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Candida ecuadorensis sp. nov., an ascomycetous yeast species found in two separate regions of Ecuador

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In the course of an on-going study aimed at cataloguing the natural yeast biodiversity found in Ecuador, two strains (CLQCA 13-025 and CLQCA 20-004^T) were isolated from samples of cow manure and rotten wood collected in two separate provinces of the country (Orellana and Bolívar). These strains were found to represent a novel yeast species based on the sequences of their D1/D2 domain of the large-subunit (LSU) rRNA gene and their physiological characteristics. Phylogenetic analysis based on LSU D1/D2 sequences revealed this novel species to belong to the *Metschnikowia* clade and to be most closely related to *Candida suratensis*, a species recently discovered in a mangrove forest in Thailand. The species name of *Candida ecuadorensis* sp. nov. is proposed to accommodate these strains, with strain CLQCA 20-004^T (=CBS 12653^T=NCYC 3782^T) designated as the type strain.

In the recently published fifth edition of '*The Yeasts, A Taxonomic Study*', nearly 1500 species of ascomycetous and basidiomycetous yeast are described. Current estimates suggest that this number may only represent approximately 1 % of all extant yeast species which, if true, would mean the expected total number would be around 150 000 species. Interestingly, and perhaps somewhat surprisingly, if the current species are categorized according to the geographical origin of their respective type strains (when known), it becomes evident that the majority of yeasts catalogued so far have been discovered in predominantly Northern hemisphere countries (Table S1 shows the geographical origins for species belonging to the ten largest ascosporogenous genera). To date, large parts of the Southern hemisphere including Africa, Antarctica, Asia, Australasia, as well as South America, remain poorly studied. This means that the many diverse and contrasting habitats found in these continental regions represent rich sources of yeast biodiversity still awaiting discovery.

In South America, for example, most attention has focused on the countries of Brazil, Chile and Argentina. Biodiversity studies have been carried out to characterize the yeast communities present in a variety of different habitats including an Atlantic rainforest site in Rio de Janeiro State, the Valdivian forest of southern Chile and *Nothofagus* (Southern beech) forests in north-western Patagonia (Argentina). All have resulted in the discovery of numerous novel yeast species, belonging to a variety of different genera including *Candida*, *Rhodotorula*, *Saccharomyces*, *Saturnispora* and *Spathaspora* (Ramírez & González, 1984a, b; Morais *et al.*, 2005; Barbosa *et al.*, 2009; Cadete *et al.*, 2009; Libkind *et al.*, 2011). Perhaps the most notable 'South American' discovery to date is that of *Saccharomyces eubayanus*, which genome sequencing has recently revealed to be the elusive cryotolerant *Saccharomyces* species that crossed with *S. cerevisiae* to produce the allotetraploid hybrid lager species *S. pastorianus* (syn. *S. carlsbergensis*) (Libkind *et al.*, 2011).

Abbreviation: LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the LSU D1/D2 and ITS sequences of CLQCA 20-004^T are FR839617 and FR839619, respectively.

The Mycobank deposit number is MB801370.

Two supplementary tables are available with the online version of this paper.

In Ecuador, in contrast, until very recently almost nothing was known about the natural native yeast diversity. However in 2007, in an effort to begin addressing this scientific shortfall, a yeast bio-prospecting project was initiated by the Colección de Levaduras Quito Católica (CLQCA), in Quito. The primary aim of the project is to catalogue, characterize and compare the indigenous yeast species present in the many differing ecological habitats found in Ecuador. Although relatively small in size (area ~272 000 km^2), Ecuador, located on the west coast of South America, possesses a number of quite distinctive and contrasting climatic regions. These include the Andean highlands, Amazon basin, the Pacific coastal plain, as well as the Galápagos Islands, the latter being recognized by UNESCO as a world heritage site. Indeed Ecuador is considered to be one of only 17 megadiverse countries, which collectively account for more than two thirds of the entire planet's biodiversity.

