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Molecular differentiation of sibling species in the *Galactomyces geotrichum* complex

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Abstract

PCR-analysis, multilocus enzyme electrophoresis and molecular karyotyping were used to characterize 52 strains belonging to the genus *Galactomyces*. The resultant data revealed that a PCR method employing the universal primer N21 and microsatellite primer $(CAC)_5$ is appropriate for the distinction of four *Ga. geotrichum* sibling species, *Ga. citri-aurantii* and *Ga. reessii*. Better separation was achieved with the UP primer N21; each species displayed a specific pattern with very low intraspecific variation. We propose to use the primer N21 for the differentiation of the six taxa composing the genus *Galactomyces*. Multilocus enzyme electrophoresis revealed genetic homogeneity of each sibling species within the *Ga. geotrichum* complex. On the other hand, the four sibling species, having from 41 to 59% of nDNA homology and similar phenotypic characteristics, are clearly distinguished based on their electrophoretic profiles using two enzymes: mannose-6-phosphate isomerase (MPI) and phosphoglucomutase (PGM). Despite the same number of chromosomal bands, different karyotype patterns were found in *Ga. geotrichum* sensu stricto and its two sibling species A and B. Within each sibling species, chromosome length polymorphism was observed, in particular for small bands, allowing discrimination to the strain level.

Introduction

The yeast-like fungus *Galactomyces* Redhead & Malloch (de Hoog et al. 1998b) is an attractive model to study the process of speciation within a clearly delimited genus. *Galactomyces* contains biological species which have, as a rule, a common mating type system (Butler et al. 1988; Smith et al. 1995, 2000; Naumov et al. 1999a,b). Judging from sequence data of 26S rRNA genes (Kurtzman & Robnett 1995, 1998; Ueda-Nishimura & Mikata 2000), the genus shows a certain relatedness to *Dipodascus* de Lagerheim (de Hoog et al. 1998a), both genera constituting the Hemiascomycetes family *Dipodascaceae*. Anamorphs of *Galactomyces* are classified in *Geotrichum*; the most important species is *Ge. candidum* Link:Fries, the anamorph of *Ga. geotrichum* (E.E. Butler & L.J. Petersen) Redhead & Malloch (de Hoog et al. 1998b). The biochemical activity of *Galactomyces* fungi is important for the food industry. In addition, some strains can be either weak plant pathogens or occasionally associated with human pulmonary disease.

Using nDNA–DNA reassociations, Guého et al. (1985) revealed heterogeneity in the anamorph *Ge. candidum. Ge. candidum* CBS 109.12 had a moderate (45%) DNA homology value with *Ga. geotrichum* CBS 775.71 (teleomorph), but showed high DNA homology with *Ge. candidum* CBS 178.53 and 149.26 (96 and 94%, respectively). De Hoog et al. (1986) further determined two different types of strains within *Ga. geotrichum* (CBS 820.71, CBS 626.83 and CBS 149.26, CBS 178.71) which had DNA homology values of 46% and 44% with the type culture of *Ga. geotrichum* CBS 772.71, respectively. Analysis of many

strains revealed a further taxon (strain CBS 866.68), which showed 57% DNA–DNA reassociation with the type culture CBS 772.71 (Smith et al. 1995). Based on DNA-DNA reassociation data, the genus *Galactomyces* now consists of *Ga. reessii* (van der Walt) Redhead & Malloch , *Ga. citri-aurantii* E.E. Butler and four closely related species of the *Ga. geotrichum* complex designated as A, B, C and *Ga. geotrichum* sensu stricto (Smith et al. 1995, 2000). Within the complex, the four species show 41–59% DNA homology while their DNA homology with *Ga. reessii* and *Ga. citri-aurantii* is 20-27%. Sibling species B and C cannot be distinguished on the basis of conventional tests used in yeast taxonomy, while species A and *Ga. geotrichum* sensu stricto can be differentiated from one another and from the former two species only by a combination of phenotypic characteristics (Smith et al. 1995, 2000). Judging from a large divergence of the 18S rRNA genes, two Japanese strains attributed to *Ge. candidum* have recently been shown not to be anamorphs of the type strain of *Ga. geotrichum* (Ueda-Nishimura & Mikata 2000).

We undertook the present study in order to obtain additional data underlining the species status of the four taxa within the *Ga. geotrichum* complex and to find molecular markers allowing their differentiation. *Ga. citri-aurantii* and *Ga. reessii* were included as references.

Materials and methods

Yeast strains

The strains and their origins are listed in Table 1.

PCR-fingerprinting

DNA extraction was performed as described by Gerrits van den Ende & de Hoog (1999). DNA concentrations were evaluated by spectrophotometer assays. A negative control without DNA was included in each experiment. PCR-fingerprinting was performed with different primers: universal primer N21, microsatellite primers and M13.

The UP-PCR technique (Universally Primed Polymerase Chain Reaction) (Bulat & Mironenko 1990) differs from RAPD (Williams et al. 1990) in primer design and reaction conditions (Bulat et al. 1998). The PCR was performed in 20 μl containing 20 mM Tris–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.6 mM each of dNTPs, 4 mM $MgCl_2$, 40 ng of a primer, between 20 and 200 ng of genomic template DNA, and 2–3 units of SuperTaq DNA polymerase. The sequence of the universal primer N21 is 5′-GGATCCGAGGGTGGCGGTTCT – 3′. Amplifications were performed in a DNA thermal cycler Amplitron® II (Barnstead/Thermolyne, USA) programmed for a denaturation step at 94°C for 3 min followed by 30 cycles of 50 s at 94°C, 80 s at 55°C, 60 s at 70°C, and a final extension of 3 min at 70°C. The PCR products were analysed by electrophoresis in agarose (1.2%) gels. The gels were stained with ethidium bromide and photographed in UV light.

