Candida halmiae sp. nov., *Geotrichum ghanense* sp. nov. and *Candida awuiai* sp. nov., isolated from Ghanaian cocoa fermentations

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During an investigation of the microbiology of Ghanaian cocoa fermentations, a number of yeast isolates with unusual pheno- and genotypic properties representing three possible novel species were isolated. Members of Group A divided by multilateral budding and ascospores were not produced. Group B strains produced true hyphae and ascospores were not produced. Group C representatives divided by budding and formed chains and star-like aggregates. Ascospores were not produced. Sequence analysis of the 26S rRNA gene (D1/D2 region) revealed that the Group A isolates were phylogenetically most closely related to *Saturnispora mendoncae* (gene sequence similarity 92.4 %), *Saturnispora besseyi* (88.8 %), *Saturnispora saitoi* (88.8 %) and *Saturnispora ahearnii* (88.3 %). Members of Group B were most closely related to representatives of the genera *Dipodascus* and *Galactomyces* and the asporogenous genus *Geotrichum*, but in all cases with 26S rRNA gene (D1/D2 region) similarities below 87 %. For Group C, the most closely related species were *Candida rugopelliculosa* (92.4 %), *Pichia occidentalis* (91.6 %) and *Pichia exigua* (91.9 %). The very low gene sequence similarities obtained for the three groups of isolates clearly indicated that they represented novel species. Repetitive Palindromic PCR (Rep-PCR) of the isolates and their closest phylogenetic relatives confirmed that the new isolates belonged to previously undescribed species. In conclusion, based on the genetic and phenotypic results, the new isolates were considered to represent three novel species, for which the names *Candida halmiae* (group A, type strain G3^T=CBS 11009^T=CCUG 56721^T); *Geotrichum ghanense* (group B, type strain G6^T=CBS 11010^T=CCUG 56722^T) and *Candida awuiai* (group C, type strain G15^T=CBS 11011^T=CCUG 56723^T) are proposed.

Cocoa beans, the principal raw material of chocolate, have to be fermented, dried and roasted to obtain the characteristic cocoa flavour and taste. The fermentation of cocoa is a microbiologically complex process involving the activities of yeasts, lactic acid bacteria and acetic acid bacteria with yeasts being particularly important during the initial phases of the fermentation process (Schwan & Wheals, 2004; Jespersen *et al.*, 2005; Nielsen *et al.*, 2007). During an investigation of the micro-organisms involved in the fermentation of cocoa beans, a number of yeast isolates with unusual pheno- and

genotypic properties were isolated from malt extract agar (Nielsen *et al.*, 2007). Preliminary pheno- and genotypic results showed that the isolates clustered in three distinct groups, all representing putatively novel yeast species. This study presents the morphological, biochemical and molecular characterization of these isolates. Group A consisted of a single isolate (G3^T=CBS 11009^T=CCUG 56721^T), group B comprised two isolates [G6^T(=CBS 11010^T=CCUG 56722^T) and G17] and group C contained four isolates [G15^T(=CBS 11011^T=CCUG 56723^T), G68, G71 and G174]. All isolates were isolated during the first 12 h of cocoa fermentation and represented between 3 and 14 % of the total yeast biota at the time of sampling (Nielsen *et al.*, 2007).

All isolates and reference strains were grown in YPG broth [yeast extract 5 g l⁻¹ (Merck), glucose 10 g l⁻¹, peptone 10 g l⁻¹, pH 5.6] at 25 °C for 2–14 days. For long-term storage, 20 % glycerol was added to the medium and cultures were stored at –80 °C.

The micro morphology of the isolates grown for 1–5 days in YM (yeast extract 3 g l⁻¹, malt extract 3 g l⁻¹, peptone

Abbreviation: Rep-PCR, repetitive palindromic polymerase chain reaction.

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA gene sequences of *Candida halmiae* sp. nov. G3^T, *Geotrichum ghanense* sp. nov. G6^T and *Candida awuiai* sp. nov. G15^T are DQ466525, DQ466527 and EU876854, respectively.

Supplementary figures showing (GTG)₅-PCR fingerprints and corresponding dendrograms, derived from UPGMA linkage of correlation coefficients, for the three novel species are available with the online version of this paper.

