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Cladosporium lebrasiae, a new fungal species isolated from milk bread rolls in France

Josiane RAZAFINARIVO[a], *Jean-Luc JANY*[a], *Pedro W. CROUS*[b], *Rachelle LOOTEN*[a], *Vincent GAYDOU*[c], *Georges BARBIER*[a], *Jerôme MOUNIER*[a], *Valérie VASSEUR*[a,*]

[a]*Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France*

[b]*CBS-KNAW Fungal Biodiversity Centre, P.O. Box 85167, 3508 AD Utrecht, The Netherlands*

[c]*MéDIAN-Biophotonique et Technologies pour la Santé, Université de Reims Champagne-Ardenne, FRE CNRS 3481 MEDyC, UFR de Pharmacie, 51 rue Cognacq-Jay, 51096 Reims cedex, France*

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ABSTRACT

The fungal genus *Cladosporium* (*Cladosporiaceae*, *Dothideomycetes*) is composed of a large number of species, which can roughly be divided into three main species complexes: *Cladosporium cladosporioides*, *Cladosporium herbarum*, and *Cladosporium sphaerospermum*. The aim of this study was to characterize strains isolated from contaminated milk bread rolls by phenotypic and genotypic analyses. Using multilocus data from the internal transcribed spacer ribosomal DNA (rDNA), partial translation elongation factor 1-α, actin, and beta-tubulin gene sequences along with Fourier-transform infrared (FTIR) spectroscopy and morphological observations, three isolates were identified as a new species in the *C. sphaerospermum* species complex. This novel species, described here as *Cladosporium lebrasiae*, is phylogenetically and morphologically distinct from other species in this complex.

Introduction

Mould contamination is of particular concern for the bakery industry and causes important economic losses due to product spoilage. Fungal spoilage results in visible growth on products, alteration of taste and texture, potential production of mycotoxins, and allergenic compounds. Since most fungal conidia and mycelia are sensitive to heat and destroyed through the baking process, fungal contamination of bakery products usually occurs during the final stages of production. Contamination is commonly due to species belonging to the genera *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Eurotium*, *Penicillium* or *Wallemia* that are frequently encountered in wheat flour, and form conidia well adapted to aerial dispersal (Berghofer *et al.* 2003; Pitt & Hocking 2009; Weidenbörner *et al.* 2000). Conidia of these different taxa may contaminate products during their final cooling stages in bakeries. Moisture on the surface of the product after packaging, and before the product is fully cooled,

* *Corresponding author*. Tel.: +33 0 290915100; fax: +33 0 290915101.
E-mail address: valerie.vasseur@univ-brest.fr (V. Vasseur).

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may also contribute to mould growth. Species pertaining to *Aspergillus*, *Cladosporium*, *Eurotium*, *Mucor*, and *Penicillium* (Guynot *et al.* 2002; Pitt & Hocking 2009; Suhr & Nielsen 2004; Tančinová *et al.* 2012), as well as *Wallemia sebi* (Deschuyffeleer *et al.* 2015), a xerophilic fungus well adapted to low moisture products, have regularly been isolated from bakery products. The pH of the product, its water activity and organic acids present are among the most important factors affecting the type and growth intensity of fungal contaminants (Guynot *et al.* 2002). Identification of the causal organism is necessary for better control against product spoilage, and requires an up to date knowledge of fungal taxonomy, with special reference to the genera encountered in bakery products.

Among these genera, the genus *Cladosporium* s. lat. comprises more than 993 names (Bensch *et al.* 2012), and is cosmopolitan in distribution. *Cladosporium* species are commonly isolated from air, food, paint, plants, soil, and textiles (Ellis 1971, 1976; Farr *et al.* 1989; Flannigan 2001; Mullins 2001; Pitt & Hocking 2009; Schubert *et al.* 2007), occur as endophytes, plant pathogens (Brown *et al.* 1998; El-Morsy 2000; Riesen & Sieber 1985), or as hyperparasites on other fungi (Heuchert *et al.* 2005).

Phylogenetic studies based on sequence data from plastid and nuclear regions have provided a framework for understanding *Cladosporium* taxonomy (Bensch *et al.* 2010, 2012; Schubert *et al.* 2007; Zalar *et al.* 2007) and supported the recognition of three main species complexes, namely *Cladosporium cladosporioides*, *Cladosporium herbarum*, and *Cladosporium sphaerospermum*. Additional studies based on multilocus analysis allowed a better resolution for *Cladosporium* species within each complex. Using 18S ribosomal DNA (rDNA), rDNA ITS (Internal Transcribed Spacer) as well as the partial sequences of the actin (*act*) and of the translation elongation factor 1-α (*tef1*), 22 species were newly described by Bensch *et al.* (2010) within the *C. cladosporioides* complex. A phylogeny of species from the *C. sphaerospermum* complex isolated from hypersaline environments was performed by Zalar *et al.* (2007), whereas Schubert *et al.* (2007) addressed the taxonomy within the *C. herbarum* species complex. A recent revision of *Cladosporium* taxonomy, employing morphological, ecological, and molecular data recognised only 169 species of the 993 names, concluding that the genus *Cladosporium* in the old broad sense is heterogeneous and polyphyletic (Bensch *et al.* 2012).