For microsatellite PCR, the primer sequences used included $(GTG)_5$, $(GACA)_4$ and $(CAC)_5$. Amplification reactions were performed in a volume of 25 μl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.2 mM each of dNTPs, 2 mM $MgCl_2$, 0.001% gelatine, 20–30 ng of primer, 25 ng of genomic template DNA, and 2.5 units of SuperTaq DNA polymerase. An Amplitron® II (Barnstead/Thermolyne, USA) thermal cycler was programmed for 30 cycles of 30 s at 94°C, 30 s at 42°C for primer $(GACA)_4$, or 50°C for primers $(GTG)_5$ and $(CAC)_5$, and 60 sec at 72°C and a final extension of 10 min at 72°C. Forty amplification cycles were performed with M13 core sequence (5′-GAGGGTGGCGGTTCT-3′): denaturation of 1 min at 94°C, annealing of 2 min at 52°C, and elongation of 3 min at 74°C with a final extension of 10 min at 74°C. The PCR products were analysed by electrophoresis in agarose (1.2%) gels. The gels were stained with ethidium bromide and photographed in UV light.

GelCompar 4.0 (Applied Maths, Kotrijk, Belgium) was used to analyse fingerprint patterns with primer $(CAC)_5$. Pearson correlation was used for clustering. A dendrogram was generated using the Unweighted Pair Group with Mathematical Average (UPGMA) method (Felsenstein 1993).

Multilocus enzyme electrophoresis

Preparation of cell lysates for isoenzyme analysis was performed as follows. Overnight cultures grown in 20 ml of YPD (1% yeast extract, 2% peptone, 2% glucose) medium at 28°C were harvested by centrifugation at 5000 × *g* for 10 min, washed once with 1 ml of Tris–glycine buffer (30 g of Trizma Base (Sigma) per litre, 144 g of glycine per litre; pH 8.0), re-suspended in 0.5 ml of the same buffer, and frozen at −80°C. Frozen cell suspensions were thawed and immediately lysed by vortexing for 20 min with 0.25-mm diameter glass beads with repeating chilling on ice for 1 min

Table 1. Strains of *Galactomyces* used in this study

Strain	Origin and/or original designation
Ga. geotrichum sensu stricto	
CBS 772.71	Type culture of *Ga. geotrichum*; neotype of *Geotrichum candidum* homothallic, soil, Puerto Rico
CBS 773.71	Soil, Puerto Rico
CBS 774.71 (MT)[a]	CBS 773.71, mating type opposite to CBS 775.71
CBS 775.71 (MT)	CBS 773.71, mating type opposite to CBS 774.71
Sibling species A	
CBS 110.12	Probable type strain of *Oidium humi*, Institut Pasteur
CBS 114.23	Possible type strain of *Oidium nubilum*
CBS 115.23	Decaying fruit of *Lycopersicon esculentum*
CBS 116.23	Authentic strain of *Oospora lactis* var. *parasitica*, fruit of *Lycopersicon esculentum*, US
CBS 121.22	Bulb of *Hyacinthus orientalis*, The Netherlands
CBS 122.22	Authentic strain of *Oospora fragrans* var. *minuta*, *Musa* sp.
CBS 144.88	Fruit, The Netherlands
CBS 149.26	Authentic strain of *Oidium asteroides*
CBS 176.28	Fruit of *Durio zibethinus*
CBS 178.30	Type culture of *Oopora lactis* var. *exuberans*, white slime flux in *Populus alba*, Germany
CBS 178.53	Type culture of *Endomyces lactis*, Germany
CBS 178.71	Soil polluted with oil, Germany
CBS 180.33	Authentic strain of *Geotrichum matelense* var. *chapmanii*
CBS 181.33	Human nail, The Netherlands
CBS 182.33	Type culture of *Geotrichum javanense*, yoghurt, Italy
CBS 184.56	Human tongue, Germany
CBS 185.56	Human sputum, The Netherlands
CBS 187.67	Camembert cheese
CBS 193.34	Type culture of *Geotrichum versiforme*
CBS 195.35	Authentic strain of *Geotrichum matelense* var. *matelense*
CBS 224.48	Fly in petroleum
CBS 240.62	Germinating grain of *Hordeum vulgare*, The Netherlands
CBS 267.51	Sent by R. Ciferri as *Geotrichum pulmoneum*
CBS 279.84	Type culture of *Geotrichum redaellii*
CBS 299.84	Fruit of *Lycopersicon esculentum*, France
CBS 357.86	Unknown
CBS 476.83	Soil, Senegal
CBS 557.83	Type culture of *Geotrichum novakii*, fruit of *Prunus persica*, Egypt
CBS 606.85	*Drosophila* sp., Cameroon
CBS 607.84	Industrial contaminant, The Netherlands
CBS 607.85	*Drosophila* sp., Cameroon
CBS 615.84	Brie cheese, France
CBS 624.85	Type culture of *Trichosporon inulinum*
Sibling species B	
CBS 100.812	Bark of beech log (*Fagus sylvatica*), Switzerland
CBS 101.161	Sewage treatment filter, England, H.A.Painter
CBS 101.162	Soil locality near Lydenburg, South Africa
CBS 101.163	Soil, South Africa
CBS 267.79	*Beta vulgaris*, The Netherlands
CBS 626.83	Type culture of *Geotrichum pseudocandidum*, stomach of elk, France
CBS 820.71	Paper pulp, France

