RESEARCH ARTICLE

Yeast diversity of Ghanaian cocoa bean heap fermentations

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Abstract

The fermentation of the *Theobroma cacao* beans, involving yeasts, lactic acid bacteria, and acetic acid bacteria, has a major influence on the quality of the resulting cocoa. An assessment of the microbial community of cocoa bean heap fermentations in Ghana resulted in 91 yeast isolates. These were grouped by PCR-fingerprinting with the primer M13. Representative isolates were identified using the D1/D2 region of the large subunit rRNA gene, internal transcribed spacer sequences and partial actin gene sequences leading to the detection of 15 species. Properties of importance for cocoa bean fermentation, namely sucrose, glucose, and citrate assimilation capacity, pH-, ethanol-, and heat-tolerance, were examined for selected isolates. *Pichia kudriavzevii* (*Issatchenkia orientalis*), *Saccharomyces cerevisiae*, and *Hanseniaspora opuntiae* formed the major components of the yeast community. *Hanseniaspora opuntiae* was identified conclusively for the first time from cocoa fermentations. Among the less frequently encountered species, *Candida carpophila*, *Candida orthopsilosis*, *Kodamaea ohmeri*, *Meyerozyma* (*Pichia*) *caribbica*, *Pichia manshurica*, *Saccharomycodes ludwigii*, and *Yamadazyma* (*Pichia*) *mexicana* were not yet documented from this substrate. *Hanseniaspora opuntiae* was preferably growing during the earlier phase of fermentation, reflecting its tolerance to low pH and its citrate-negative phenotype, while no specific temporal distribution was recognized for *P. kudriavzevii* and *S. cerevisiae*.

Introduction

Cocoa-based products are to be considered as fermented foods. The raw materials, cocoa beans, undergo a spontaneous fermentation, carried out traditionally and usually close to the site of harvest in tropical regions throughout the world, with the Ivory Coast and Ghana as the leading exporting countries (Wood & Lass, 2001; Schwan & Wheals, 2004; Thompson *et al.*, 2007). Fermentations may be performed in heaps, boxes, baskets, or on trays according to different processing practices. In Ghana, fermentation in heaps is by far the most dominant method (Baker *et al.*, 1994). In the pods, the cocoa beans are embedded in a mass of mucilaginous white pulp, mainly consisting of pectin, citric acid, and the main carbohydrates sucrose, glucose, and fructose (Roelofsen, 1958; Pettipher, 1986). Once the pulp-bean mass is exposed to the environment by cutting the pods and removal of beans and pulp, a spontaneous fermentation starts, during which successive microbial activities of yeasts, lactic acid bacteria, and acetic acid bacteria lead to the formation of a range of metabolic end products, such as alcohols, lactic acid, and acetic acid, which are precursors of cocoa flavour formation (Ardhana & Fleet, 2003; Schwan & Wheals, 2004; Camu *et al.*, 2007; Nielsen *et al.*, 2007). Yeasts proliferate in the early stages and decline upon prolonged fermentation due to exhaustion of appropriate energy sources, production of ethanol and its conversion to acetic acid, and a temperature increase of up to 50 °C due to aerobic oxidation reactions (Schwan & Wheals, 2004; Camu *et al.*, 2007).

The microbial diversity of cocoa bean fermentations has been shown to vary with location and process parameters, such as nutrient availability, temperature, pH, and oxygen tension, and with metabolic activities (Baker *et al.*, 1994;

Camu *et al*., 2007, 2008a). Several classical studies revealed the involvement of an abundant yeast diversity, encompassing *Candida* spp. (including former *Torulopsis* spp.), *Cryptococcus* spp., *Hanseniaspora* spp. (and their anamorphs *Kloeckera* spp.), *Kluyveromyces* spp. (including *Lachancea thermotolerans*), *Meyerozyma* (former *Pichia* spp.), *Millerozyma* (former *Pichia* spp.), *Pichia* spp. (including former *Issatchenkia* spp. and *Hansenula* spp.), *Rhodotorula* spp., *Saccharomyces* spp. (including *Kazachstania exigua*), *Saccharomycopsis* spp., *Schizosaccharomyces* spp., *Torulaspora* spp., and *Wickerhamomyces* spp. (including former *Pichia* spp.) (Schwan *et al*., 1995; Fowler *et al*., 1998; Ardhana & Fleet, 2003; Schwan & Wheals, 2004; Jespersen *et al*., 2005; Lagunes-Gálvez *et al*., 2007; Nielsen *et al*., 2007; Thompson *et al*., 2007). Potentially due to its rapid growth, pectinolytic activity, and ethanol tolerance, *Saccharomyces cerevisiae* is the most often detected species and also the most abundant species during cocoa bean fermentations, followed by *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*) and *Pichia membranifaciens*, generally after an initial fermentation phase dominated frequently by *Hanseniaspora guilliermondii* (Schwan *et al*., 1995; Ardhana & Fleet, 2003; Jespersen *et al*., 2005; Nielsen *et al*., 2005, 2007).

