Research article

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Isolation of *Rhizopus arrhizus* from Albanian barley

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Fungal contamination of barley can have a negative impact on malt quality. This work was undertaken to identify the fungi associated with barley kernels, which have been implicated in turbidity problems. *Rhizopus arrhizus* was isolated from barley and identified by morphological analysis together with molecular approaches. © 2018 The Institute of Brewing & Distilling

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Introduction

In terms of the global production of cereal grains, barley is ranked fourth after maize, rice and wheat. Barley is grown in temperate climates and tolerates poorer soils and lower temperatures than wheat. Accordingly, its cultivation has spread throughout Europe. In Albania, barley is grown in an area of 3104 ha with 9000 tonnes produced *(1)*.

Being rich in proteins, carbohydrates, dietary fibre, minerals and vitamins, barley contributes to food for human consumption and as animal fodder *(2)*. It is a source of fermentable material for beers and some distilled beverages. Malting of barley is a biological process involving complex biochemical and physiological reactions, in which microbial communities on barley grains play a crucial role *(3)*. Together with bacteria and yeasts, filamentous fungi from the genera *Fusarium*, *Alternaria*, *Cladosporium*, *Penicillium*, *Aspergillus*, *Rhizopus*, *Epicoccum*, *Gonatobotrys*, *Mucor* and *Helminthosporium* have been reported *(4)*. Estimation of fungal contamination of barley grain is important, as some fungi proliferating during malting can confer unwanted characteristics, such as turbidity. During steeping *(5)*, barley grains are soaked in water to stimulate germination. During this phase, the kernel absorbs water, and the initial moisture increases from 12–14 to 42–46% and in turn promotes the active growth of microbial communities. Further, the presence of broken kernels can encourage contamination as sugar is released during the wetting phase. Owing to several cases of high turbidity beer in Albania, a study of the microbial contamination of barley destined for beer production was conducted.

Materials and methods

Fungal isolation

Thirty barley samples were collected in Albania during March–May 2017. They were surface sterilised by washing with either ethanol–water (80:20 *v*/v) or sodium hypochlorite–water (3.5% *w*/*v*). The kernels were rinsed with tap water, air-dried and plated (12 kernels/plate) on potato dextrose agar (PDA, Oxoid, Basingstoke, UK). Two plates per sample were prepared. After incubation for 2–5 days at 25°C, the primary fungal colony was transferred to a PDA plate, purified as required and stored on PDA slants at 4°C in the fungal collection of the Department of Plant Protection of the Agricultural University of Tirana, Albania.

Morphological identification

Collected isolates were grown for 5–7 days on PDA at 25°C in the dark. Colonies were characterised (colour, margin, diameter and texture) and microscopic features recorded. Microscopic examinations were performed at 40× magnification (Optika, Ponteranica, BG, Italy). Branching patterns were observed with a Nikon SMZ1500 stereomicroscope. As all isolates were morphologically similar to each other, one isolate was randomly selected and used for the molecular identification.

Molecular identification and phylogenetic reconstruction

The isolate was grown for seven days on potato dextrose broth (PDB) at 25°C in the dark, in anagitated culture (150 rpm). The mycelium was collected and stored at −20°C. Genomic DNA was extracted as reported by Baroncelli *et al. (6)*. The ITS1-5.8 s-ITS2 region of rDNA was sequenced using ITS1 and ITS4 primers *(7)*. PCR was carried out in a 100 µL reaction mixture containing 100 ng of DNA, 10 µM of each primer and 5 U of Taq Polymerase (EmeraldAmp PCR master mix, Takara, Clontech, USA), in a MyCycler thermal cycler (BioRad, Hercules, CA, USA), using reported cycling conditions *(8)*. Macrogen (Seoul, Republic of Korea) synthesised all primer pairs. Amplicon was resolved in 1.7% agarose gel in TAE buffer (1×) and visualised by UV illumination.

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DNA was recovered from agarose by Isolate II Genomic DNA kit (Bioline, London, UK) according to the manufacturer's recommendations. Macrogen Europe (Amsterdam, The Netherlands) sequenced the purified product with both forward and reverse primers.

Evolutionary analyses of the sequence were conducted in MEGA6 *(9)*, using the Maximum Likelihood method based on the Tamura and Nei model *(10)*. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with superior log likelihood values. The analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 448 positions in the final dataset.

Results and discussion

Isolation and morphological identification

When kernels from the 30 barley samples were incubated at 25°C on PDA, a white mycelium with a net-like structure was observed. Thirty-three isolates with similar morphologies were transferred to new plates, showing similar features. The mycelium later changed to brownish-grey (Fig. 1a), and the reverse side of the colony was light brown in colour. Colony growth was very rapid and typically cotton-like in texture. Colonies were about 5–8 mm high, with some tendency to collapse. Non-septate or scarcely septate broad hyphae, rhizoids, sporangia and sporangiospores were observed. Sporangia were round and 80–300 μm in diameter (Fig 1b). Hyaline to brown unicellular spores were observed and their size was 6–9 μm in diameter (Fig. 1c). The observed characteristics matched those of *Rhizopus arrhizus s.l.* A. Fisch. *(11,12)*. *Rhizopus* was isolated following both disinfectant solutions; however, the kernels surface sterilised using ethanol gave the highest percentage (60%) of *Rhizopus* colonies. Rabie *et al. (13)*, who isolated *R. arrizhus s.l.* from barley kernels particularly after ethanol disinfection, obtained similar results.