Table 1. Continued

Strain	Origin and/or original designation
Sibling species C	
CBS 866.68	Wheat field soil, Germany
Ga. citri-aurantii	
CBS 175.89 (MT)	Type culture of *Ga. citri-aurantii*, soil of orange orchard, Zimbabwe, mating type A1
CBS 176.89 (MT)	Type culture of *Ga. citri-aurantii*, soil of orange orchard, California, mating type A2
CBS 228.38 (MT)	*Citrus limonium*, Argentina, mating type A1
CBS 605.85 (MT)	*Citrus paradisi*, mating type A2 *Ga. reessii*
CBS 179.60	Type culture of *Endomyces reessii*, cold-water retting of *Hibiscus cannabinus*
CBS 295.84	From E. Guého
CBS 296.84	Dominican Republic, from Institut Pasteur

[a] MT, mating type.

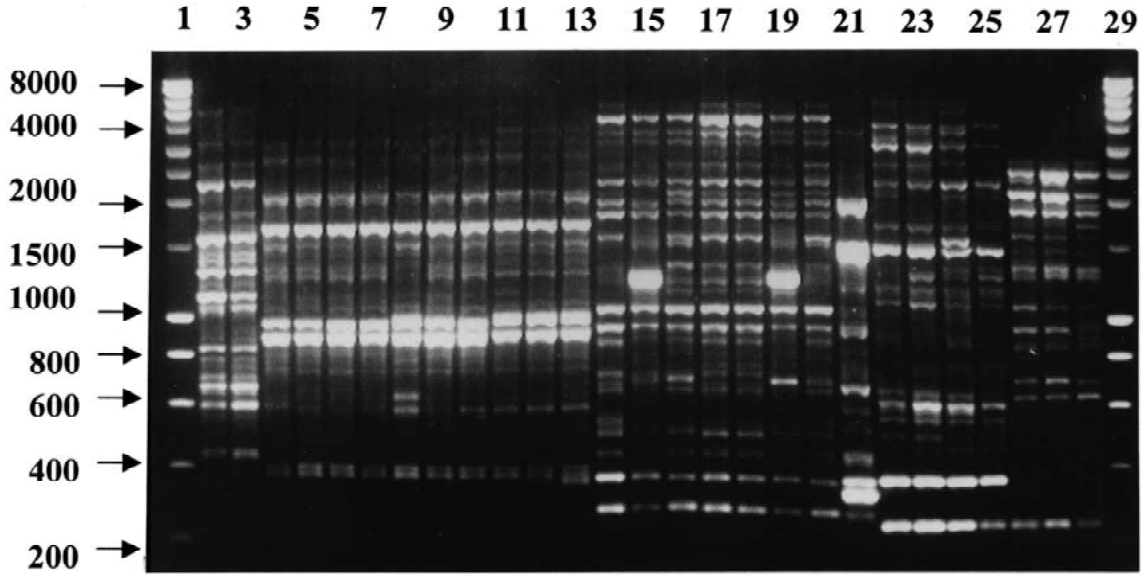

Figure 1. PCR banding profiles for four sibling species in the *Galactomyces geotrichum* complex, *Ga. citri-aurantii* and *Ga. reessii* generated with universal primer N21. Lanes 2, 3: *Ga. geotrichum*, CBS 772.71 and 773.71, respectively. Lanes 4–13: species A — CBS 110.12, CBS 121.22, CBS 116.23, CBS 149.26, CBS 178.30, CBS 180.33, CBS 182.33, CBS 476.83, CBS 557.83 and CBS 606.85, respectively. Lanes 14–20: species B — CBS 636.83, CBS 820.71, CBS 267.79, CBS 101.162, CBS 101.163, CBS 101.161 and CBS 100.812, respectively. Lane 21: species C — CBS 866.68. Lanes 22–25: *Ga. citri-aurantii* - CBS 175.89, CBS 176.89, CBS 228.38 and CBS 605.85, respectively. Lanes 26–28: *Ga. reessii* — CBS 179.60, CBS 295.84 and CBS 296.84, respectively. Lanes 1, 29: molecular weight SmartLadder marker (EUROGENTEC, Belgium) is indicated in base pairs.

after each 5 min of shaking. The mixtures were then centrifuged at 12 000 × *g* at 4°C to remove debris; cell lysates were distributed into chilled Eppendorf tubes in 100-μl aliquots and stored at −80°C until further use.