The assessment of microbial diversity relies on an accurate identification of the isolated species. The constantly growing sequence database of the 5′ end of the large subunit rRNA gene (D1/D2 LSU), encompassing virtually all known yeast species, makes this partial gene sequence a first reference point for yeast identification (Kurtzman & Robnett, 1998; Fell *et al*., 2000). However, some distinct species show low sequence divergence in this region. Therefore, the use of D1/D2 LSU sequences as the only means of molecular identification is limiting the resolution in certain groups of closely related species, such as species related to *H. guilliermondii* (Cadez *et al*., 2003). Identification can be refined by the grouping of isolates using fingerprint methods based on PCR amplification of repetitive DNA elements (e.g. Lieckfeldt *et al*., 1993) and by the determination of additional gene sequences such as the internal transcribed spacer (ITS) region of the rRNA gene cluster (ITS1/5.8S/ITS2; Scorzetti *et al*., 2002) or protein-coding genes such as the actin gene (*ACT1*) (Daniel & Meyer, 2003). The evaluation of conventional classification criteria, namely morphological observations and assimilation/fermentation of different carbon and nitrogen sources (Robert *et al*., 1997; Barnett *et al*., 2000) allows the comparison of molecular with conventional identification results and conclusions on the physiological adaptation of the yeast community to the investigated environment.

In recognition of the artificial nature of several yeast genera, a series of taxonomic changes will gradually transform the current phenotype-based classification towards a phylogeny-based classification. The encountered taxa concerned by these changes are the genus *Issatchenkia*, which has been shown by multigene sequence analyses to be closely related to the genus *Pichia*, into which it has been integrated (Kurtzman *et al*., 2008); *Pichia caribbica*, which, together with *Pichia guilliermondii*, has been assigned to the new genus *Meyerozyma* (Kurtzman & Suzuki, 2008); and *Pichia mexicana*, which has been confirmed by Kurtzman & Suzuki (2008) to be appropriately classified in the genus *Yamadazyma* (Billon-Grand, 1989).

This study aimed at the molecular identification and phenotypic characterization of yeasts from cocoa bean heap fermentations performed in Ghana in the framework of a detailed analysis of the microbial community of these fermentations and its possible impact on cocoa and chocolate flavours (Camu *et al*., 2007, 2008a, b).

Materials and methods

Cocoa bean fermentations

Samples were taken during two field experiments of cocoa bean heap fermentations in Ghana during mid-crop harvest season (June–July 2004, heaps 1–3) and during the main-crop (October–November 2004, heaps 4–7). The heaps were set up at two small farms (A and B), located about 15 km from each other near New Tafo and Old Tafo, respectively. The fermentation and sampling conditions have been described in detail before (Camu *et al*., 2007).

Isolation of yeasts

Fresh cocoa bean samples were homogenized and serially diluted in 1% (w/v) peptone water; aliquots were plated on malt extract agar (MEA; Oxoid, Basingstoke, UK) with 100 mg L^{-1} oxytetracycline. After incubation at 37 °C for 1–4 days, morphologically different yeast colonies were picked up and purified for further identification. Also, swab samples corresponding to 25 cm^2 each were taken from cocoa pod surfaces, baskets, and farmers' hands. Selected yeast isolates, including all sequenced isolates, were deposited at the BCCM/MUCL culture collection with the numbers MUCL 51767–51812.

DNA extraction and PCR-fingerprint analyses

High-molecular mass DNA was extracted using the Invisorb® Spin Plant Mini Kit (Invitek GmbH, Berlin, Germany) as described by Groenewald *et al*. (2008). PCR-based fingerprinting with the single primer M13, a ubiquitous microsatellite sequence (Vassart *et al*., 1987), was applied as described before (Groenewald *et al*., 2008). The resulting M13-based PCR-fingerprint profiles were grouped by cluster analysis using BIONUMERICS version 5.1 (Applied Maths NV, Sint-Martens-Latem, Belgium).