Over the past five years, the CLQCA has collected, and subsequently preserved, more than 2000 yeast isolates from the 24 provinces of Ecuador. So far, the predominant species registered are *Candida tropicalis* and *S. cerevisiae*, although yeasts from many other genera including *Hanseniaspora*, *Pichia* and *Rhodotorula* are also well represented. In addition, in 2008, a further 800 isolates were collected from four of the Galápagos Islands (namely Floreana, Isabela, San Cristóbal and Santa Cruz). To date, more than 15 putative novel species have been identified and of these, three have been fully characterized and formally described. The first, *Candida carvajalis*, was isolated from rotting wood and leaf debris samples collected at separate sites in the central Amazonian region of Orellana province (James *et al.*, 2009). The second, *Saturnispora quitensis*, was isolated from the fruit of an unidentified species of bramble (*Rubus* sp.) collected in the Maquipucuna Cloud Forest Reserve in the north-west of the country (James *et al.*, 2011); while the third, *Candida theae*, was isolated independently in Taiwan from an Indonesian-produced tea drink, and in Ecuador during a microbial archaeological study of ancient Indian chicha vessels (Chang *et al.*, 2012). In the present study, we describe the discovery of two additional novel strains (CLQCA 13-025 and CLQCA 20-004^T) and the formal taxonomic description of a fourth Ecuadorian yeast species, *Candida ecuadorensis* sp. nov. to accommodate them.

The two strains, CLQCA 13-025 and CLQCA 20-004^T, were isolated from samples of cow manure and rotten wood collected at two separate sites in Ecuador. Strain CLQCA 13-025 was collected in the central province of Bolívar. at 2668 m above sea level (GPS coordinates: 01° 32.278′ S, 079° 00.471′ W), while CLQCA 20-004^T was collected in the eastern province of Orellana at 310 m above sea level (GPS coordinates: 00° 29.187′ S, 077° 06.325′ W).

Yeast sampling was carried out using sterile cotton wool swabs. The sampled material was inoculated in 1.5 ml micro-tubes containing enrichment medium. The enrichment medium consisted of YPD (yeast extract, peptone, glucose) broth supplemented with ampicillin (100 μg ml^{-1}) to suppress bacterial growth. Inoculated broth cultures were incubated at 30 °C for 36 h, after which time 100 μl aliquots were removed and plated onto YPD agar, and incubated for an additional 36 h (at 30 °C). Selected representative colonies were picked and replated onto YPD agar. If visible contamination was detected, a further round of colony purification was carried out. Samples of each purified yeast isolate were also examined by light microscopy to check for signs of possible bacterial and/or fungal contamination. The strains were characterized morphologically, biochemically, and physiologically according to the standard methods described by Kurtzman *et al.* (2011). Growth temperature testing was determined by cultivation on YM (yeast extract-malt extract) agar. Sporulation tests were performed on cornmeal agar, Gorodkowa agar, potassium acetate agar and YM agar, and plates were incubated at 20 °C and 25 °C for 1 month in pure and mixed cultures.

The variable D1/D2 domains of the LSU rRNA gene and ribosomal ITS region were amplified by PCR directly from whole yeast cell suspensions as described previously by James *et al.* (1996). The LSU D1/D2 domain was amplified and sequenced using primers NL1 and NL4 (O'Donnell, 1993), and the ITS region amplified using primers ITS5 and ITS4, and sequenced using these primers as well as internal primers ITS2 and ITS3 (White *et al.*, 1990). The amplified DNA was checked by agarose gel electrophoresis, purified and concentrated using QIAquick PCR purification columns (Qiagen), and sequenced using a Life Technologies 3730XL sequencer at The Genome Analysis Centre (TGAC; Norwich, UK). Sequence traces were edited manually and consensus sequences generated using the program SEQMAN, version 7 (DNASTAR). The LSU D1/D2 sequences were compared pairwise using a FASTA similarity search (Pearson & Lipman, 1988) and were aligned with the sequences of closely related taxa, retrieved from EMBL, using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994), included in the DNAMAN software package, version 5.1.5 (Lynnon BioSoft). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), with the Jukes–Cantor distance measure, and *Candida torresii* was used as the outgroup species. Confidence limit values were estimated from bootstrap analyses of 1000 replicates (Felsenstein, 1985).

The LSU D1/D2 and ITS sequences of CLQCA 13-025 and CLQCA 20-004^T were found to be identical. A FASTA sequence similarity search of the EMBL fungal sequence database revealed no other yeast taxon with a LSU D1/D2 sequence identical to these strains. In fact, in terms of pairwise sequence similarity, the two Ecuadorian strains were most closely related to *Candida* sp. LCF-121, *Candida* sp. 11-507 and *C. suratensis*, displaying 8.4 % (35 nt substitutions and two indels), 8.8 % (37 nt substitutions and two indels) and 8.5 % (38 nt substitutions and two indels) sequence divergence, respectively. Interestingly, the LSU D1/D2 sequences of *Candida* sp. LCF-121 and *C. suratensis* were found to differ by only three (T/C) base