Enzymes were separated on 1.2% native polyacrylamide gels by using the PhastSystem^{TM} (Pharmacia, Sweden). Gel straining was done as described by Hebert & Beaton (1993). The following five enzymes were studied: alcohol dehydrogenase (ADH) (EC 1.1.1.1), glucose-6-phosphate isomerase (GPI) (EC 5.3.1.9), mannose-6-phosphate isomerase (MPI) (EC 5.3.1.8), glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49), and phosphoglucomutase (PGM) (EC 5.4.2.2). We used side-by-side electrophoretic comparisons to confirm equivalence in enzyme mobility between strains. For each enzyme, distinct electromorphs were numbered in order of increasing anodal mobility. The electromorph with the greatest anodal mobility was designated electromorph

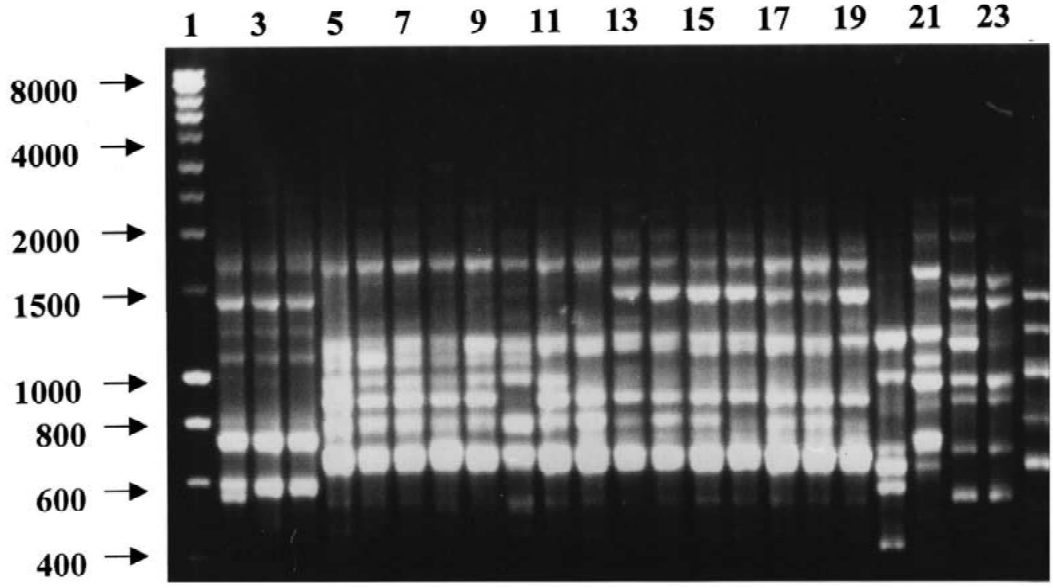

Figure 2. PCR banding profiles for four sibling species in the *Galactomyces geotrichum* complex, *Ga. citri-aurantii* and *Ga. reessii* generated with microsatellite primer $(CAC)_5$. Lanes 2–4: *Ga. geotrichum* - CBS 772.71, 773.71 and 774.71, respectively. Lanes 5-19: species A — CBS 178.30, CBS 476.83, CBS 557.83, CBS 607.84, CBS 606.85, CBS 607.85, CBS 176.28, CBS 279. 94, CBS 110.12, CBS 121.22, CBS 116.23, CBS 149.26, CBS 182.33, CBS 615. 84 and CBS 180.33, respectively. Lane 20: species B – CBS 626.83. Lane 21: species C – CBS 866.68. Lanes 22,23: *Ga. citri-aurantii* - CBS 175.89 and CBS 176.89, respectively. Lanes 24: *Ga. reessii* - CBS 179.60. Lane 1: molecular weight SmartLadder marker (EUROGENTEC, Belgium) is indicated in base pairs.

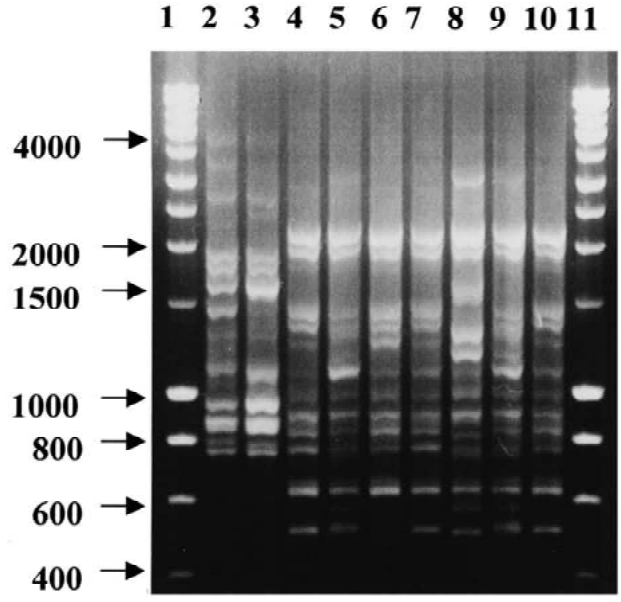

Figure 3. PCR banding profiles of strains from sibling species A and B generated with primer M13. Lanes 2, 3: species A - CBS 557.83 and CBS 180.33, respectively. Lanes 4–10: species B – CBS 636.83, CBS 820.71, CBS 267.79, CBS 101.161, CBS 101.163, CBS 101.161 and CBS 100.812, respectively. Lanes 1 and 11: molecular weight SmartLadder marker (EUROGENTEC, Belgium) is indicated in base pairs.

1, and the more slowly migrating variants were subsequently assigned increasing numbers. Each strain was characterized by its combination of electromorphs for the five enzymes. *S. cerevisiae* strain YNN 295 (=ATCC 200358) was used in all gels as the standard.