rRNA and actin gene amplification, sequencing, and data analysis

The primer pairs LR0R-LR6 and NS7-ITS4 (White *et al.*, 1990, http://www.biology.duke.edu/fungi/mycolab/primers.htm) were used to amplify the D1/D2 LSU and ITS regions, respectively. Parts of the *ACT1* gene were amplified using either the primer combination CA1-CA5R or Act1F-CA22R (Daniel & Meyer, 2003; Cadez *et al.*, 2006). Successful PCR amplifications resulted in a single band observed on a 0.8% (w/v) agarose gel, corresponding to *c.* 600 bp for D1/D2 LSU and 1000 bp for ITS and *ACT1*. PCR products were cleaned using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA), following the manufacturer's protocol. Sequencing reactions were performed using a CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA), according to the manufacturer's recommendations, with the primers ITS2, ITS3, and ITS4 for the ITS region and LR0R and LR3 for the D1/D2 LSU region. *ACT1* fragments were sequenced using the respective amplification primers and CA8 and CA9R as internal primers (Daniel & Meyer, 2003). LSU and ITS nucleotide sequences were determined using a CEQ 2000 XL capillary automated sequencer (Beckman Coulter). Partial *ACT1* gene sequences were determined through the services of Macrogen Inc. (Seoul, South Korea). The sequences were assembled and edited in SEQUENCHER 4.8 (Gene Codes Corp., Ann Arbor, MI). After initial BLAST searches for the most similar sequences, alignments were performed to compare type strain sequences with the query sequences. The number of substitutions and potential insertions or deletions (indels) were determined from these pairwise comparisons using BIOEDIT 7.0.5.3 (Hall, 1999). The sequences were deposited in the EMBL databank (http://www.ebi.ac.uk/EMBL; Hinxton, UK) with accession numbers FM180529–FM180555, FM864198–FM864200 (D1/D2 LSU), FM199951–FM199972, FM864202, FM864202 (ITS), and FM199986–FM200007, FM864203, FM864204 (*ACT1*).

Physiological and morphological properties as conventional classification criteria

Physiological and morphological profiles of the identified yeast species were determined with the automated microplate method Allev/BIOLOMICS (BioAware SA, Hannut, Belgium) of Robert *et al.* (1997), a yeast identification system based on standard taxonomic criteria (Van der Walt & Yarrow, 1984; Kreger-van Rij, 1987).

Cocoa fermentation-related physiological characterization of yeast isolates

To investigate the physiological adaptation of the yeast community to the cocoa bean fermentation environment, the following physiological characteristics of selected isolates representing all detected species were determined in duplicate on a 10-mL scale in glass tubes: citrate assimilation capacity, pH tolerance, ethanol tolerance, and heat tolerance. Tubes were incubated at 30 °C for 48 h with periodic agitation unless stated otherwise. The test medium used was composed of 6.2 g L^{-1} of yeast nitrogen base (YNB, Difco, Basingstoke, UK) and 5 g L^{-1} of carbohydrate (glucose or sucrose) or citrate. YNB without added carbon source was used as a negative control. Citrate consumption was verified by measuring residual citrate concentrations through high-performance anion exchange chromatography with conductivity measurement under ion suppression, as described previously (Camu *et al.*, 2007). To test pH tolerance, YNB medium with glucose was adjusted to pH 2.5, 3.5, and 5.0 with 5 M HCl. To test ethanol tolerance, YNB medium with glucose was supplemented with 5%, 10%, or 15% (v/v) ethanol. To test heat tolerance, YNB medium with glucose, adjusted to pH 5.5, was incubated at 25, 35, and 45 °C. All tubes were inoculated with 1% (v/v) of a yeast culture grown at 30 °C for 24 h. Growth was determined by measurements of $OD_{600\,nm}$.

Results

Identification

Ninety-one yeast isolates were obtained and identified in the context of an assessment of the microbial community of seven cocoa bean heap fermentations carried out in Ghana (Camu *et al.*, 2007). PCR-based fingerprinting with the primer M13 and numerical cluster analysis of the 91 yeast isolates resulted in 16 clusters, referred to as CA01–CA16 (Fig. 1 and Supporting Information, Table S1). A large degree of variability was noted in clusters CA06 and CA09, indicating strain variation. One to nine isolates per PCR-fingerprinting group, resulting in 33 isolates, were selected for sequence-based identification. For these isolates, 30 sequences of the D1/D2 LSU, 24 sequences of the ITS1/5.8S/ITS2 rRNA, and 24 partial *ACT1* sequences were generated (Table S1). At least two gene sequences for at least one isolate per cluster were compared with type strain sequences, with the exceptions of *Candida carpophila*, *Candida ethanolica*, *Candida stellimalicola*, *Meyerozyma caribbica* (formerly *Pichia caribbica*), and *Pichia occidentalis* (formerly *Issatchenkia occidentalis*), for which no or not all three targeted gene sequences were available for the respective type strains in the public databases or they could not be determined (Table S1). Morphological and physiological characteristics were determined for 20 isolates and used for an independent validation of the sequence-based identification (Table S2). The most frequently detected species were *P. kudriavzevii* (27 isolates), *S. cerevisiae* (22 isolates), *Hanseniaspora opuntiae* (18 isolates), *M. caribbica* (five

