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**Research Article**

# Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis

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## Abstract

**Samples of *Coffea arabica* were collected during the different stages of the fermentation from two production sites in Tanzania. The yeasts community was identified by genotyping using ITS–PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene. For confirmation, denaturating gradient gel electrophoresis (DGGE) of PCR-amplified 26S rRNA gene was performed to detect yeast directly from coffee samples without cultivation. Yeast counts were in the range $4.0 \times 10^4 - 5.0 \times 10^7$ CFU/g with an increase during fermentation. Three yeasts species were dominant. The predominant yeast found during fermentation and drying was *Pichia kluyveri. Pichia anomala* was found in high numbers during drying of coffee beans. *Hanseniaspora uvarum* was the predominant yeast during fermentation but decreased during drying. *Kluyveromyces marxianus, Candida pseudointermedia, Issatchenkia orientalis, Pichia ohmeri* and *Torulaspora delbrueckii* occurred in concentrations of $10^3$ CFU/g or below in coffee samples. *Saccharomyces cerevisiae* and *Candida xestobii* were not isolated by cultivation, but by the DGGE technique. A good agreement was found between the sequence analysis of the D1/D2 domain of the 26S rRNA gene and sequencing of the DGGE bands. Sequences of yeast isolates determined in this study have been deposited in the GenBank database under Accession Nos AY305664–AY305669 and AY305672–AY305683. Sequences of DGGE bands have also been deposited in the GenBank database under Accession Nos AY314789–AY314802.** 

**Keywords:** ***Coffea arabica*; wet fermentation; *Pichia kluyveri*; *Pichia anomala*; *Hanseniaspora uvarum*; DGGE**

## Introduction

The main objective of coffee processing is removal of the pulp, mucilage, parchment and silver skin surrounding the coffee beans, which leaves the so-called 'green' coffee beans (Fowler *et al*., 1998). Coffee can be processed by two different methods, referred to as the 'wet' or the 'dry' method. The wet method is used for *arabica* coffee and involves removal of the outer skin and part of the mucilage by machines. The remaining mucilage is then removed by fermentation in water for 24–48 h, followed by drying (Fowler *et al*., 1998). The dry method is mainly used for *robusta* coffee, which has a thin pulp that allows direct drying (Fowler *et al*., 1998).

During processing of *Coffea arabica*, Gram-negative and Gram-positive bacteria, yeast and filamentous fungi are present in high numbers during the different stages (*Silva et al*., 2000). Limited information is available in the yeast community of coffee fermentation. *Kluyveromyces marxianus* (Agate and Bhat, 1966; Van Pee and Castelein, 1971), *Saccharomyces cerevisiae* (Agate and Bhat, 1966; Van Pee and Castelein, 1971; Silva *et al*., 2000) and *Schizosaccharomyces* spp. (Agate and

Bhat, 1966; Silva *et al*., 2000) have been isolated. In addition, *Pichia* spp. and *Arxula* spp. (Silva *et al*., 2000), *Kloeckera* spp. and *Cryptococcus* spp. (Avallone *et al*., 2001) have been isolated from *arabica* coffee.

It is assumed that degradation of the pectin-rich mucilage adhering to coffee beans is the major role of yeasts during coffee fermentation. In a study of fermented *robusta* coffee cherries harvested in India, Agate and Bhat (1966) have isolated yeasts identified as *K. marxianus*, *S. bayanus*, *S. cerevisiae* and *Schizosaccharomyces* spp., which were found to have strong pectinolytic activity. In another study, it has been reported that yeasts isolated were non-pectinolytic (Avallone *et al*., 2001). In a recent study, Avallone *et al*. (2002) reported that the mucilage degradation seems to be due to acidification rather than to microbial pectinolytic enzymes.