substitutions from each other, and by four and five base substitutions, respectively, from *Candida* sp. 11-507. *Candida* sp. LCF-121 was isolated from a leaf collected in Taiwan, *Candida* sp. 11-507 from palm fruit collected in Laos, while *C. suratensis* was discovered in Thailand (Limtong & Yongmanitchai, 2010). Based on the limited degree of sequence divergence, *Candida* sp. LCF-121 and *Candida* sp. 11-507 either represent sister taxa or are geographical variants of *C. suratensis*. Overall, these results clearly indicated that strains CLQCA 13-025 and CLQCA 20-004^T belong to a novel and as yet un-described species. A phylogenetic analysis based on LSU D1/D2 sequences showed that the two Ecuadorian strains along with *Candida* sp. LCF-121, *Candida* sp. 11-507 and *C. suratensis* form a distinct (bootstrap value, 100 %) species group closely related to members of the *C. intermedia* subclade (Fig. 1), which in turn belongs to the much larger *Metschnikowia* clade (Lachance, 2011; Lachance *et al.*, 2011). The closest known ascosporogenous species is *Metschnikowia koreensis*. However, this species not only differs by over 17 % sequence divergence (85 nt substitutions and four indels), but also belongs to a quite separate and distinct subclade which includes *Metschnikowia bicuspidata*, the type species of the genus (Fig. 1).

At present, it is difficult to generalize on the ecological niche of *C. ecuadorensis* sp. nov. as only two strains have so far been recovered, one from cow manure and the other from rotten wood. However, an inspection of the other member species of the *C. intermedia* subclade would suggest a possible insect and/or plant association. For instance, *C. blattae*, *C. dosseyi* and *C. thailandica* have all been isolated from either insect guts (e.g. *Corydalus cornutus*) and/or insect frass, while *C. citri*, *C. flosculorum*, *C. pseudointermedia*, *C. suratensis* and *C. tsuchiyae* have all been isolated from plants (e.g. *Acacia*, *Citrus*, *Gliricidia* and *Heliconia* spp.) (Jindamorakot *et al.*, 2007; Nguyen *et al.*, 2007; Rosa *et al.*, 2007; Lachance *et al.*, 2011; Sipiczki, 2011). In the case of *C. akabanensis* and *C. intermedia*, both species have been isolated from plants (e.g. *Cornus*, *Gliricidia* and *Musa* spp.) as well as from insect frass (Lachance *et al.*, 2011).

Although no phenotypic data are currently publicly available for either *Candida* sp. LCF-121 or Candida sp. 11-507, *C. ecuadorensis* sp. nov. can be readily differentiated from its next closest relative, *C. suratensis*, based on a variety of fermentation and assimilation tests. This perhaps reflects the fact that the two species were isolated from quite different sources as well as from different continents. The two strains of *C. ecuadorensis* sp. nov. were isolated from rotten wood and cow manure samples collected in Ecuador, whereas the single representative strain of *C. suratensis* was isolated from a decaying leaf submerged in water collected from a mangrove forest in Thailand (Limtong & Yongmanitchai, 2010). A complete list of the phenotypic tests which can be used to differentiate between the two *Candida* species is shown in Table S2. Likewise, the