Molecular identification

The nucleotide sequence of the ITS region from a representative isolate (RO1) was deposited in the NCBI database (accession no. MG599472). BLAST analysis revealed 100% sequence similarity with a *R. arrhizus* strain present in Genbank (accession no. KM225290). A phylogenetic tree was built with *Rhizopus* spp. present on barley *(14,15)* and the representative isolate was placed within a clade containing reference isolates of *R. arrhizus* (Fig. 2). Moreover, it clustered separately from *R. arrhizus* var. *tonkinensis* and *R. arrhizus* var. *delemar*. Thus, it could be confidently identified as *R. arrhizus* var. *arrhizus*. Indeed, the existence of varieties within *R. arrhizus s.l.* has been recently reported *(16)*.

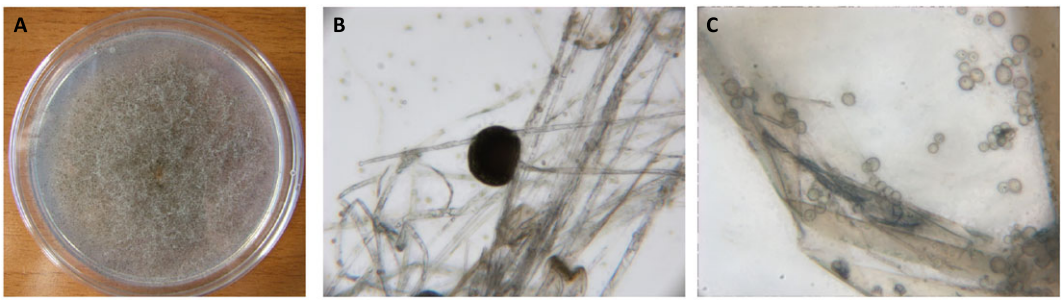

Figure 1. Macro- and microscopic characteristics of *Rhizopus arrhizus* on PDA at 25°C in the dark. [Colour figure can be viewed at wileyonlinelibrary.com]

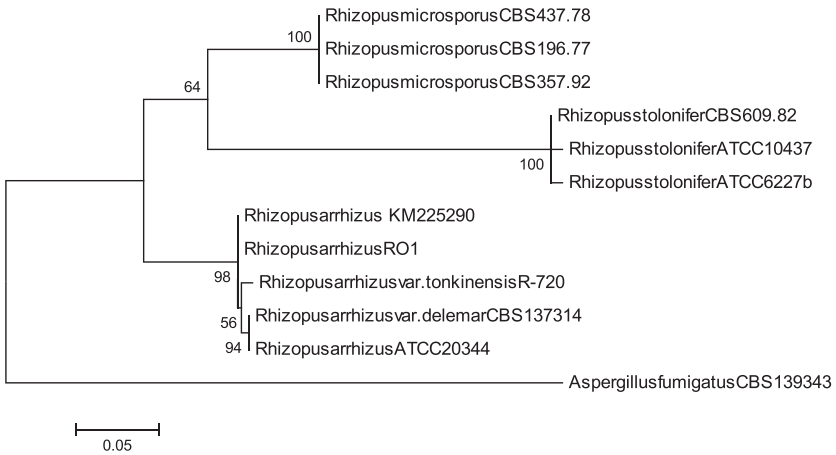

Figure 2. Phylogenetic analysis of *Rhizopus arrhizus* isolated in this study (RO1) and related sequences from GenBank. *Aspergillus fumigatus* was used as the out-group taxon. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (−1685.5953) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6.

R. arrhizus is a filamentous fungus classified in the order of Mucorales in the phylum Zygomycota. The genus *Rhizopus* was first recognised in 1820 *(17)* and produces fermentation products such as ethanol, L-(+)-lactic acid, and fumaric acid. *R. arrhizus* is ubiquitous in nature, since it is able to grow on a wide range of carbon sources including lactic acid, glucose, fructose, sucrose, xylose, cellobiose, fatty acids and oils *(18–21)*, at wide temperature range (up to 40°C) and between pH 4 and 9. Owing to this great adaptability to suboptimal conditions, *Rhizopus* can cause premature yeast flocculation (PYF) during wort preparation from barley. Indeed, antimicrobial peptides induced by microorganisms during growth in the field and/or malting process are able to disrupt the membrane integrity and function of yeast and consequently may impair sugar uptake during fermentation *(22)*. It has been reported that, among the fungi colonising barley, *Fusarium* and *Rhizopus* contribute greater PYF than genera such as *Aspergillus (23)*. PYF is a serious problem that has been observed more frequently in the brewing industry in recent times. This phenomenon results in a poor product quality with undesirable flavour characteristics and can cause losses. Furthermore, other negative effects have been reported, such as lower carbon dioxide evolution during fermentation and increasing sulphur dioxide *(24)*, leading to additional blending and potentially negative consumer reaction with a compromised brand identity *(25)*.

Conclusions

The present investigation confirmed the presence *R. arrizhus* from barley destined for brewing. Because of putative involvement of *Rhizopus* in beer turbidity issues, the results highlight the need to minimize additional microbial contamination.

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