Cultivation and isolation of yeasts as applied in previous studies can be combined with culture-independent methods to give a complete picture of the microbial diversity. Culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), have been developed to differentiate rRNA genes directly purified from complex microbial communities (Muyzer *et al*., 1993). Recently, DGGE has been shown to be a good tool for monitoring microbial dynamics, such as in fermentation of Mexican fermented maize dough (Ampe *et al*., 1999), wine (Cocolin *et al*., 2000) and sausages (Cocolin *et al*., 2001). It was demonstrated that the DGGE of PCR-amplified 26S rRNA genes provided a qualitative assessment of the yeast diversity in wine fermentation (Cocolin *et al*., 2000). So far, culture-independent techniques seem not to have been used for studies on coffee fermentation.

The objective of the present study was to investigate the yeast community in samples from different coffee fermentation sites in Tanzania. Conventional microbiological cultivation methods were combined with DGGE of PCR-amplified 26S rRNA gene.

## Materials and methods

### Coffee samples

Samples of *Coffea arabica* were collected during different stages of the wet processing method from two processing sites in Arusha, Tanzania (Table 1). Samples were placed aseptically in sterile plastic bags and transferred in ice boxes to a nearby laboratory in Arusha for immediate analysis by cultivation and isolation of yeasts. For extraction of DNA for the DGGE analysis, coffee samples were frozen at $-20\,^{\circ}C$ until analysed.

### Conventional microbiological analysis and yeasts isolation

Ten g of each sample were added to 90 ml diluent saline peptone (SPO) [0.1% bactopeptone (Difco,

**Table 1.** The number of colony forming units (CFU/g) of yeast species isolated from coffee samples collected from two processing sites in Tanzania

| | Number of colony forming units (CFU/g) of yeast species[1] | | | | | | |
|---|---|---|---|---|---|---|---|
| **Coffee processing stage** | ***P. kluyveri*** | ***C. pseudointermedia*** | ***P. anomala*** | ***K. marxianus*** | ***I. orientalis*** | ***T. delbrueckii*** | ***H. uvarum*** |
| Cherries[2] | $4.5 \times 10^5$ | —[4] | $3.0 \times 10^4$ | —[4] | —[4] | —[4] | $7.5 \times 10^5$ |
| Pulp[3] | $5.2 \times 10^5$ | —[4] | —[4] | —[4] | —[4] | —[4] | $6.5 \times 10^5$ |
| Fresh beans[2] | $6.0 \times 10^5$ | —[4] | $1.5 \times 10^4$ | —[4] | —[4] | —[4] | $6.0 \times 10^5$ |
| Beans during 1st day of fermentation[3] | $2.0 \times 10^6$ | —[4] | $1.0 \times 10^3$ | $1.3 \times 10^3$ | —[4] | —[4] | $1.6 \times 10^6$ |
| Beans during 2nd day of fermentation[3] | $3.4 \times 10^7$ | —[4] | —[4] | —[4] | —[4] | —[4] | $1.4 \times 10^7$ |
| Beans after fermentation and washing[3] | $2.1 \times 10^6$ | —[4] | —[4] | —[4] | —[4] | $1.4 \times 10^3$ | $1.7 \times 10^6$ |
| Beans during 3rd day of drying[3] | $2.6 \times 10^5$ | $1.3 \times 10^3$ | $6.5 \times 10^5$ | —[4] | —[4] | —[4] | $2.6 \times 10^5$ |
| Beans during 7th day of drying[3] | $6.4 \times 10^4$ | —[4] | $9.6 \times 10^4$ | —[4] | —[4] | —[4] | —[4] |
| Beans > 7 days of drying[3] | $1.6 \times 10^4$ | —[4] | $2.0 \times 10^4$ | —[4] | $4.0 \times 10^3$ | —[4] | —[4] |

[1] Identified by sequencing of D1/D2 domain of the 26S rRNA gene (see Table 2).
[2] Samples collected from processing site e.
[3] Samples collected from processing site f.
[4] $<10^3$ cfu/g.