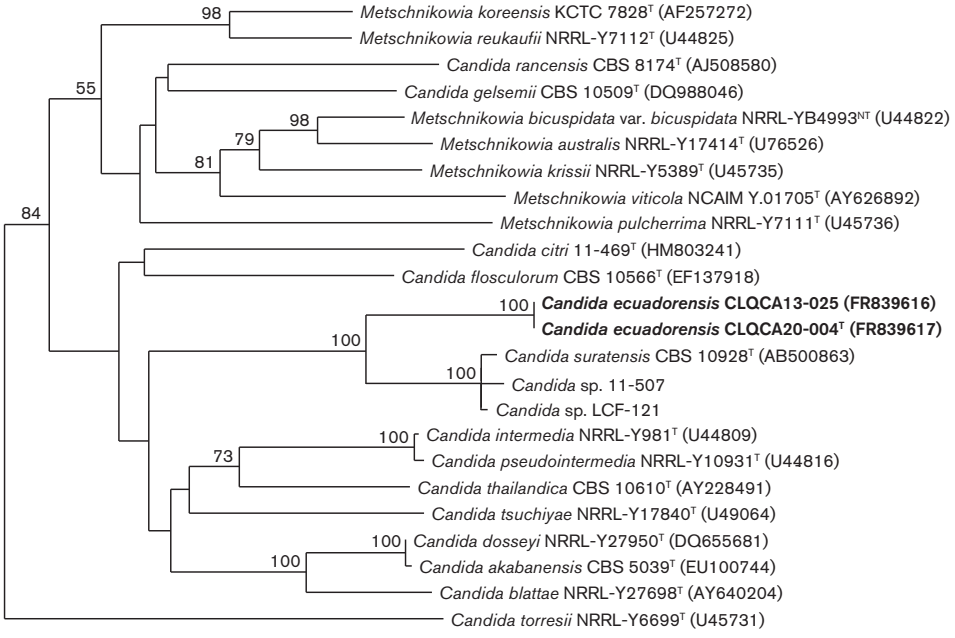

Fig. 1. Neighbour-joining dendrogram based on sequences of the D1/D2 domain of the LSU rRNA gene of *Candida ecuadorensis* sp. nov. and close relatives belonging to the *Candida intermedia* clade. *Candida torresii* was used as the outgroup species for the analysis. Bootstrap values $\geqslant$50 %, determined from 1000 replicates, are shown at branch nodes. Bar, 2 base substitutions per 100 nt.

two strains of *C. ecuadorensis* sp. nov. can also be distinguished from one another based on their differing abilities to assimilate L-arabinose, erythritol, ribitol and D-glucosamine, as well as differing salt tolerance. Once again, these differing phenotypes may perhaps be attributable to the fact that the two strains were isolated from quite different sources, namely cow manure (CLQCA 13-025) and rotten wood (CLQCA 20-004^T).

Based on the molecular and phenotypic data presented here, we conclude that the two Ecuadorian strains represent a novel species. As neither sporulation nor conjugation was observed in either mixed or pure cultures on a variety of different sporulation media, the novel species was assigned to the asexual genus *Candida* with the name *Candida ecuadorensis* sp. nov. The type strain is CLQCA 20-004^T.

Description of *Candida ecuadorensis* sp. nov.

Candida ecuadorensis (e.cua.dor.en.sis. N.L. fem. adj. *ecuadorensis* of or belonging to Ecuador).

In YM broth, after 2 days at 25 °C, cells are ovoid (2–5 × 3–7 μm) and occur singly, in pairs, in short chains or in groups (Fig. 2). Budding is multilateral. Sediment is formed after 1 month, but no pellicle is observed. Pseudomycelia or true mycelia are not formed. No sexual state is observed from either mixed or pure cultures grown for 1 month at 25 °C on cornmeal agar, Gorodkowa agar, potassium acetate agar and YM agar. Glucose, sucrose, raffinose and cellobiose (variable) are fermented, but not galactose, maltose, lactose, trehalose, melibiose melezitose, methyl α-D-glucoside, inositol, starch or xylose. Glucose, inulin (latent), sucrose, raffinose, galactose, trehalose, maltose, melezitose, methyl α-D-glucoside, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylose, L-arabinose (variable), ethanol, glycerol, erythritol (variable), ribitol (variable), D-mannitol, D-glucitol, succinate, citrate, xylitol (latent) and D-glucosamine (variable) are assimilated. No growth occurs on melibiose, lactose, soluble starch, D-arabinose, D-ribose, methanol, galactitol, inositol or DL-lactate. Ethylamine hydrochloride, lysine and cadaverine are assimilated, but not nitrate. Growth occurs at 30 °C, but not at 37 °C. Growth occurs on YM agar with 10 % (w/v) NaCl, but not in 50 % glucose/yeast extract (0.5 %). Starch-like compounds are not produced. Growth occurs in 10 μg cycloheximide ml^{-1}, but not in 100 μg cycloheximide ml^{-1}.

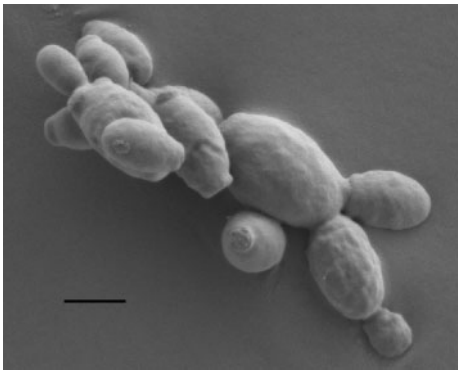

Fig. 2. Scanning electron microscopic image of vegetative cells of *Candida ecuadorensis* sp. nov. CLQCA 20-004^T grown in YM broth for 2 days at 25 °C with agitation. Bar, 2 μm.

The type strain, CLQCA 20-004^T (=CBS 12653^T=NCYC 3782^T), was isolated from rotten wood, collected in Guaranda, Bolívar province, Ecuador. The Mycobank deposit number is MB801370.

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