The present study focuses on the characterization of *Cladosporium* isolates obtained from contaminated milk bread rolls. Isolates were initially identified according to macro- and microscopic criteria, combined with an analysis of rDNA ITS sequence data. Initial data indicated that these isolates could represent a previously undescribed species, requiring comprehensive molecular analyses using a multilocus approach. Isolates were also investigated by using phenotypic (macroscopic and microscopic morphological examinations), and Fourier-transform infrared (FTIR) spectroscopy features, which have been successfully applied for differentiation of moulds in the past.

Materials and methods

Cladosporium strains and culture conditions

Visible olivaceous brown fungal colonies were observed on the surface of commercial milk bread rolls. Pieces of contaminated product sampled from the edge of fungal colonies were deposited on yeast malt agar medium (2 % malt extract, 0.3 % yeast extract, and 1.5 % agar) supplemented with penicillin G (50 mg L^{-1}) and streptomycin (50 mg L^{-1}). Plates were incubated for 10 d at 25 °C prior to isolation. Three isolates were identified as *Cladosporium* sp. according to microscopic features (Bensch *et al.* 2012), and kept for further study. Isolates were deposited in the culture collection of the Université de Bretagne Occidentale (Culture Collection of University of Western Brittany, LUBEM Plouzané, France) as strains UBOCC-A-112061, UBOCC-A-112062, and UBOCC-A-112063 and in addition in the culture collection of the CBS-KNAW Fungal Biodiversity Centre (CBS, Utrecht, The Netherlands) as CBS 138280, CBS 138281, and CBS 138283. Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org; Crous *et al.* 2004). An additional 34 *Cladosporium* reference strains for *Cladosporium fusiforme*, *Cladosporium velox*, *Cladosporium sphaerospermum*, *Cladosporium dominicanum*, *Cladosporium langeronii*, *Cladosporium psychrotolerans*, and *Cladosporium halotolerans* were obtained from diverse collections to append to the dataset used in the phylogenetic study (Table 1). All strains were maintained on yeast malt agar medium at 25 °C in the dark.

DNA extraction and sequencing

DNA was extracted using the FastDNA® SPIN Kit (MP Biomedicals, Irvine, CA) following the manufacturer's instructions. For each strain, partial nuclear rDNA ITS region and partial translation elongation factor 1-α gene sequence (*tef1*), partial actin (*act*), and β-tubulin (*tub*) genes were amplified using respectively primers ITS4 and ITS5 (White *et al.* 1990), EF1-728F and EF1-986R (Bensch *et al.* 2012), ACT-512F and ACT-783R (Carbone & Kohn 1999), and T1 and T22 (O'Donnell & Cigelnik 1997). PCR was performed under the conditions listed in Table 2.

Each PCR product was sequenced using both primers at the 'plateforme Biogenouest' (Roscoff, France, http://www.sb-roscoff.fr/SG/). Sequence analyses were carried out with DNA Baser (Heracle Software, Germany), and the contig was manually edited using MESQUITE v.7.2 (Maddison & Maddison 2009). Sequences were deposited in GenBank under the accession numbers listed in Table 1.

Phylogenetic analyses

In addition to sequences obtained in the present study from 20 *Cladosporium* strains, 20 rDNA ITS sequences, 34 *tef1* sequences, 22 *act* sequences, and 20 *tub* sequences pertaining to 17 different *Cladosporium* strains used in Zalar *et al.* (2007), along with the seven reference strains (Table 1) were obtained from the GenBank database. Sequences for each taxon and locus, including both introns and exons, were aligned using MAFFT v.6 (Katoh *et al.* 2005) using the E-INS-i strategy and refined manually using MESQUITE v.7.2 (Maddison & Maddison 2009).

Incongruence Length Difference (ILD) tests were performed as implemented in PAUP v.4.0b10 (hompart option) (Farris *et al.* 1995). Maximum Likelihood (ML), Maximum Parsimony (MP), and Bayesian Inference (BI) analyses were performed on separate and concatenated datasets. MODELTEST v.3.7