Strains with identical multilocus genotypes were grouped into electrophoretic types (ETs), each representing a particular combination of alleles. Pairwise genetic similarities among multilocus electrophoretic types (ETs) were estimated by calculating simple matching coefficients (Selander et al. 1986). Genetic distances were calculating by subtracting these coefficients from 1. The NEIGHBOR program from the PHYLIP phylogeny inference package (Felsenstein, 1993), version 3.572, was used to produce an Unweighted Pair Group with Mathematical Average (UPGMA) dendrogram from the matrix of pairwise genetic distances.

Pulsed-field gel electrophoresis (CHEF)

Preparation of chromosomal DNA was done as described in Boekhout & Bosboom (1994). A CHEF-DR^{TM} II apparatus (Bio-Rad, Richmond, CA, USA) was used for the separation of chromosomal DNA. The electrophoresis buffer was 0.5 × TBE cooled to 14°C. Electrophoresis was carried out at 80 V for 48 h with a switching time of 300 s and then for 20 h with a 300–600 s ramping switching interval. To separate large chromosomes, electrophoresis was carried out at 80 V for 48 h with a switching time of 300 s and then for 48 h with a 300–1000-s ramping interval. The largest chromosomes were separated by running for 94 h with increased switching intervals. The sizes of chromosomes were estimated with reference to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomal standards (Bio-Rad). Standard sets of chromosomes of *S. cerevisiae* (YNN 295) and *Sch. pombe* (972 h^{-}) were obtained commercially (Bio-Rad). After electrophoresis, the gels were stained with ethidium bromide to visualize the chromosomes.

Results

PCR-fingerprinting

The five primers used generated complex banding patterns in the strains studied. Many bright bands ranging in size from 200 to 5000 bp were obtained with universal primer N21. Based on these profiles, the strains can be divided into six groups corresponding to the four sibling species of the *Ga. geotrichum* complex (sensu stricto, A, B and C), *Ga. citri-aurantii* and *Ga. reessii* (Fig. 1). Despite different geographical origins and source of isolation (Table 1), 33 strains from species A displayed nearly identical profiles, some of which are shown in Fig. 1 (lanes 4–13). Seven strains of species B (Fig. 1, lanes 14–20) showed mutually similar patterns, with the exception of CBS 820.71 and CBS 101.161, having a very bright band of about 1300 bp (Fig. 1, lanes 15 and 19, respectively). Strain CBS 866.68 displayed a unique pattern very different from those of the other strains studied (Fig. 1, lane 21). Four *G. citri-aurantii* (Fig. 1, lanes 22–25) and three *G. reesii* (Fig. 1, lanes 26–28) strains showed weak intraspecific variations.

The microsatellite primer $(CAC)_5$ also enabled the distinction between the members of the *Ga. geotrichum* complex, *Ga. citri-aurantii* and *Ga. reessii* (Fig. 2). With this primer more pronounced polymorphism among strains of the species A, *Ga. geotrichum* sensu stricto, *Ga. citri-aurantii* and *Ga. reessii* was observed. Strain CBS 772.71 had two separate bands of 600 bp, while in CBS 773.71 and CBS 774.71 the bands co-migrated (Fig. 2, lanes 2, 3 and 4, respectively). Two *Ga. citri-aurantii* strains, CBS 175.89 and CBS 176.89, having different mating types can be distinguished by this primer (Fig. 2, lanes 22 and 23, respectively). Thirty-three strains of species A displayed two major patterns with primer $(CAC)_5$ these differed by absence or presence of a major band of about 1500 bp (Fig. 2, lanes 5–12 and 13–19, respectively). Within each profile type, some variation was observed for the bands of 800-1100 bp. The strains were isolated from different sources originating from various geographical regions: Cameroon, Egypt, Germany, France, The Netherlands, US, etc. (Table 1). However, a UPGMA dendrogram resulting from the amplification profiles with primer $(CAC)_5$ did not show any significant correlation between the fingerprints and origin of strains or source of isolation (not shown). For example, two strains, CBS 606.85 and CBS 607.85, isolated from *Drosophila* sp. in Cameroon, showed different patterns (Fig. 2, lanes 9 and 10, respectively). Variations were also found among the $(CAC)_5$ profiles of sibling species B strains but these were much slighter than with primer M13. All seven strains of species B can be differentiated by the latter primer (Fig. 3, lanes 4–10). In contrast, patterns obtained with the M13 core sequence were similar among all strains of sibling species A. Two representative strains are shown in Fig. 3. With primers

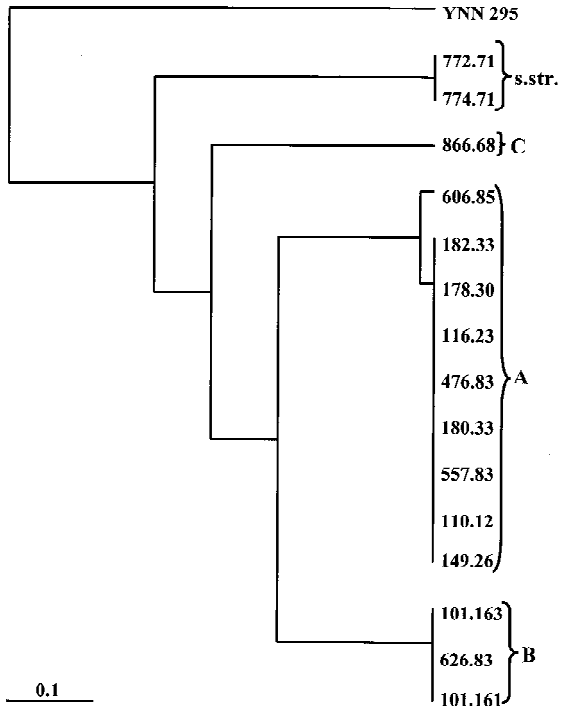

Figure 4. UPGMA cluster analysis showing differentiation among four sibling species strains within the *Galactomyces geotrichum* complex based on the combination of electromorphs for the five enzymes: ADH, G6PDH, MPI, PGI and PGM.