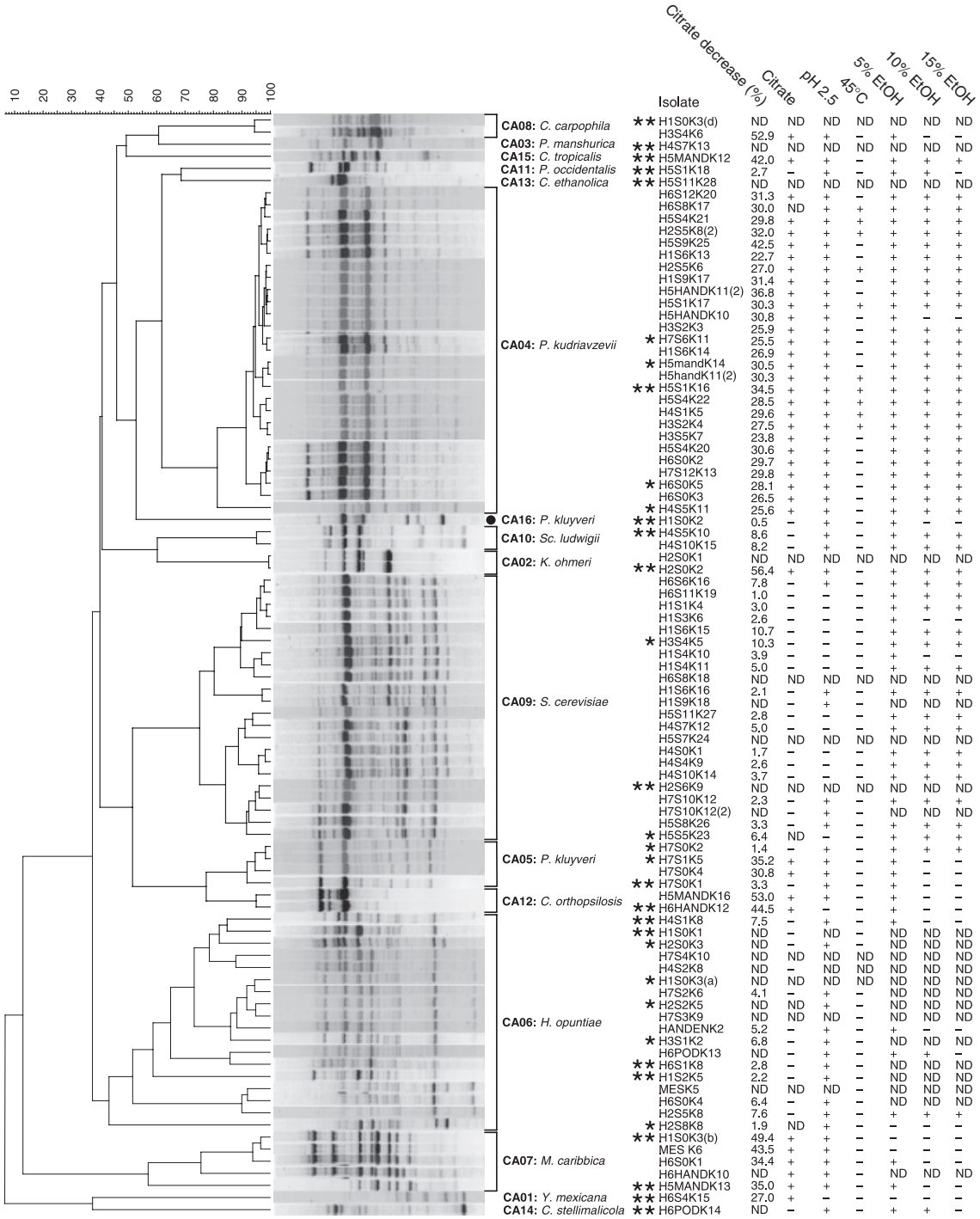

Fig. 1. Dendrogram generated by the unweighted pair-group method with arithmetic averages (UPGMA) based on M13 PCR-fingerprints of yeasts obtained from cocoa bean heap fermentations. Correlation levels are expressed as percentage values of the Pearson correlation coefficient (scale bar). The fingerprint clusters CA01 to CA16 were assigned to 15 species by sequence analyses of selected isolates, marked by single asterisks. One profile was separated from the major cluster of the species as which it was identified: *Pichia kluyveri* var. *kluyveri* (CA16, marked by filled circle). A second asterisk was added to mark isolates that were physiologically characterized by the microplate method. Results of citrate consumption (chromatographically measured as citrate decrease in % of original concentration and determined by growth on citrate as carbon source), growth at pH 2.5, at 45 °C and in the presence of 5%, 10%, and 15% ethanol, respectively, are indicated. Isolate designations use the following abbreviations: H, heap; S, sample (corresponding to fermentation time given in Table 1); MAND, basket; HAND, hand; POD, cocoa pod; K, colony picked up from the isolation plate. Other abbreviations are: ND, not determined; *C.*, *Candida*; *H.*, *Hanseniaspora*; *K.*, *Kodamaea*; *M.*, *Meyerozyma*; *P.*, *Pichia*; *S.*, *Saccharomyces*; *Sc.*, *Saccharomycodes*; *Y.*, *Yamadazyma*.