5 g l⁻¹, glucose 10 g l⁻¹, pH 5.6) and YPG broth (25 °C) was determined using phase-contrast microscopy (Fig. 1). Colony morphology was determined after 4–14 days of growth on YGP and MYGP agar (25 °C). Spore formation was examined on YM agar, V8 agar, 5 % malt agar and acetate agar (Robert *et al.*, 2008) incubated for 4–28 days (20 and 25 °C). Spore formation was determined using bright-field microscopy. None of the isolates investigated produced ascospores.

The carbohydrate assimilation and fermentation patterns of the new isolates were determined following the protocol of Yarrow (1998) aided by use of the API ID32C identification system (bioMérieux). The results of these analyses are presented in Tables 1, 2 and 3 and the species descriptions.

Fig. 1. Phase-contrast micrographs of cells of (a) *Candida halmiae* sp. nov. G3^T; (b) *Geotrichum ghanense* sp. nov. G6^T and (c) *Candida awuuii* sp. nov. G15^T grown in YPG broth for 3 days. Bars, 10 µm.

Table 1. Key phenotypic characteristics of *Candida halmiae* sp. nov. and its closest phylogenetic relatives

Taxa: 1, *Candida halmiae* sp. nov.; 2, *Candida diversa*; 3, *Candida silvae*; 4, *Saturnispora ahearnii*; 5, *Saturnispora besseyi*; 6, *Saturnispora dispora*; 7, *Saturnispora hagleri*; 8, *Saturnispora mendoncae*; 9, *Saturnispora saitoi*; 10, *Saturnispora zaruensis*. +, Positive; –, negative; v, variable, strain dependent; s, slow; w, weak. Data partially adopted from Kurtzman (1998a), Meyer *et al.* (1998), Morais *et al.* (2005) and Kurtzman (2006).

Characteristic	1	2	3	4	5	6	7	8	9	10
Assimilation of:										
Cellobiose	w	–	–	–	–	–	–	–	–	–
Glycerol	–	v	+	–	–	–	s	v	–	–
Maltose	s	–	–	–	–	–	–	–	–	–
DL-Lactic acid	+	–	v	+	+	w/–	+	+	+	+
Trehalose	w	–	–	–	–	+	+	–	+	+
D-Xylose	w	v	–	–	–	–	–	–	–	–

For 26S rRNA gene sequencing, DNA was extracted using the Instagene Matrix DNA isolation kit following the manufacturer's instructions (Bio-Rad). The D1/D2 region of the 26S rRNA gene was amplified using primers NL1 and NL4 as outlined previously (Jespersen *et al.*, 2005). Following purification (Qiagen PCR Purification kit), the fragments were sequenced in both directions by a commercial sequencing facility (DNA Technology, Aarhus, Denmark). Sequences were manually corrected and aligned using Vector NTI Suite 10 software (Informax). The closest phylogenetic relatives were determined by aligning the corrected sequences to 26S rRNA gene sequences in the GenBank database using the BLAST algorithm (Altschul *et al.*, 1997).

Comparison of the similarity of the 26S rRNA gene sequence (D1/D2 region) of isolate G3^T with type strains from the GenBank database revealed that isolate G3^T was most closely related to *Saturnispora mendoncae* [26S rRNA gene (D1/D2 region) gene sequence similarity 92.4 %], *Saturnispora besseyi* (88.8 %), *Saturnispora saitoi* (88.8 %) and *Saturnispora ahearnii* (88.3 %). Isolates G6^T and G17 were most closely related to *Dipodascus albidus* (86.2 %), *Dipodascus geniculatus* (85.3 %) and *Dipodascus australiensis* (84.7 %). Isolates G15^T, G68, G71 and G174 were most closely related to *Candida rugopelliculosa* (92.3 %), *Pichia occidentalis* (91.6 %) and *Pichia exigua* (91.9 %). Until recently *Pichia occidentalis* and *Pichia exigua* were considered to belong to the genus *Issatchenkia*. However, convincing phylogenetic evidence that the genus *Issatchenkia* should be transferred to the genus *Pichia* was recently published (Kurtzman *et al.*, 2008); this nomenclature is used in the present manuscript.