$(GTG)_5$ and $(GACA)_4$, differences between the taxa studied were mainly found in the distribution of minor bands (not shown).

Isoenzyme analysis

Fourteen strains belonging to sibling species A (nine), B (two), C (one) and *Ga. geotrichum* sensu stricto (two) were analysed for their electrophoretic profiles of five enzymes: ADH, G6PDH, PGM, MPI and GPI. All enzyme extracts were electrophoretically examined at least three times and produced consistent zymograms. Each strain was characterized by a combination of electromorphs for the five enzymes (Table 2). Within each of the four sibling species, all the strains investigated presented a single ET, with CBS 606.85 of species A, lacking one ADH band, as the only exception (Table 2, ET3). PGI patterns were similar in all four species. Strains of sibling species A, B and C had a single band for G6PDH; however, in sibling species B a faster migrating band was observed. Strains of *Ga. geotrichum* sensu stricto showed two bands for G6PDH. Four species can be differentiated from one another by a combination of enzyme profiles. *Ga. geotrichum* sensu stricto displayed a unique pattern with four enzymes: ADH, PGM, G6PDH and MPI. Sibling species A had different patterns with three enzymes: ADH, MPI and PGM. Sibling species B can be distinguished from each of the other three species by the enzymes MPI, PGM and G6PDH. Two enzymes MPI and PGM can clearly differentiate all four sibling species. To further evaluate the relatedness among the sibling species, the UPGMA cluster analysis was performed (Fig. 4). The 14 strains are clearly separated into four clusters corresponding to four sibling species: A, B, C and *Ga. geotrichum* sensu stricto.

Molecular karyotyping

To study intraspecific variation, karyotypes of *Ga. geotrichum* sensu stricto (four strains), sibling species A (16 strains) and B (two strains) were compared by CHEF electrophoresis. Sibling species C was not included in the electrophoretic karyotyping, since it is represented by a single strain CBS 866.68. To achieve a good separation of small, middle-sized and large chromosomes, different running conditions were used. Chromosomal patterns within each of the sibling species were similar, but did allow clear differentiation of sibling species A, B and *Ga. geotrichum* sensu stricto. Fig. 5 shows separation by small and some middle-sized chromosomal bands. Unlike *Ga. geotrichum* sensu stricto and species B, strains of species A do not have small chromosomal bands (Fig. 5, lanes 6–10, 12). Within *Ga. geotrichum* sensu stricto, strain CBS 772.71 displayed only one short chromosome of 800 kb (Fig. 5, lane 2), while the other three strains (Fig. 5, lanes 3–5) showed identical patterns characterized by the presence of two small chromosomes and an additional middle-sized chromosome of about 2800 kb. Strain CBS 820.71 of sibling species B also had a small chromosome, but of faster migration (Fig. 5, lane 11). All strains showed many bands ranging in size from 1500 to 3500 kb (Fig. 6). Some chromosome length polymorphism was present among the strains of the same species. For example, different strains from species A displayed from five to nine chromosomal bands (Fig. 6, lanes 2–7). Judging from their relative intensity, some bands contained two or even three chromosomes (Fig. 6, lane 7). Strain CBS 820.71 showed eight middle-sized chromosomes (Fig. 6, lane 8). Representative strains of the *Ga. geotrichum* complex are shown in Fig. 7. The range of chromosomal bands is different in three of the species studied: from

