. This technique allowed the differentiation of the newly described species which were previously grouped in the same complex [12, 47]. The observed differences in the genetic profiles of *G. geotrichum* and *G. candidum*, already reported [5, 49], reinforce the creation of a new teleomorph.

The second part of this work involved molecular typing of *G. candidum* strains. The intraspecific differences observed with the M13 primer on 77 strains, chosen according to their biotope and their morphotype, led us to investigate the biodiversity of strains of *G. candidum* isolated from food and environment (25/77) (raw milk, cheeses, breweries, grass, tomato, silage and commercial starter cultures) as well as from human samples (stools, sputum, tongue, throat, nails, gluteal fold, penis, leg ulcer or ascitic fluid). Strains were typed, with the aim of developing a standardized, reproducible, reliable and easy protocol, using a DNA kit, to minimize the effect of using buffers made in different laboratories. Preliminary tests with ten strains showed that the DNeasy Plant Mini kit (Qiagen) gave similar profiles to those obtained with a classical DNA extraction method. Although *G. candidum* is officially considered as a yeast, the DNeasy tissue kit (Qiagen) generally used for yeast could not be used in this case. Indeed, the wall of *G. candidum* is similar to that of filamentous fungi: rich in cellulose and chitin, thicker and denser than those of "classical" yeasts [30, 57]. For this reason, plant kits are more suitable.

Only four of the 20 RAM-PCR primers tested were discriminatory (*D* varied between 0.3 and 0.94). The poor discrimination obtained with most of the RAM-PCR primers tested resulted either from a small number of amplified bands, or from a multiband profile but with a very small number of variations or from variations affecting only low-intensity bands.

The GATA4 primer showed a discriminatory power of 0.94 with the first ten strains tested. The addition of 67 new strains, including 52 human strains, decreased the discriminatory power to 0.87 when the dendrogram was constructed using all 77 strains. This decrease may be due to disproportionate numbers of strains from certain biotopes. Indeed, the GATA4 primer can mostly identify strains belonging to the species *G. candidum* and classify them according to their ecological niche [24]. Extension of the genetic study to human strains showed that strains with a 600-bp band came from dairy products and stools, whereas those with an 800-bp band were isolated exclusively from human substrates. This band may be characteristic of human strains, suggesting the existence of a human ecological niche.

Some strains possessing both bands were isolated from stools, sputum and the tongue. Thus, passage in the digestive tract seems to be accompanied by genetic modifications that can be visualized by RAM-PCR. Alternatively, it is possible that only adapted strains (i.e. strains with certain genetic profiles) [40] are able to survive in the gastrointestinal tract.

The quality assurance step that should be carried out when typing *G. candidum* strains by RAM-PCR has been described in this study. Quality control strains have been identified: they must systematically be included for interlaboratory comparisons. The optimized protocols make it possible to obtain repeatable profiles in the same laboratory and to compare results between laboratories. The technique has been shown to be robust: the dendrograms generated by typing the quality control strains in two different laboratories were similar. This technique should help to demonstrate the biodiversity of yeast strains by means of simple, rapid, universal methods. The robustness of the RAM-PCR technique makes it possible to compare the profiles of *G. candidum* strains obtained at different stages in a given laboratory, the stability of starter during production and subsequent use. It makes it possible to create and to manage profile databanks, to constitute a collection of strains presenting a certain biodiversity and to compare strains from different biotopes.

Conclusion

In this study, methods for the species identification and the typing of *G. candidum* strains, respectively, by M13-PCR and by RAM-PCR were developed. These procedures generate profiles that are repeatable within a given laboratory. A procedure facilitating the interlaboratory comparison of profiles of *G. candidum* strains was also developed.

Similar studies have been carried out for the molecular typing of strains of technological importance such as members of the species *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Issatchenkia orientalis*, together with other laboratories (Laboratory of Food Microbiology of the University of Caen-Basse Normandie, the INRA Collection of Yeasts of Biotechnological Interest, ADRIA Normandie, ADRIA Dévelopment). A database of the genetic profiles of these strains of yeasts is now maintained by several laboratories, making it possible for the agro-food industry to evaluate the biodiversity of strains of technological interest.