The 26S rRNA gene sequences (D1/D2 region) of the new isolates and their closest phylogenetic relatives retrieved from the GenBank database were aligned and phylogenetic trees were constructed by the neighbour-joining method

Table 2. Key phenotypic characteristics of *Geotrichum ghanense* sp. nov. and its closest phylogenetic relatives

Taxa: 1, *Geotrichum ghanense* sp. nov.; 2, *Geotrichum silvicola*; 3, *Geotrichum klebahnii*; 4, *Galactomyces geotrichum*; 5, *Dipodascus albidus*; 6, *Dipodascus armillariae*; 7, *Dipodascus australiensis*; 8, *Dipodascus geniculatus*; 9, *Dipodascus macrosporus*. +, Positive; –, negative; v, variable, strain dependent; w, weak; ND, not determined. Data partially adopted from de Hoog *et al.* (1998a, b, c) and Pimenta *et al.* (2005).

Characteristic	1	2	3	4	5	6	7	8	9
Fermentation of:									
Glucose	+	–	+	v	–	–	–	w/–	–
Galactose	+	ND	w/–	v	–	–	–	–	–
Assimilation of:									
Cellobiose	+	–	–	–	–	+	–	–	+
Maltose	+/w	–	–	–	–	–	–	+	–
D-Mannitol	+	+	+	v	+	+	+	+	+
Ribitol	+	–	v	v	–	–	v	–	v
Sucrose	w	–	–	–	–	–	–	–	–
Trehalose	w	–	–	–	–	–	–	–	–
D-Xylose	+	+	+	+	+	+	+	+	+

using BioNumerics version 4.5 (Applied Maths). Unknown bases were discarded for the analysis. The statistical reliability of the topology of the phylogenetic trees was evaluated using bootstrap resampling of the data (1000 resamplings) (Figs 2, 3 and 4).

Additionally, the isolates and their closest phylogenetic neighbours were genotypically investigated by Repetitive Element Palindromic (rep)-PCR using the primer GTG₅ (5'-GTGGTGGTGGTGGT-3') as previously described (Andrade *et al.*, 2006; Nielsen *et al.*, 2007). Isolate G3^T (group A) was clearly separated ($r \leq 68\%$) from type strains representing the phylogenetically closest relatives (see Supplementary Fig. S1 in IJSEM Online). Isolates G6^T and G17 (group B) clustered closely together ($r > 90\%$) and were only distantly related ($r \leq 52\%$) to type strains representing the phylogenetically closest relatives (see Supplementary Fig. S2). Isolates G15^T, G68, G71 and G174 (group C) formed two closely related ($r = 82\%$) but distinct groups with the four isolates clearly clustering ($r = 61\%$) away from the closest phylogenetic relatives (see Supplementary Fig. S3).

Based on the above data, it can be concluded that strain G3^T is genetically and phenotypically distinct from all currently recognized species with *S. mendoncae* and *S. ahearnii* being the closest phylogenetic relatives. Phenotypically, strain G3^T can be differentiated from the closest phylogenetic neighbours on the basis of its ability to assimilate cellobiose, D-xylose and maltose (slow reaction) as seen from Table 1. The results obtained in the present study clearly indicate that strain G3^T represent a novel species that is genetically closely related to the genus *Saturnispora*. However, as ascospore production was not

Table 3. Key phenotypic characteristics of *Candida awuuii* sp. nov. and its closest phylogenetic relatives

Taxa: 1, *Candida awuuii* sp. nov.; 2, *Candida rugopelliculosa*; 3, *Pichia occidentalis*; 4, *Pichia exigua*. +, Positive; –, negative; v, variable, strain dependent; s, slow; w, weak. Data partially adopted from Kurtzman (1998b) and Meyer *et al.* (1998).

Characteristic	1	2	3	4
Fermentation of maltose	+	–	–	–
Assimilation of:				
Cellobiose	+	–	–	–
Maltose	v	–	–	–
Ribitol	v	–	–	–
Sucrose	s	–	–	–
Trehalose	v	–	–	–
D-Xylose	w	–	–	+

observed in the new isolate, the novel species must belong to the anamorphic ascomycetous yeasts. The name *Candida halmiae* sp. nov. is proposed for the new isolate, with strain G3^T (=CBS 11009^T=CCUG 56721^T) being the type strain.