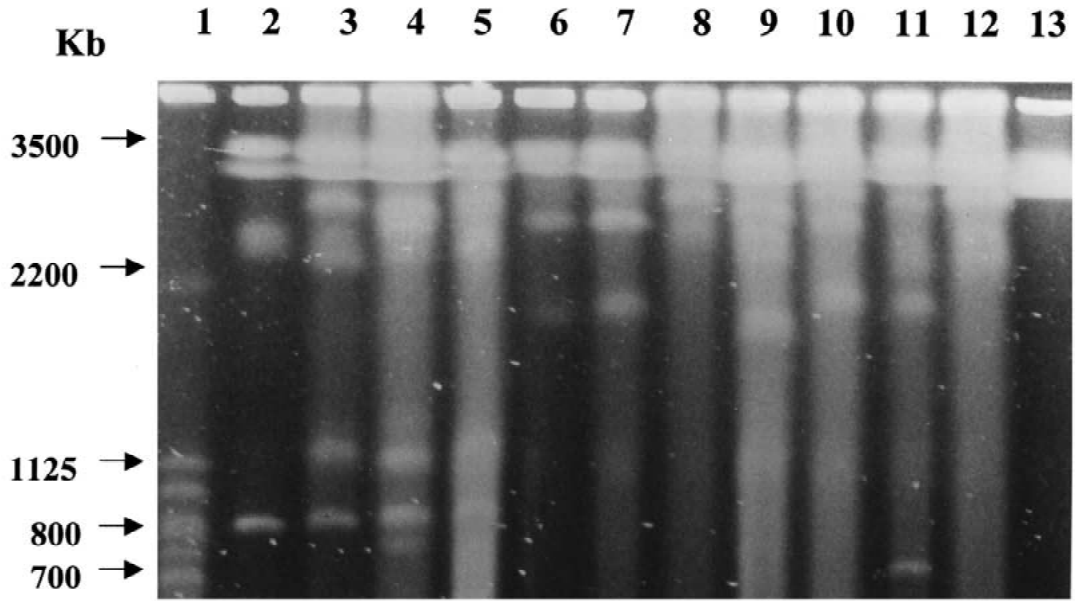

Figure 5. Karyotypic analysis of chromosomal DNAs of three sibling species within the *Galactomyces geotrichum* complex. Lane 1: *Saccharomyces cerevisiae* YNN 295. Lanes 2-5: *Ga. geotrichum* sensu stricto – CBS 772.71, CBS 773.71, CBS 774.71 and CBS 775.71, respectively. Lanes 6–10, 12: species A – CBS 122.22, CBS 607.85, CBS 615.84, CBS 116.23 and CBS 178.30, respectively. Lane 11: species B – CBS 820.71. Lane 13: *Schizosaccharomyces pombe* 972 h^-. CHEF was run at 80 V under the following conditions: 300 s for 48 h, then 300-600 s ramping interval for 20 h. The chromosome sizes of the standard strains are indicated to the left.

800 to 3500 kb in *Ga. geotrichum* sensu stricto (Fig. 7, lane 1), from 1500 to 3500 kb in species A (Fig. 7, lane 2) and from 700 to 3500 kb in species B (Fig. 7, lane 3). Thus, strains of the same sibling species have similar karyotype patterns with some chromosome length polymorphism allowing differentiation of individual isolates. *Ga. geotrichum* sensu stricto and its two sibling species B and C appeared to have the same number of chromosomal bands. However, the sizes of individual chromosomes are different.

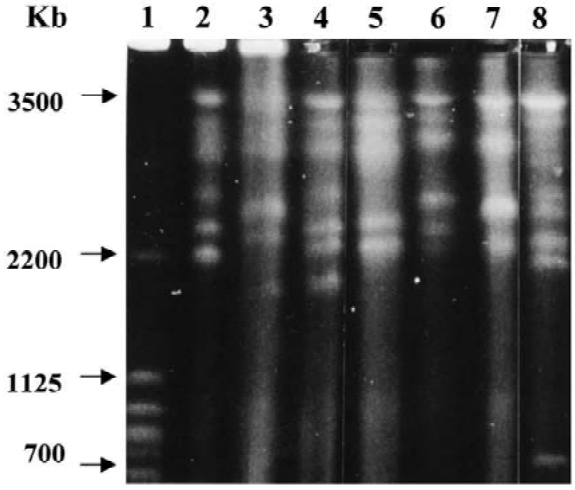

Figure 6. Karyotype patterns of strains from sibling species A and B. Lane 1: *Saccharomyces cerevisiae* YNN 295. Lanes 2–7: species A — CBS 122.22, CBS 115.23, CBS 116.23, CBS 181.33, CBS 178.33 and CBS 184.56, respectively. Lane 8: species B — CBS 820.71. CHEF was run at 80 V under the following conditions: 300 s for 36 h, then 300-1000 s ramping interval for 36 h. The chromosome sizes of the standard strains are indicated to the left.

Discussion

Two different revisions (Redhead & Malloch 1977; von Arx 1977) determined the modern classification of the teleomorph genus *Galactomyces*. Based on ecological and cytological data, Redhead & Malloch transferred two species from the genus *Endomyces* to the novel genus *Galactomyces*. In contrast, von Arx considered that these two species belonged to the known genus *Dipodascus*. Based on sequences of the 26S rRNA genes, Kurtzman & Robnett (1995, 1998) revealed that *Galactomyces* species are part of the genus *Dipodascus*, with the remaining *Dipodascus* species in another subclade. Studying the nucleotide sequences of the 18S rRNA gene from species in the genera *Dipodascus, Galactomyces* and *Geotrichum*, Ueda-Nishimura and Mikata (2000) found a similar

Table 2. Electrophoretic isoenzyme profiles of the sibling species composing the *Ga. geotrichum* complex

Sibling species	ET	Alleles observed at enzyme loci				
		ADH	G6PDH	PGI	MPI	PGM
Ga. geotrichum	1	1,2,3,5,6	1,2	1,2,3	1,5	1,4
Sibling species A	2	3,4	2	1,2,3	3,4	2,3
	3	4	2	1,2,3	3,4	2,3
Sibling species B	4	2,3	1	1,2,3	3,6	2,5
Sibling species C	5	1,2	2	1,2,3	2,7	1