Detroit, MI, USA), 0.85% (w/v) NaCl (Merck, Darmstadt, Germany), 0.03% $Na_2H_2PO_4$, $2H_2O$ (Merck), adjusted with 1 M NaOH (Merck) and 1 M HCl (Merck) to pH 5.6]. After mixing in a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 30 s, 10-fold dilutions were prepared and spread onto malt yeast glucose peptone (MYGP) agar [3 g yeast extract (Difco), 3 g malt extract (Difco), 5 g bactopeptone (Difco), 10 g glucose (Merck), 100 mg chloramphenicol (Oxoid) and 20 g Agar (Difco)] per litre of distilled water, adjusted with 1 M NaOH and 1 M HCl to pH 5.6] and incubated at 25 °C for 5 days. The number of colony forming units (CFU) was recorded. From a suitable dilution of each sample, 10 representative colonies were picked and recultivated in MYGP broth at 25 °C for 2 days, and further purified by streaking onto MYGP agar. A total of 110 yeast isolates were obtained.

## Typing of yeast isolates by ITS PCR

From a colony of a pure culture on MYGP agar incubated at 25 °C for 5 days, a loop-full was transferred to 200 µl Tris–EDTA (TE) buffer [10 mM Tris–HCl (Sigma), 1 mM EDTA (Sigma) (Merck)], boiled for 15 min and centrifuged at 1400 × *g* for 2 min. The supernatant was used as a template for the PCR reaction. ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers were used for amplification of the ITS1–5.8S rDNA–ITS2 region (White *et al*., 1990). DNA amplification was performed in a 100 µl volume containing 10 µl template, 1 µM of each primer, 200 µM of each nucleotide [dATP, dCTP, dGTP, dTTP (Promega, Madison, WI)], 2.5 U *Taq* polymerase (Amersham Pharmacia Biotech), 10 µl 10× PCR buffer (Amersham Pharmacia Biotech) and adjusted to 100 µl by addition of MilliQ water. The reactions were performed in an automatic thermal cycler (GeneAmp® PCR System 9700, Perkin Elmer, Norwalk, CT) with initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s; final extension at 72 °C for 7 min. The PCR products were analysed by electrophoresis in 2% (w/v) Nusieve agarose (FMC BioProducts, Rockland, ME, USA) gel in 1× TBE [89 mM Tris-base (Sigma), 89 mM boric acid (Sigma), 2 mM EDTA (Sigma)] at 80 V for 2 h. GenRuler DNA ladder mix (Fermentas, Vilnius, Lithuania) was used as a marker. The amplified fragments were visualized by ethidium bromide staining and UV transillumination. The sizes of the amplified ITS1–5.8S rDNA–ITS2 regions were determined with Fragment Manager (Amersham Pharmacia Biotech).

## Sequence analysis of the D1/D2 domain of the 26S ribosomal RNA gene for selected yeast isolates

For amplification of the D1/D2 domain, the external primers NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman and Robnett, 1998) were used. The reactions were performed in an automatic thermal cycler as described above. The amplified products were purified using the QIAGEN PCR purification kit (QIAGEN, Dorking, UK). Sequencing of the purified PCR products was performed using the CEQ 2000 Dye Terminator Cycle Sequencing kit (Beckman Coulter, Fullerton, USA) and the primers NL-1 and NL-4, NL-2A (5′-CTT GTT CGC TAT CGG TCT C-3′) and NL-3A (5′-GAG ACC GAT AGC GAA CAA G-3′) (Kurtzman and Robnett, 1998) were used for cycle sequencing following the instructions of the manufacturer. Sequences were aligned to the 26S rRNA gene sequences obtained from the National Center for Biotechnology Information (NBCI) Genbank database, using the BLAST algorithm.