Acknowledgments This work was carried out within the framework of several research programs funded by the ACTIA and ARILAIT-Recherches: ''Molecular keys for the identification of yeasts and molds and traceability of yeasts'' and ''MATEASy'' (molecular and analysis tools for easy and simple identification). This work was supported by the Association de Coordination Technique des Industries Alimentaires (ACTIA), the Association de Recherche des Industries Laitières (ARILAIT RECHERCHE), the Ministère de l'Education Nationale, de l'Enseignement Supérieur, et de la Recherche (MENESR, France) and by the Institut National de la Recherche Agronomique (INRA, France). We thank the Institut Français des Boissons de la Brasserie Malterie for the malting environment strain and F. Grenouillet (Parasitology Laboratory, Besançon University Hospital) for providing the human strains of *G. candidum*.

References

1. Ayed L, Assas N, Sayadi S, Hamdi M (2005) Involvment of lignin peroxydase in the decolourization of black olive mill wastewaters by *Geotrichum candidum*. Lett Appl Microbiol 40:7–11
2. Barnett JA, Pankhurst RJ (1974) A new key to the yeasts. American Elsevier, North Holland
3. Barnett JA, Payne RW, Yarrow D (2000) Yeasts: characteristics and identification. 3rd edn. Cambridge university press, Cambridge
4. Berger C, Khan JA, Molimard P, Martin N, Spinnler HE (1999) Production of sulfur flavors by ten strains of *Geotrichum candidum*. Appl Environ Microbiol 65:5510–5514
5. Blanchard V (2000) [Contribution to the molecular characterization of *Geotrichum candidum*]. Universite Claude Bernard LYON I
6. Boivin P, Malanda M (1997) Improvement of malt quality and safety by adding starter culture during the malting process. Tech Q Master Brew Assoc Am 34:96–101
7. Borja R, Martin A, Maestro R, Alba J, Fiestas JA (1992) Enhancement of the anaerobic digestion of olive mill wastewater by the removal of phenolic inhibitors. Process Biochem 27:231–237
8. Boutrou R, Gueguen M (2005) Interests of *Geotrichum candidum* for cheese technology. Int J Food Microbiol 102:1–20
9. Bruford MW, Wayne RK (1993) Microsatellites and their application to population genetic studies. Curr Opin Genet Dev 3:939–943
10. Cichowicz SM, Bandler R (1980) Determination of Geotrichum mold in citrus juices: collaborative study. J Assoc Off Anal Chem 63:483–484
11. Coulabaly L, Gourene G, Agathos NS (2003) Utilization of fungi for biotreatment of raw wastewaters. Afr J Biotechnol 2:620–630
12. De Hoog GS, Smith MT (2004) Ribosomal gene phylogeny and species delimitation in Geotrichum and its teleomorphs. Stud Mycol 50:489–515
13. Demarigny Y, Berger C, Desmasures N, Gueguen M, Spinnler HE (2000) Flavour sulphides are produced from methionine by two different pathways by *Geotrichum candidum*. J Dairy Res 67:371–380
14. Desmasures N, Bazin F, Gueguen M (1997) Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. J Appl Microbiol 83:53–58
15. Engel G (1986) Hefen in silagen und rohmilch. Milchwissenschaft 41:633–637
16. Engel G, Teuber M, Hoffmeister G, Neumann KI (1980) Entwicklung von Hefen und Schimmelpilzen in Speisequark. Milchwissenschaft 35:13–16
17. Espinel-Ingroff A, Stockman L, Roberts G, Pincus D, Pollack J, Marler J (1998) Comparison of RapID Yeast Plus System with API 20C System for identification of common, new, and emerging yeast pathogens. J Clin Microbiol 36:883–886