Isolates G6^T and G17 formed a genetically and phenotypically distinct group that was most closely related to *D. albidus*, *D. geniculatus* and *D. australiensis* but as ascospore formation was not observed, the isolates belong to the anamorphic ascomycetous yeasts. Phenotypically, the ability to ferment galactose and assimilate sucrose and trehalose differentiated strains G6^T and G17 from their closest phylogenetic neighbours (Table 2). The results obtained in the present study clearly indicate that isolates G6^T and G17 represent a novel species in the anamorphic genus *Geotrichum*, for which we propose the name *Geotrichum ghanense* sp. nov. with strain G6^T (=CBS 11010^T=CCUG 56722^T) being the type strain.

Isolates G15^T, G68, G71 and G174 formed a genetic and phenotypic distinct group phylogenetically closest related to *C. rugopelliculosa*, *P. occidentalis* and *P. exigua*. Phenotypically, the ability to ferment maltose and assimilate cellobiose and ribitol clearly differentiated strains G15^T, G68, G71 and G174 from their closest phylogenetic neighbours (Table 3). In conclusion, the results obtained in the present study clearly indicate that isolates G15^T, G68, G71 and G174 represent a novel species in the genus *Candida*, for which we propose the name *Candida awuuii* sp. nov. with strain G15^T (=CBS 11011^T=CCUG 56723^T) being the type strain.

Latin diagnosis of *Candida halmiae* Nielsen, Jakobsen et Jespersen sp. nov.

In liquido YM, *post dies* 1–3 *ad* 25 °C, *cellulae vegetativae ovoidae* (2.5–5.0 µm × 3.8–7.5 µm), *singulae aut binae*. *Per gemmationem multipolarem reproducentes*. *In agar* MYGP *post dies* 4–6 *ad* 25 °C *cultura* 2–3 mm, *candida et cremea*,

Fig. 2. Phylogenetic tree (neighbour-joining method) based on almost complete 26S rRNA gene sequences (D1/D2 region) showing the phylogenetic position of isolate G3^T (GenBank accession nos in parentheses). *Schizosaccharomyces pombe* was included as the outgroup. Bootstrap values (%) based on 1000 replications are given on each node. Bar, 2 % sequence divergence.

butyrosa, *glabra*, *infima-convexa* et *margo integra*. *Ascosporae* non formantur. *Glucosum* et *maltosum* fermentatur. *Sucrosum*, D-galactosum, D-trehalosum, D-lactosum, D-raffinose non fermentatur. *Assimilantur* D-glucosum, ribitolum (*exigüe*), D-xylosum (*exigüe*), cellobiosum (*exigüe*), D-mannitolum, maltosum (*exigüe*), trehalosum (*exigüe*), et DL-acidum lacticum. *Non assimilatur* L-sorbosum, sucrosum, D-galactosum, D-sorbitolum, D-ribosum, N-acetylglucosaminum, L-rhamnosum, L-arabinosum, erythritolum, D-raffinose, D-melibiosum, D-melezitosum, 2-ketogluconatum, gluconatum, methyl D-pyranosidum, D-lactosum, inositolum et glycerolum. *Non assimilatur* nitratum. *Aesculinum* hydrolysatur. *Crescentiae* (*exigüe*) 37 °C. *Typus* G3^T (=CBS 11009^T=CCUG 56721^T).

Description of *Candida halmiae* Nielsen, Jakobsen & Jespersen sp. nov.

Candida halmiae (hal'mi.æ. N.L. fem. gen. n. *halmiae* of Halm, named in honour of Dr Mary Halm, a Ghanaian microbiologist who has contributed significantly to our understanding of the importance of yeasts in indigenous African fermented foods).