isolates), and *Pichia kluyveri* var. *kluyveri* (five isolates). Two isolates of *C. carpophila*, *Candida orthopsilosis, Kodamaea ohmeri*, and *Saccharomycodes ludwigii* were found. *Candida ethanolica*, *C. stellimalicola*, *Candida tropicalis*, *Pichia manshurica*, *P. occidentalis*, and *Yamadazyma mexicana* were represented by single isolates. The sequence variation in comparison with type strains of existing species or their synonyms were 0–5 nucleotides for D1/D2 LSU, 0–18 nucleotides for ITS, and 0–6 nucleotides for partial actin gene sequences. The isolate H1S0K2 was assigned to the species *P. kluyveri* var. *kluyveri*, based on highly similar D1/D2 LSU and ITS sequences in comparison with the type strain of this species, despite its atypical PCR-fingerprint profile. No *ACT1* gene fragment could be amplified from this isolate after repeated PCR experiments. The species assignments of isolate H4S7K13, showing 11 nucleotide differences in the ITS region in comparison with the type strain of *P. manshurica*, and of isolate H4S5K10, showing five substitutions in the D1/D2 LSU and 18 nucleotide differences (nine substitutions and four contiguous indels) in the ITS region in comparison with the type strain of *S. ludwigii* were confirmed by PCR-fingerprint comparisons with the respective type strains (Fig. 2).

Distribution

The distribution of yeast species throughout the fermentation process and the different fermentation heaps is shown in Table 1. The three most frequently isolated species (*P. kudriavzevii*, *S. cerevisiae*, and *H. opuntiae*) were also the only species present in each of the seven analysed fermentation heaps, with exception of heap 5, where *H. opuntiae* was isolated only from a basket. The species *C. carpophila*, *C. ethanolica*, *P. kluyveri* var. *kluyveri*, *P. manshurica*, *P. occidentalis*, *S. ludwigii*, and *Y. mexicana* were found only during single fermentations, while the species *C. orthopsilosis*, *C. stellimalicola*, *C. tropicalis*, *K. ohmeri*, and *M. caribbica* were isolated at the start of the fermentations and from associated materials such as the hands of the workers, the baskets, and the cocoa pod surfaces.

Cocoa fermentation-related physiological characterization

All tested cocoa yeast isolates assimilated glucose and sucrose. All tested isolates of *C. carpophila*, *C. orthopsilosis*, *C. tropicalis*, *K. ohmeri*, *M. caribbica*, *P. kudriavzevii*, and *Y. mexicana* were citrate positive (Fig. 1). All tested isolates of *H. opuntiae*, *P. occidentalis*, *S. cerevisiae*, and *S. ludwigii* were citrate negative. Two isolates of *P. kluyveri* var. *kluyveri* were citrate positive; two isolates were citrate negative. All yeasts tested grew at pH 3.5 and pH 5.0. At pH 2.5, 13 out of 20 *S. cerevisiae* isolates, one out of two *C. orthopsilosis* isolates, and the one isolate of *Y. mexicana* were not capable