## DNA extraction from coffee samples

Portions (10 g) of each sample resuspended in 90 ml SPO were homogenized with a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 2 min; 30 ml each suspension were centrifuged at 1000 × *g* for 15 min; 500 µl lysis buffer [2% Triton X-100, 1% SDS (Sigma), 100 mM NaCl (Merck), 10 mM Tris–HCl (Sigma), 1 mM EDTA (Sigma)], 500 µl phenol : chloroform : isoamylalcohol (50 : 48 : 2) and 0.30 g glass beads were added to each pellet and the mixtures were shaken in a bead mill (MM 2000, Retsch, Germany) for 20 min. The aqueous phase was obtained by centrifugation at 12 000 × *g* for 10 min. The DNA was precipitated with 2.5 vols 96% ethanol, centrifuged at 16 000 × *g* for 10 min; after which the pellets were washed with 70% ethanol, dried and resuspended in MilliQ water containing 2 IU RNAse (Sigma).

### DNA extraction from pure cultures

From a 72 h culture in MYGP broth, 1.5 ml was centrifuged at $16000 \times g$ for 10 min. The pellet were resuspended in 200 µl lysis buffer, 200 µl phenol : chloroform : isoamylalcohol (50 : 48 : 2) and 0.30 g glass beads; the mixture was homogenized in a bead mill (MM 2000, Retch) for 20 min. The DNA was obtained as described previously.

### PCR–DGGE analysis

For amplification of the D1/D2 domain of the 26S rRNA gene, an indirect approach with two types of nested PCR (a and b) was performed. Type (a) included a first PCR round using primers NL-1A (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and NL-4 (indicated above) (O'Donnell, 1993). Type (b) included a first PCR round, using primers NL-1A and LS2 (5′-ATTT CCC AAA CAA CTC GAC TC-3′) according to Cocolin *et al*. (2000). Templates obtained from either (a) or (b) were used in the second PCR round; primers NL1-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3′) (the GC clamp sequence is underlined) and LS2 were used to amplify the 253 bp fragment with the GC clamp.

The PCR products were then analysed by DGGE using a DCode system apparatus (Bio-Rad, Hercules, CA, USA). Polyacrylamide gels (8% w/v acrylamide–bisarylamide) were prepared with a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad) using solutions containing 40% and 60% denaturant (100% denaturant corresponds to 7 M urea and 40% v/v formamide). Gels were run at 60 °C for 16 h at 70 V. The amplified fragments were visualized by SYBR-GOLD (Molecular Probes, Eugene, OR) staining and UV transillumination.

### Sequence analysis of DGGE bands

Selected bands were excised from the DGGE gels with a sterile scalpel and the DNA was eluted in 50 µl distilled water overnight at 4 °C. DNA was reamplified using the same reaction mixture described above for the second PCR round. To confirm their electrophoretic mobility relative to the fragment from which they were excised, the PCR products were analysed by DGGE, as described previously. PCR products that migrated as the original bands were sequenced using CEQ 200 Dye Terminator Cycle Sequencing kit (Beckman Coulter) following the manufacturer's instructions.

## Results

### Yeast species determined by cultivation, ITS–PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene

Yeasts counts were in a range $4.0 \times 10^4 - 5 \times 10^7$ CFU/g with an increase during fermentation (Table 1). According to fragment sizes obtained by amplification of the ITS1–5.8S rDNA–ITS2 region, a total of 110 yeast isolates were divided into eight groups, with three being dominant (Table 2). The square root of the number of yeast

**Table 2.** Groups of yeast isolates obtained from coffee samples according to PCR amplification of the ITS1-5.8S rDNA-ITS2 region and further identified by sequencing D1/D2 domain of the 26S rRNA gene

| ITS-Group | Number of isolates | Band size (bp) | 26S rRNA gene sequence | Homology[1] (%) | GenBank Accession No. |
|---|---|---|---|---|---|
| G1[2] | 37 | 439 | *Pichia kluyveri* | 99.4 | AY305664 |
| G2[3] | 3 | 399 | *Candida pseudointermedia* | 99.1 | AY305666 |
| G3[2] | 39 | 604 | *Pichia anomala* | 99.5 | AY305668 |
| G4[3] | 3 | 679 | *Kluyveromyces marxianus* | 99.4 | AY305673 |
| G5[3] | 1 | 520 | *Issatchenkia orientalis* | 99.4 | AY305674 |
| G6[3] | 1 | 831 | *Torulaspora delbrueckii* | 100 | AY305680 |
| G7[2] | 25 | 746 | *Hanseniaspora uvarum* | 99.3 | AY305678 |
| G8[3] | 1 | 410 | *Pichia ohmeri* | 98.9 | AY305683 |