In YM and YGP broth after 1–3 days at 25 °C, cells are ovoidal (2.5–5.0 µm × 3.8–7.5 µm), occur singly and in pairs, and divide by multilateral budding. On YPG and MYGP agar (4–6 days, 25 °C) colonies are circular with a

diameter of 2–3 mm, tannish-white, butyrous, smooth, glistening, low convex and with an entire margin. Formation of ascospores is not observed. Glucose and maltose are fermented. Sucrose, D-galactose, trehalose, lactose and raffinose are not fermented. Glucose, ribitol (weak), D-xylose (slow), cellobiose (slow), D-mannitol, maltose (slow), trehalose (weak) and DL-lactic acid are assimilated. L-Sorbose, sucrose, D-galactose, D-sorbitol, D-ribose, N-acetylglucosamine, L-rhamnose, L-arabinose, erythritol, raffinose, melibiose, melezitose, 2-ketogluconate, gluconate, methyl D-glucopyranoside, D-lactose, inositol and glycerol are not assimilated. Aesculin is hydrolysed. Nitrate is not assimilated. No growth in the presence of 0.01 % cycloheximide. Grows at 37 °C, but growth is weak and delayed.

The type strain, G3^T (=CBS 11009^T=CCUG 56721^T), was isolated from cocoa fermentations in Tafo, Ghana. The description of the type strain corresponds to the description of the species.

Latin diagnosis of *Geotrichum ghanense* Nielsen, Jakobsen et Jespersen sp. nov.

In liquido YM, *post dies* 1–5 *ad* 25 °C *pellicula* formantur et *cellulae* ovoidae aut *elongatae* (3.5–6.3 × 6.3–12.5 µm), *singulae* *authyphae*. *Hyphae ramosae cum arthroconidiis* formantur. *In agaro* MYGP *post dies* 4 *ad* 25 °C *cultura* 5–10 mm, *candida*, *farinosus*, *marginis ciliata*. *Ascosporae* non

Fig. 3. Phylogenetic tree (neighbour-joining method) based on almost complete 26S rRNA gene sequences (D1/D2 region) showing the phylogenetic position of isolate G6^T. For further details, see Fig. 2.

Fig. 4. Phylogenetic tree (neighbour-joining method) based on almost complete 26S rRNA gene sequences (D1/D2 region) showing the phylogenetic position of isolate G15^T. For further details, see Fig. 2.

formantur. Glucosum et D-galactosum fermentatur. Sucrosum, maltosum, D-trehalosum, D-lactosum, D-raffinose non fermentatur. Assimilantur D-glucosum, L-sorbose, ribitolum (exiguum), sucrosum (exiguum), maltosum (exiguum), D-xylosum, cellobiosum, D-mannitolum, trehalosum (exiguum), D-galactosum glycerolum et levulitum. Non assimilatur D-ribosum, N-acetylglucosaminum, L-rhamnosum, L-arabinosum, D-raffinose, D-melibiosum, gluconatum, D-melezitosum, 2-ketogluconatum, methyl D-pyranosidum, D-lactosum et inositolum. Assimilantur DL-acidum lacticum variabile. Non assimilatur nitratum. Aesculinum hydrolysat. Crescentiae 37 °C. Typus G6^T (=CBS 11010^T=CCUG 56722^T) non assimilatur DL-acidum lacticum.

Description of *Geotrichum ghanense* Nielsen, Jakobsen & Jespersen sp. nov.

Geotrichum ghanense (ghan.en'se. N.L. neut. adj. *ghanense* pertaining to Ghana where the organism was first isolated).

A pellicle is formed in YM and YGP broth after 1–5 days at 25 °C. Cells are ovoid to elongate (3.5–6.3 × 6.3–12.5 µm) occurring singly and as hyphae disarticulating into arthroconidia. On YPG and MYGP agar (4 days, 25 °C), true mycelium with branching hyphae is produced. Colonies are 5–10 mm in diameter, whitish, dry, farinose and with creeping hyphae. Formation of ascospores is not observed. Glucose and galactose are fermented. Sucrose, trehalose, lactose, maltose and raffinose are not fermented. Glucose, L-sorbose, ribitol, sucrose (weak), maltose (weak), D-xylose, cellobiose, D-mannitol, trehalose (weak), galactose, levulinate and glycerol are assimilated. D-Ribose, N-acetylglucosamine, L-rhamnose, L-arabinose, raffinose, melibiose, gluconate, melezitose, 2-ketogluconate, methyl D-glucopyranoside, lactose, inositol and glucosamine are not assimilated. Assimilation of DL-lactic acid is strain dependent. Nitrate is not assimilated. Aesculin is hydrolysed. Able to grow in the presence of 0.01 % cycloheximide. Growth occurs at 37 °C.