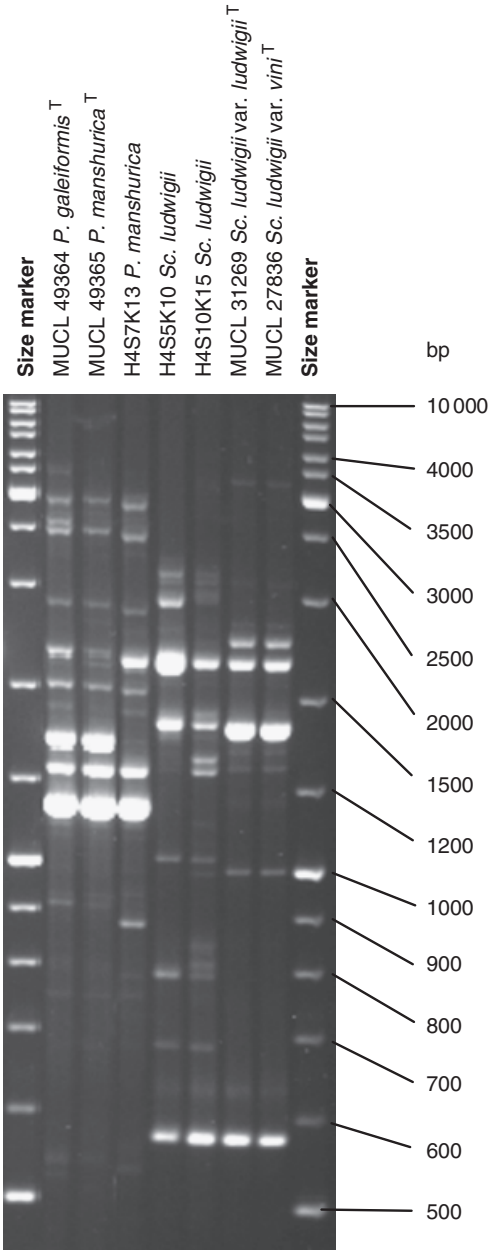

Fig. 2. PCR-fingerprint profiles with the primer M13 showing high similarities between H4S7K13, the type strain of *Pichia manshurica* and its synonym *Pichia galeiformis*, as well as H4S5K10, H4S10K15, the type strain of *Saccharomycodes ludwigii* var. *ludwigii*, and its synonym *Saccharomycodes ludwigii* var. *vini*.

to grow. Almost all tested isolates of *S. cerevisiae* (except H1S4K10), *P. kudriavzevii* (except H5HANDK10), and all of *S. ludwigii* grew in the presence of ethanol in concentrations of up to 15%. Growth of selected *H. opuntiae* isolates in the presence of different ethanol concentrations was variable. The single isolate of *C. stellimalicola* grew in the presence of up to 10% ethanol. Isolates of *C. carpophila*, *C. orthopsilosis*, *M. caribbica*, and *P. kluyveri* var. *kluyveri* tolerated the

Table 1. Distribution of isolates during the different cocoa bean heap fermentations

				Fermentation time (h)															
Heap	Farm	Crop*	Species	0	6	12	18	24	30	36	42	48	54	60	66	72	Hand	Basket	Pod
1	A	Mid	*P. kudriavzevii*							2			1						
			S. cerevisiae		1		1	2		2			1						
			H. opuntiae	2		1													
			P. kluyveri	1															
			M. caribbica	1															
			C. carpophila	1															
2	B	Mid	*P. kudriavzevii*						2										
			S. cerevisiae							1									
			H. opuntiae	1		1			1			1							
			K . ohmeri	2															
3	A	Mid	*P. kudriavzevii*			2			1										
			S. cerevisiae					1											
			H. opuntiae		1														
			C. carpophila					1											
4	A	Main	*P. kudriavzevii*		1				1										
			S. cerevisiae	1				1			1			1					
			H. opuntiae		1	1													
			P. manshurica								1								
			Sc. ludwigii						1					1					
5	B	Main	*P. kudriavzevii*		2			3					1				2	1	
			S. cerevisiae						1		1	1			1				
			H. opuntiae															1	
			M. caribbica															1	
			P. occidentalis		1														
			C. orthopsilosis															1	
			C. ethanolica												1				
			C. tropicalis															1	
6	A	Main	*P. kudriavzevii*	3								1				1			
			S. cerevisiae							1		1			1				
			H. opuntiae	1	1														1
			M. caribbica	1													1		
			C. orthopsilosis														1		
			C. stellimalicola																1
			Y. mexicana					1											
7	B	Main	*P. kudriavzevii*							1						1			
			S. cerevisiae											2					
			H. opuntiae			1	1	1											
			P. kluyveri	3	1														

*Mid-crop, June–July 2004; main-crop, October–November 2004.

Fermentation time 0 refers to the time when the heap was completed. Numbers following species names indicate the number of isolates per species from the same time point. Three isolates obtained from equipment were not assigned to a particular fermentation heap and are not shown in this table. *C.*, *Candida,*; *H.*, *Hanseniaspora*; *K.*, *Kodamaea*; *M.*, *Meyerozyma*; *P.*, *Pichia*; *S.*, *Saccharomyces*; *Sc.*, *Saccharomycodes*; *Y.*, *Yamadazyma*.

presence of only 5% ethanol in the growth medium (except for the *P. kluyveri* var. *kluyveri* H7S0K2 isolate that still grew at 15% ethanol). The single isolate of *Y. mexicana* did not grow at 5% ethanol. All tested isolates grew at 25 and 35 °C. Only some *P. kudriavzevii* isolates grew at 45 °C.