[1] Percentage of identical nucleotides of the closest relative found in the GenBank database.
[2] The square root of the number of yeasts isolates were sequenced.
[3] One isolate was sequenced.

isolates in each group were identified by sequencing the D1/D2 domain of the 26S rRNA gene, and the three dominant groups, G1, G3 and G7, were identified as *Pichia kluyveri, Pichia anomala* and *Hanseniaspora uvarum*, respectively. For each yeast species, the number of CFU was calculated for the various samples (Table 1). *Pichia kluyveri* was isolated from cherries, pulp and beans during fermentation and drying; it accounted for $1.6 \times 10^4 - 3.4 \times 10^7$ CFU/g. *Pichia anomala* was found in coffee cherries, pulp, fresh beans and beans during the first day of fermentation in the range $1.0 \times 10^3 - 3.0 \times 10^4$ CFU/g. Later in fermentation, *P. anomala* was not detected, but during drying it was found to be predominant, accounting for $2.0 \times 10^4 - 6.5 \times 10^5$ CFU/g in drying coffee samples. *Hanseniaspora uvarum*, the third dominating yeast, was isolated from cherries and during fermentation and during the third day of drying; it accounted for $2.6 \times 10^5 - 1.5 \times 10^7$ CFU/g, but decreased to less than $10^3$ CFU/g later in the drying process (Table 1). Other yeast species, which included *Kluyveromyces marxianus, Candida pseudointermedia, Issatchenkia orientalis, Pichia ohmeri* and *Torulaspora delbruekii*, were found at low levels and accounted for about $10^3$ CFU/g or less in the coffee samples examined.

### Yeast species detected by PCR–DGGE profiles

As seen from Figure 1, no differences in the intensity of the bands were observed for the two types of nested PCR used, indicating that both primer sets used gave PCR products that allowed differentiation by DGGE. Sequence analysis of excised DGGE bands showed that *H. uvarum* was observed in all samples examined by DGGE. *Pichia kluyveri* was also identified in all samples and it was present as three bands that migrated very close to each other. Sequencing of the three bands showed that all the three bands were belonging to *P. kluyveri* with a homology of 98.5% for the first band, 99.0% for the second band and 99.4% for the third band. The sequences of the three bands were aligned together and it was found that the first band was different in two base pairs from the second band and in one base pair from the third band. *Pichia anomala* was not detected in any of the samples examined. It should be noted that dried samples were not included. *Eremothecium coryli* was

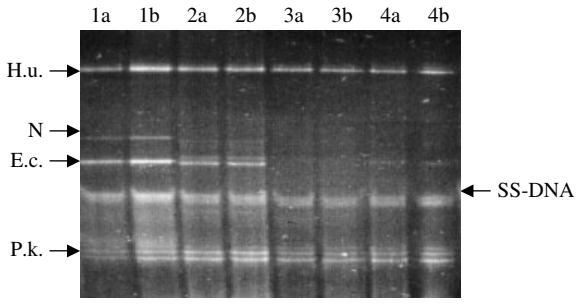


**Figure 1.** DGGE profiles of PCR-products of the 26S rRNA gene fragments obtained from selected coffee samples. The lane number refers to the coffee sample: lane 1, beans during the first day of fermentation; lane 2, pulp; lane3, beans during the second day of fermentation; lane 4, beans after fermentation and washing. The lane letter corresponds to the PCR conditions: a, NL-1A and NL-4 primers were used; b, NL-1A and LS2 primers were used. The bands common to all samples (SS-DNA) are single-stranded DNA artefacts. H.u., *Hanseniaspora uvarum*; E.c., *Eremothecium coryli* (a yeast plant parasite); P.k., *Pichia kluyveri*; N, unsequenced

detected in the pulp and beans during the first day of fermentation. We did not succeed in sequencing a band (Figure 1, N) found in the yeast profiles of pulp and beans during the first day of fermentation.