18. Fiddy C, Trinci AP (1976) Nuclei, septation, branching and growth of *Geotrichum candidum*. J Gen Microbiol 97:185–192
19. Fitzgibbon FJ, Nigam P, Singh D, Marchant R (1995) Biological treatment of distillery waste for pollution - remediation. J Basic Microbiol 35:293–301
20. Foszcynska B, Dzubia E, Stempniewicz R 2004 The use of *Geotrichum candidum* starter culture for protection of barley and its influence on biotechnological qualities of malts. Electronic journal of polish agricultural universities
21. Garcia IG, Venceslada JLB, Pena PRJ, Gomez ER (1997) Biodegradation of phenol compounds in vinasse using *Aspergillus terreus* and *Geotrichum candidum*. Water Res 31:2005–2011
22. Garcia IG, Pena PRJ, Venceslada JLB, Martin AM, Santos MAM, Gomez ER (2000) Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. Process Biochem 35:751–758
23. Gente S, Desmasures N, Jacopin C, Plessis G, Beliard M, Panoff JM, Gueguen M (2002) Intra-species chromosome-length polymorphism in *Geotrichum candidum* revealed by pulsed field gel electrophoresis. Int J Food Microbiol 76:127–134
24. Gente S, Desmasures N, Panoff JM, Gueguen M (2002) Genetic diversity among *Geotrichum candidum* strains from various substrates studied using RAM and RAPD-PCR. J Appl Microbiol 92:491–501
25. Grigoriu D, Delacretaz J, Borelli D (1986) Traité de mycologie médicale Paris pp 482
26. Guessous Z, Ouhssine M, Mokhtari A, Faid M, El Yachioui M (2000) Isolement et caractérisation de *Geotrichum candidum* pour la production d'une polygalacturonase extracellulaire. Sci Aliments 20:309–320
27. Heinic GS, Greenspan D, MacPhail LA, Greenspan JS (1992) Oral *Geotrichum candidum* infection associated with HIV infection. A case report. Oral Surg Oral Med Oral Pathol 73:726–728
28. Hennequin C, Thierry A, Richard GF, Lecointre G, Nguyen HV, Gaillardin C, Dujon B (2001) Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains. J Clin Microbiol 39:551–559
29. Hunter PR (1991) A critical review of typing methods for *Candida albicans* and their applications. Crit Rev Microbiol 17:417–434
30. Jacobsen T, Jensen B, Olsen J, Allermann K (1985) Preparation of protoplasts from mycelium and arthroconidia of *Geotrichum candidum*. Can J Microbiol 31:93–96
31. Kantardjiev T, Kuzmanova A, Baikushev R, Zisova L, Velinov T (1998) Isolation and identification of *Geotrichum candidum* as an etiologic agent of geotrichosis in Bulgaria. Folia Med 40:42–44
32. Katti MV, Ranjekar PK, Gupta VS (2001) Differential distribution of simple sequence repeats in eukaryotic genome sequences. Mol Biol Evol 18:1161–1167
33. Kim SJ, Shoda M (1999) Decolorization of molasses and dye by a newly isolated strain of the fungus *Geotrichum candidum* Dec 1. Biotechnol Bioeng 62:114–119
34. Kreger van Rij NTW (1984) The yeasts: a taxonomic study. Elsevier, Amsterdam
35. Kurtzman CP, Fell JW (1999) The yeasts: a taxonomic study. 4th edn. Elsevier, Amsterdam
36. Laville E (1974) La pourriture des agrumes due à *Geotrichum candidum* Link var. *citri-aurentii* (Ferr.). Fruits 29:35–38
37. Lo Curto RB, Tripodo MM, Leuzzi U, Giuffre D, Vaccarino C (1992) Flavonoids recovery and SCP production from orange peel. Bioresour Technol 42:83–87
38. Lodder J, Kreger van Rij NTW (1952) The yeasts: a taxonomic study. North Holland Publishing Company, Amsterdam, pp 713
39. Manivannan PVG, Chand R, Aggarwal PK (2001) Screening of lindane degrading milk mould. Indian J Dairy Biosci 12:80–83