Discussion

Ninety-one yeast isolates from cocoa bean heap fermentations, isolated from MEA, were typed by PCR-fingerprinting with a single primer derived from the core sequence within the protein III gene of the bacteriophage M13 (M13-based PCR fingerprinting). Based on this hypervariable minisatellite, groups of yeast isolates can be determined, generally corresponding to species (Lieckfeldt *et al.*, 1993; Groenewald *et al.*, 2008). According to the currently most often detected intraspecies sequence variability of 0–3 nucleotide differences in the D1/D2 LSU rRNA region, 0–4 differences in the ITS region, 0–11 differences in the *ACT1* gene, and some fingerprint comparisons with type strains, the 16 fingerprint

groups obtained in the present study were assigned to 15 species. The variability seen in ITS and *ACT1* sequences as well as in the fingerprint profiles of *H. opuntiae* was interpreted as intraspecies variation. The isolate of *P. kluyveri* var. *kluyveri* showing an atypical fingerprint profile will be included in a future taxonomic re-evaluation of this species. Molecular analysis in combination with morphological and physiological observations showed that isolates of some species would have been misidentified, if phenotypic identifications were applied solely. In particular, *P. manshurica* would have been phenotypically identified as *P. membranifaciens* (Mikata & Ueda-Nishimura, 2000), *H. opuntiae* as *H. guilliermondii* (Cadez *et al.*, 2003), *M. caribbica* and *C. carpophila* as *Meyerozyma* (formerly *Pichia*) *guilliermondii* (Vaughan-Martini *et al.*, 2005), *C. orthopsilosis* as *Candida parapsilosis* (Tavanti *et al.*, 2005), and *C. stellimalicola* as *Candida silvae* (Meyer *et al.*, 1998). As an example, an identification of *P. manshurica* as *P. membranifaciens* could be excluded based on the highly divergent *ACT1* sequences (< 60 substitutions). Conversely, the identification of *C. ethanolica* was only possible with the help of phenotypic data. Indeed, *Pichia deserticola* and *C. ethanolica* have been assumed to be synonymous based on their highly similar D1/D2 LSU sequences (Meyer *et al.*, 1998). However, ITS sequence divergence suggests that they do constitute different species (Wu & Bai, 2005). No *C. ethanolica* ITS sequence could be obtained during this study, due to double peaks in the sequence electropherograms, and no matching *ACT1* sequence was available. As *P. deserticola* and *C. ethanolica* can be distinguished by the ability of the latter to grow in vitamin-free medium, the investigated isolate was identified based on its morphological/phenotypic profile as *C. ethanolica*. Using D1/D2 LSU sequences alone, the identification of *C. carpophila*, *C. ethanolica*, *C. orthopsilosis*, *H. opuntiae*, and *M. caribbica* would have been problematic, because of no or low sequence variability among sibling species.

Pichia kudriavzevii (30% of the total isolates), *S. cerevisiae* (24%), and *H. opuntiae* (20%), of which the first two species are known to be present in Ghanaian cocoa bean fermentations (Jespersen *et al.*, 2005; Nielsen *et al.*, 2005, 2007), formed the major components of the yeast isolates identified during this study. Although the number of yeast isolates (91) may be rather low, they represent morphologically different colony types derived from samples taken every 6 h during the first 72 h of the fermentations and yet allowed 15 different species to be identified. This number of species is in the same range as previous findings based on more extensive isolate numbers from two and three fermentations (Jespersen *et al.*, 2005; Nielsen *et al.*, 2007). Jespersen *et al.* (2005) hesitated to report several of their strains as *H. opuntiae*, described by Cadez *et al.* (2003), and referred to them as *H. guilliermondii*. The distinction of *H. opuntiae* from *H. guilliermondii* and other species was made by PCR-fingerprints and DNA–DNA relatedness (Cadez *et al.*, 2003). Cadez *et al.* (2006) established the small but consistent differences of D1/D2 LSU and ITS sequences between *H. guilliermondii* and *H. opuntiae* to be indicative of different species by additional data from the elongation factor-1α and *ACT1* genes. Comparisons of the sequences deposited by Jespersen *et al.* (2005) showed higher similarities to *H. opuntiae* than to *H. guilliermondii* and suggested the presence of *H. opuntiae* in their study. In the context of cocoa bean fermentations, the most frequently mentioned species are *S. cerevisiae* (synonym *Saccharomyces chevalieri*), *H. guilliermondii* (anamorph *Kodamaea apis*), *P. kudriavzevii* (anamorph *Candida krusei*), and *P. membranifaciens* (Schwan *et al.*, 1995; Ardhana & Fleet, 2003; Schwan & Wheals, 2004; Jespersen *et al.*, 2005; Nielsen *et al.*, 2005, 2007; Lagunes-Gálvez *et al.*, 2007). Two of these, *H. guilliermondii* and *P. membranifaciens* were not detected in the current study, while *H. opuntiae* was identified for the first time from cocoa bean fermentations. Among the less frequently encountered species identified during this study (1–5% of the total isolates), *M. caribbica*, *C. carpophila*, *C. orthopsilosis*, *K. ohmeri*, *S. ludwigii*, *P. manshurica*, and *Y. mexicana* have not yet been documented from cocoa bean fermentations. This may or may not indicate that they have not yet been isolated from this ecosystem, due to the steadily improving sensitivity of identification procedures, followed by an accordingly evolving taxonomy (Meyer *et al.*, 1998; Mikata & Ueda-Nishimura, 2000; Cadez *et al.*, 2003; Tavanti *et al.*, 2005; Vaughan-Martini *et al.*, 2005).