For comparison, the DGGE profiles for one sample of coffee beans during the fourth day of drying and five yeasts cultivated and isolated from the same sample are shown in Figure 2. Six bands were obtained from the coffee sample. Sequence analysis of the bands from the DGGE gel revealed that *P. anomala* and *P. burtonii* were detected in samples by DGGE and by cultivation. The other yeast species identified by DGGE were *Candida xestobii* and *Saccharomyces cerevisiae*. One band was identified as *Mitchella repens*, which is a plant that belongs to the family Rubiaceae (Greth Van Wijk, 1962). However, the homology of this band to *M. repens* was only 97.2%.

## Discussion

In the present study, increasing yeasts counts in the range $4.0 \times 10^4 - 4.8 \times 10^7$ CFU/g were observed during coffee fermentation, which indicates that yeasts might play a role. An increase of yeasts counts during fermentation of *Coffea arabica* in Brazil has also been reported by Silva *et al*. (2000). In the present work, *Pichia kluyveri* was a predominant yeast during the whole process,

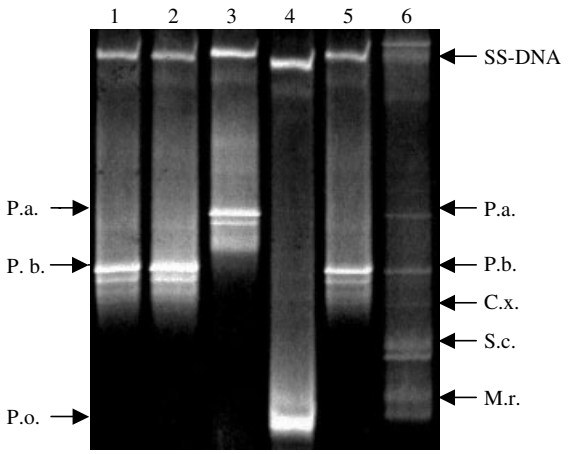


**Figure 2.** DGGE profiles of PCR products of the 26S rRNA gene fragments obtained from coffee beans, isolated during the fourth day of drying. Lanes 1–5 correspond to the bands obtained from pure cultures isolated from the same coffee sample: lanes 1, 2, 5, P.b. (*Pichia burtonii*); lane 3, P.a. (*Pichia anomala*); lane 4, P.o. (*Pichia ohmeri*). Lane 6 refers to the bands obtained from DNA extracted from the coffee sample: P.a., *Pichia anomala*; P.b., *Pichia burtonii*; C.x., *Candida xestobii*; S.c., *Saccharomyces cerevisiae*; M.r., *Mitchella repens* (a plant belonging to the botanical genus *Mitchella* of the family Rubiaceae, to which coffee also belongs); P.o., *Pichia ohmeri*. The bands common to all samples (SS-DNA) are single-stranded DNA artefacts

including drying. This yeast species has not previously been isolated from coffee; however, it was found in cocoa (Kurtzman, 1998) and in fruits (Spenser *et al*., 1992; Abranches *et al*, 2000). *Hanseniaspora uvarum* was found to be the predominant yeast on coffee cherries and during fermentation but it decreased in numbers during drying. *Kloeckera apicullata*, which is the imperfect form of *H. uvarum*, has also been isolated from coffee fermentation (Avallone *et al*., 2001) and from fruits (Arias *et al*., 2002; Heras-Vazquez *et al*., 2003). *Pichia anomala* was isolated from cherries, pulp, fresh beans and beans during the first day of fermentation but it disappeared later in the fermentation. However, *P. anomala* dominated during the drying stage; it constituted $2.0 \times 10^4 - 6.5 \times 10^5$ CFU/g in drying beans. *Pichia* spp., including *P. anomala*, were reported to constitute 39.0% of 107 yeasts isolated from *Coffea arabica* in Brazil (Silva *et al*., 2000).