40. McCullough M, Ross B, Reade P (1995) Characterization of genetically distinct subgroup of *Candida albicans* strains isolated from oral cavities of patients infected with human immunodeficiency virus. J Clin Microbiol 33:696–700
41. Meyer W, Latouche GN, Daniel HM, Thanos M, Mitchell TG, Yarrow D, Schönian G, Sorrell TC (1997) Identification of pathogenic yeasts of the imperfect genus *Candida* by polymerase chain reaction fingerprinting. Electrophoresis 18:1548–1559
42. Mislivec PB, Cichovicz SM, Bruce VR (1974) Incidence and proliferation of *Geotrichum candidum* in refrigerated cake yeast and factors affecting its growth and detection. J Milk Food Technol 37:350
43. Mo K, Hayashida S (1988) Production and characterization of two types od endocellulase from *Geotrichum candidum*. Agric Biol Chem 52:1675–1682
44. Molimard P, Buchet C, Boivin P (2005) Use of *Geotrichum candidum* starters during the malting process for malt bioprotection. 30th EBC congress Prague
45. Monfort A, Blasco A, Sanz P, Prieto JA (1999) Expression of LIP1 and LIP2 genes from *Geotrichum* species in Baker's yeast strains and their application to the bread-making process. J Agric Food Chem 47:803–808
46. Nagao T, Shimada Y, Sugihara A, Tominaga Y (1993) Cloning and sequencing of two chromosomal lipase genes from *Geotrichum candidum*. J Biochem (Tokyo) 113:776–780
47. Naumova ES, Smith MT, Boekhout T, de Hoog GS, Naumov GI (2001) Molecular differentiation of sibling species in the Galactomyces geotrichum complex. Antonie Van Leeuwenhoek 80:263–273
48. Nelson MV (1953) Nutritional factors influencing growth and lipase production by *Geotrichum candidum*. J Dairy Sci 36:143–151
49. Prillinger H, Molnar O, Eliskases-Lechner F, Lopandic K (1999) Phenotypic and genotypic identification of yeasts from cheese. Antonie Van Leeuwenhoek 75:267–283
50. Quinn JP, Marchant R (1980) The treatment of malt wiskey distillery waste using the fungus *Geotrichum candidum*. Water Res 14:545–551
51. Raeder U, Broda P (1985) Rapid preparation of DNA filamentous fungi. Lett Appl Microbiol 1:17–20
52. Shimada Y, Sugihara A, Tominaga Y, Iizumi T, Tsunasawa S (1989) cDNA molecular cloning of *Geotrichum candidum* lipase. J Biochem (Tokyo) 106:383–388
53. Skoropad FI (1968) [Yeasts in fruit juices]. Mikrobiol Zh 30:502–506
54. Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. Univ Kansas Sci Bull 22:1409–1438
55. Splittstoesser DF, Bowers J, Kerschner L, Wilkinson M (1980) Detection and incidence of *Geotrichum candidum* in frozen branched vegetables. J Food Sci 45:511–513
56. Tiunova NA, Rodionova NA, Martinovich LI (1980) The cellulose hydrolysing enzyme system of *Geotrichum candidum*. Appl Biochem Microbiol 16:40–45
57. Trinci APJ (1971) Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. J Gen Microbiol 67:325–344
58. Vaccarino C, Lo Curto RB, Tripodo MM, Patane RM, Lagana G, Schachter S (1989) SCP from orange peel by fermentation with fungi. Submerged and "surface" fermentations. Biol Wastes 29:279–287
59. Vauterin L, Vauterin P (1992) Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur Microbiol 1:37–41
60. Walmsley M, Barrie M, Kong TH (1989) Genetic fingerprinting for yeasts. Biotechnology 7:1168–1170
61. Wouters JTM, Ayad EHE, Hugenholtz J, Smit G (2002) Microbes from raw milk for fermented dairy products. Int Dairy J 12:91–109
62. Yoshii H, Furuta T, Ikeda M, Ito T, Lefuji H, Linko P (2001) Characterization of the cellulose-binding ability of *Geotrichum* sp. M111 cells and its applications to dehydratation of the distilled waste of sweet potato shouchu. Biosci Biotechnol Biochem 65:2187–2192