The type strain, G6^T (=CBS 11010^T=CCUG 56722^T), and all other known strains were isolated from cocoa fermentations in Tafo, Ghana. The description of the type strain corresponds to the description the species, except that the type strain does not assimilate DL-lactic acid.

Latin diagnosis of *Candida awuuii* Nielsen, Jakobsen et Jespersen sp. nov.

In liquido YM, post dies 2–5 ad 25 °C, cellulae vegetativae ovoidae (2.5–3.8 µm × 3.8–6.3 µm), singulae, binae et aggregatae. Per gemmationem multipolarem reproducentes. In agar MYGP post dies 4 ad 25 °C cultura 2–3 mm, candida et crenea, butyrosa, convexa et margo non integra. Ascospores non formantur. Glucosum et maltosum fermentatur. Sucrosum, D-galactosum, D-trehalosum, D-lactosum, D-raffinose non fermentatur. Assimilantur D-glucosum, glycerolum, D-xylosum (exiguum), cellobiosum (exiguum), L-sorbose (exiguum), sucrosum (exiguum). Non assimilatur D-galactosum, N-acetylglucosaminum, D-sorbitolum, L-rhamnosum, L-arabinosum, erythritolum, D-raffinose, D-melibiosum, D-melezitosum, 2-ketogluconatum, gluconatum, methyl D-pyranosidum, D-lactosum, inositolum et glucosaminum. Assimilantur maltosum (exiguum), ribitolum, D-mannitolum et trehalosum variabile. Non assimilantur nitratum. Aesculinum hydrolysat. Crescentiae (exiguum) 37 °C. Typus G15^T (=CBS 11011^T=CCUG 56723^T) assimilantur ribitolum et trehalosum; D-mannitolum non assimilatur.

Description of *Candida awuuii* Nielsen, Jakobsen & Jespersen sp. nov.

Candida awuuii (a.wuai'i. N.L. masc. gen. n. *awuuii* of Awua, named in honour of Dr Wisdom K. Amoa-Awua, a Ghanaian microbiologist who has contributed significantly to our understanding of the importance of yeasts in indigenous African fermented foods).

In YM and YGP broth after 2–5 days at 25 °C, cells are ovoid (2.5–3.8 µm × 3.8–6.3 µm), divide by multilateral budding, occur singly, in pairs and form chains and star-like aggregates. On MYGP agar (4 days, 25 °C) colonies are 2–3 mm in diameter, tannish-white, circular, butyrous, convex and with a slightly fringed margin. After 14 days the colony edge is fringed and the centre of the colonies becomes yellowish. Formation of ascospores is not observed. Glucose and maltose are fermented. Sucrose, galactose, trehalose, lactose, and raffinose are not fermented. Glucose, cellobiose, L-sorbose (weak), sucrose (slow), D-xylose (weak) and glycerol are assimilated. Galactose, D-sorbitol, N-acetylglu-

cosamine, L-rhamnose, L-arabinose, erythritol, raffinose, melibiose, melezitose, 2-ketogluconate, gluconate, methyl D-glucopyranoside, lactose, inositol and glucosamine are not assimilated. Assimilation of maltose (slow), ribitol, D-mannitol (slow) and trehalose (slow) are strain dependent. Nitrate is not assimilated. No growth in the presence of 0.01 % cycloheximide. Growth occurs at 37 °C.

The type strain, G15^T (=CBS 11011^T=CCUG 56723^T), and all other known strains of the species have been isolated from cocoa fermentations in Tafo, Ghana. The description of the type strain corresponds to the description the species except that ribitol (slow) and trehalose (slow) are assimilated and D-mannitol is not assimilated.

Acknowledgements

The work performed was partly financed through the EU INCO project: 'Developing biochemical and molecular markers for determining quality assurance in the primary processing of cocoa in West Africa – COCOQual' (ICA4-CT-2002-10040). The cooperation of the Cocoa Research Institute of Ghana (Dr J. S. Takrama) and the excellent technical assistance of Janne Benjaminsen are highly appreciated.

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