The isolation frequency and distribution of yeast species suggest that *P. kudriavzevii*, *S. cerevisiae*, and *H. opuntiae* played important roles in the analysed cocoa bean heap fermentations. All three species and *P. kluyveri* var. *kluyveri* were represented by multiple strains, as minor sequence variation among different isolates was observed. Species solely isolated during single fermentations, at the start of the fermentations, and from the associated materials, might be considered as members of the surrounding ecosystems that occasionally become established during a fermentation, if their physiological properties favour growth under the fermentation conditions in place. Heap fermentation 5 seems remarkable in this regard, as of the five species isolated from the baskets used to build the heap, only one (*P. kudriavzevii*, also isolated from workers hands) became established in the fermentation together with *S. cerevisiae*. Interestingly, this fermentation was dominated by *Weissella ghanensis* instead of *Lactobacillus plantarum* (Camu *et al.*, 2007) and chocolate produced of the corresponding beans displayed a fruity flavour, indicating yeast dominance (Camu *et al.*, 2008b). It may indicate a mutual coexistence of *P. kudriavzevii* and *W. ghanensis*, as is the case, for instance, between *Candida humilis* and *Lactobacillus*

sanfransiscensis during spontaneous wheat sourdough fermentation (Gobbetti *et al*., 1994).

The temporal distribution observed during this study indicated that *H. opuntiae* was preferably growing during the earlier phase of fermentations with the last isolate at 48 h, while no specific temporal distribution was recognized for *P. kudriavzevii* and *S. cerevisiae*. This yeast distribution was reflected in the citrate fermentation capacity of yeasts and lactic acid bacteria and their coexistence in the beginning of the cocoa bean fermentation process. Whereas lactic acid bacteria are responsible for a fast citrate consumption at the start of the fermentation (Camu *et al*., 2007), *H. opuntiae* was not able to assimilate citrate and grew – together with the other yeasts – on sucrose as an energy source. The tolerance of low pH values of *H. opuntiae* was in accordance with environmental conditions prevailing during cocoa bean fermentation (low pH at the start of the fermentation due to a high-citrate concentration), *S. cerevisiae* being the most sensitive yeast species towards the lowest pH values and hence proliferating after *H. opuntiae*. Higher temperatures and ethanol concentrations towards the end of a cocoa bean fermentation, due to increased microbial activities, influence survival of cocoa-specific yeast species, as their tolerance towards these factors was variable (Ardhana & Fleet, 2003; Jespersen *et al*., 2005; Nielsen *et al*., 2005, 2007). The limited number of yeasts capable to grow at 45 °C explains the disappearance of the yeast population once ethanol oxidation by acetic acid bacteria, causing a substantial temperature increase during cocoa bean fermentation, has started (Camu *et al*., 2007).

To conclude, the use of M13-based PCR-fingerprinting allowed the reliable grouping of cocoa bean yeast isolates from Ghanaian cocoa bean heap fermentations at the species and sometimes population level. Sequence comparisons of representative isolates, using not only rRNA gene cluster sequences but also a faster evolving protein-coding gene, *ACT1*, provided the currently most comprehensive identification of the yeasts involved in the cocoa bean heap fermentations sampled. Although only 91 yeast isolates were identified from seven fermentations, each sampled at 13 time points, 15 different species were found. However, the predominant yeast community of the Ghanaian cocoa bean heap fermentations is restricted to three species representing 74% of the total isolates. These few yeast species seem to be important for the initiation of the cocoa bean fermentation process. Citrate assimilation capacity, pH tolerance, ethanol tolerance, and heat tolerance are determining factors for the (temporal) yeast distribution during cocoa bean fermentations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. DNA sequence-based identification and morphological/physiological characterization of selected yeast strains.

Table S2. Physiological and morphological profiles of 20 isolates selected to represent the 16 M13-based PCR-fingerprint groups.

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