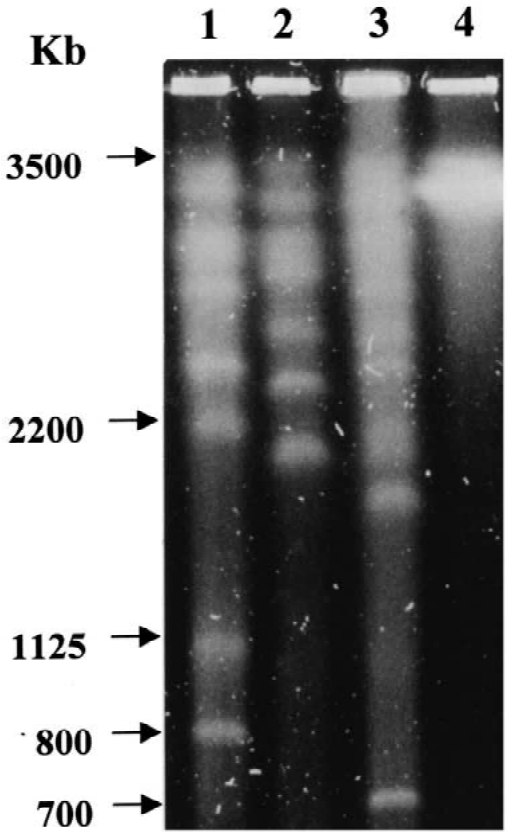

Figure 7. Karyotype patterns of three sibling species within the *Galactomyces geotrichum* complex. Lanes 1: *Ga. geotrichum* sensu stricto – CBS 773.71. Lanes 2: sibling species A – CBS 122.22. Lane 3: sibling species B – CBS 820.71. Lane 4: *Schizosaccharomyces pombe* 972 h^-. CHEF was run at 80 V under the following conditions: 300 s for 48 h, then 300-1000 s ramping interval for 48 h. The chromosome sizes of the standard strains are indicated to the left.

structure. The genus *Galactomyces* is genetically well defined, viz. it includes closely related species having a common mating type system, as a rule, and DNA-DNA homology values among its species are below 60%. We consider the maintenance of the genus *Galactomyces* (de Hoog et al. 1998b) as justified.

Within the well-studied *Saccharomyces cerevisiae* complex, DNA–DNA homology values of 50% (*S. cerevisiae* × *S. paradoxus*) correspond to different biological species. Such species do not have normal genetic exchange since the meiotic products of their hybrids are non-viable ascospores (Naumov 1987b, 1996; Naumov et al. 2000). If, in general, DNA homology values of 50–60% and below delineate different biological species, we consider that all five taxa of the complex *Ga. geotrichum-Ga. citri-aurantii* (Smith et al. 1995) represent separate taxa. *Galactomyces* basically meets the concept of a genetic genus in fungi, which suggests that member species possess the same system of mating types responsible for their crossing (Naumov 1979, 1988). This concept of a genetic genus may be applicable to many ascomycetous yeasts: *Saccharomyces* sensu stricto (Naumov 1987b, 1996; Naumov et al. 2000), *Metschnikowia* sensu stricto (Pitt & Miller 1970), *Metschnikowia* sensu lato (Lachance et al. 1998), *Williopsis* (Naumov 1987a), *Arthroascus* (Naumov et al. 1985, 1999a,b) and *Zygofabospora* (Naumov 1986). The genetic genera form well separated clusters in phylogenetic trees based on sequence data of rRNA genes (Kurtzman & Robnett 1998). Many yeast genera are heterogeneous, and, within them, there are groups of closely related species corresponding to a genetic genera, a good example being the *Hansenula polymorpha* complex of sibling species (Naumov et al. 1997).

The molecular methods used in the present study confirmed that *Ga. reessii, Ga. citri-aurantii* and the four sibling species of the *Ga. geotrichum* complex (A, B, C and *Ga. geotrichum* sensu stricto) are closely related but distinct species. With conventional taxonomic tests, only *Ga. reessii* can be distinguished. The other five entitles have very similar physiological

properties. The two sibling species B and C cannot be differentiated by phenotypic characteristics, while species A, *Ga. geotrichum* sensu stricto and *Ga. citri-aurantii* can be recognized only by a combination of phenotypic properties (Smith et al. 1995, 2000). In the present study, we found that by using molecular markers a clear distinction of all six fungi is possible. The best differentiation of *Ga. geotrichum* sensu stricto and its three sibling species was achieved by using UP-PCR with primer N21. In addition, this primer allowed a clear distinction of the four *Ga. geotrichum* sibling species from *Ga. citri-aurantii* and *Ga. reessii*. Each species displays a specific pattern with very low intraspecific variation. Species-specific fingerprints were also obtained with microsatellite primer $(CAC)_5$, however with higher intraspecific variation. Regarding the isoenzyme analysis, within each of the four *Ga. geotrichum* sibling species, all the strains studied showed a single pattern for MPI, PGI, G6PDH and PGM. The electrophoretic profiles of PGM and MPI are good molecular markers for delineation of sibling species A, B, C and *Ga. geotrichum* sensu stricto (Table 2 and Fig. 4). More pronounced intraspecific variation was observed in karyotype patterns. Three sibling species, A, B and *Ga. geotrichum* sensu stricto, have probably the same number of chromosomal bands (nine); however, the sizes of individual chromosomes, especially of the small ones, are quite different.

In anticipation of a formal taxonomic description of three new species (A, B and C) in the *Ga. geotrichum* complex, it would be expedient to conduct a comparative analysis of their mitochondtrial DNAs and rRNA genes.

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