With regard to the predominant yeast species (*P. kluyveri* and *H. uvarum*) identified in coffee samples, a good agreement was found between sequence analysis of the D1/D2 region of the 26S rRNA gene for yeast obtained by cultivation and sequencing of the DGGE bands obtained from the same samples (Figure 1). In the present study, *P. kluyveri* was found as three bands that migrated very close to each other. The sequences of the three bands belonging to *P. kluyveri* were found to have differences of one to two base pairs between the three bands, indicating that the three bands might represent three different strains of *P. kluyveri*. According to Nübel *et al*. (1996) and Rosado *et al*. (1998), the presence of such triple bands for the same strain indicates that the DNA molecules have slightly different migration behaviour, which might be due to incomplete extension of the same template caused by the GC clamp. *Pichia anomala*, which was isolated from the pulp and during the first day of fermentation, was not detected by DGGE analysis of the same samples. This could be due to the low numbers of this yeast species encountered in the samples investigated. It has been demonstrated that the DGGE patterns are related to the numerically dominant species (Ampe *et al*., 1999; Boon *et al*., 2002). In addition, Cocolin *et al*. (2000) found that during wine fermentation, the PCR–DGGE was unable to detect yeast species when their number was $<10^3$ cells/ml. *Kluyveromyces marxianus, T. delbrueckii* (Table 1) and *P. ohmeri* (Figure 2) were isolated from some coffee samples but they were not detected from the DGGE profiles of the same samples. This might be explained by the fact that these yeast species were encountered occasionally in some samples and only in low numbers. On the other hand, *S. cerevisiae* and *C. xestobii* were identified in the DGGE profile for a sample of drying beans, but they were not detected by cultivation. This was also the case for *Eremothecum coryli*, which was detected from the DGGE profiles of two coffee samples examined. According to Cocolin *et al*. (2000), reasons could be that the yeast cells have entered a viable non-cultural (VBNC) state, or the presence of the DNA of the yeast in question as a result of cell lysis. *Eremothecum coryli* has been reported to be a yeast plant parasite in tropical and subtropical areas (Hoog *et al*., 1998). It has not been previously isolated from coffee fermentation. The sequence of band 5, lane 6, in the DGGE profile of drying beans (Figure 2) was found to have a homology of 97.2% with five different nucleotides from the

sequence of *Mitchella repens*, which indicates that the sequence of this band does not belong to *M. repens*, which is a plant that belongs to the botanical genus *Mitchella* of the family Rubiaceae (Greth Van Wijk, 1962), to which the genus *Coffea* belongs. The sequence of this band might belong to *Coffea arabica*. Unfortunately, no sequence for the D1/D2 domain of the 26S rRNA gene for *Coffea* spp. was found in the GenBank database.

In the present work, *P. kluyveri, P. anomala* and *H. uvarum* were found to dominate during different stages of coffee processing. These yeast species are reported to be fermentative and have been found in soil, fruits and trees (Kurtzman, 1998; Smith, 1998). Furthermore, *P. kluyveri* and *P. anomala* are able to grow on pectin as a sole carbon source (unpublished results). Therefore, it is assumed that they may play a role in coffee fermentation among other yeast species identified, which were encountered occasionally in few samples and in low numbers. However, further studies are needed to investigate the technological properties of the predominant yeast species and their interactions with other microorganisms, such as lactic and acetic acid bacteria and Gram-negative bacteria present during fermentation. With regard to predominant yeast species in coffee samples, a good agreement was found between the profiles obtained by the DGGE and the findings obtained by traditional isolation and further identification of yeasts. DGGE seems to be an efficient tool for studying microbial diversity during natural fermentation. In addition, DGGE is a fast technique compared to time-consuming cultivation and isolation methods, especially when investigating large numbers of samples.

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