Site Visit Report

The site visit process is a sample on a particular day of an installation's compliance with some of its licence conditions. Where non-compliance against a particular condition has not been reported, this should not be construed to mean that there is full compliance with that condition of the licence.

Instructions and actions arising from the visit shall be addressed, or where applicable noted, by the licensee in order to ensure compliance, to improve the environmental performance of the installation and to provide clarification on certain issues.

The licensee shall take the actions specified to close out the non-compliances and observations raised in this Site Visit Report.

The licensee may also be requested to provide a response to the Environmental Protection Agency (hereafter referred to as the Agency) in relation to the site visit report findings.

Licensee

Name of Installation	Irish Distillers Limited
Licensee	Irish Distillers Limited
Licence Register No.	P0442-02
CRO Number	23732
Site Address	Midleton Distilleries, Midleton, Cork
Site Visit Reference No.	SV25861

Report Detail

Issue Date	30/01/2023
Prepared By	Pól O'Seasnain

Site Visit Detail

Date Of Inspection	25/07/2022		
Time In	14:30	**Time Out**	16:10
EPA Inspector(s)	Pól O'Seasnain		
Additional Visitors	Prof. Alan Dobson UCC and behalf of EPA.		
Licensee Personnel and Role	Ronan Holt, Environmental & Facilities Manager. Paul Wickham, Head of Production.		

Summary

The site visit was conducted in response to a complaint of black discoloration of tiles and external surfaces of the complainant's house at The Park, Midleton which was initially viewed on 21/07/2022. On 25/07/2022 samples were taken for analysis and identification by Prof. Alan Dobson Professor of Environmental Microbiology, UCC from the black discoloration on the house of the complainant and from two nearby houses. Samples were also taken by Prof. Dobson from the external surfaces of one of the warehouses (ref. no. 29A) at Irish Distillers Limited.

Site Areas Inspected

- External areas of Irish Distillers Ltd warehouses.
- External surfaces of 3 residences.

Documents Inspected

No documents were inspected.

1. Site Specific Issues

		Answer	Condition Number	Non Compliance	Observation
1.1	Microbiological Assessment	Checked	5.2		

Comment / Action Required

A microbiological assessment was carried out to determine the presence of a fungus, Baudoinia spp. commonly referred to as whiskey fungus , at Irish Distillers warehouse buildings and at three nearby residential locations outside the licence boundary. One of the residences was the subject of a complaint in relation to Irish Distillers . The outcome of the investigation was that Baudoinia spp. was identified at the IDL warehouse location but not at any of the three residential locations sampled. The full details of the assessment are attached to this report.

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Background

Professor Dobson from the School of Microbiology at University College Cork (UCC) was requested by the EPA to carry out an assessment of discoloration of buildings in the vicinity of the Irish Distillers facility in Midleton, Co Cork. Sampling was undertaken to identify a fungus potentially causing a discoloration problem.

Report (Compiled on December 2nd, 2022).

This report on samples (D) taken from the Irish Distillers facility in Midleton and neighbouring properties (A, B) and C (the complainant) located in The Park, Midleton (P25TD28) to identify a fungus potentially causing a discoloration problem; was prepared by Professor Dobson who is Professor of Environmental at UCC and who has worked in the area of Fungal physiology and genetics for over 33 years. Analysis of the samples was conducted at Professor Dobson's laboratory under controlled conditions which facilitate the growth of fungi and their characterisation using molecular based approaches. This laboratory has appropriate quality control systems in place for the sampling and analysis that was performed.

Sampling

The samples (A, B, C & D) were taken on 25th July 2022 between 2:30pm and 4:05pm, with sampling involved taking scraping from areas where visible blackening was apparent into 20 ml sterilin tubes until further analysis, which occurred within 24 hours. The samples were taken in the presence of an EPA inspector. The scrapings were then plated onto agar plates [Modified Leonian's Medium (Scott et al., 2016) or Modified Ewaze's Medium (MEM)] and incubated at room temperature and at 26°C, in an attempt to grow any fungi that might be present in the samples. *Baudoinia* spp is often known as "Whiskey Fungus" as it commonly grows in the environs of distillery sites. Therefore MEM was used as it is a semi-selective media for the growth of *Baudoinia* spp. where the only carbon source present is ethanol (Ewaze et al., 2008b). The focus on *Baudoinia compniacensis* was due to the fact that this fungus is well known to be associated

with growth on surfaces that are subject to periodic exposure to low levels of ethanol (distillery alcohol) vapour, such as those in the vicinity of distillery aging warehouses.

Growth

Growth was observed on MEM in the samples taken from all four locations (Figure 1). Discrete fungal colonies were then picked from these plates. In all cases attempts were made to pick fungal colonies resembling a *Baudoinia compniacensis* phenotype. In some cases the colonies that were picked were then sub-cultured in an attempt to further purify them.

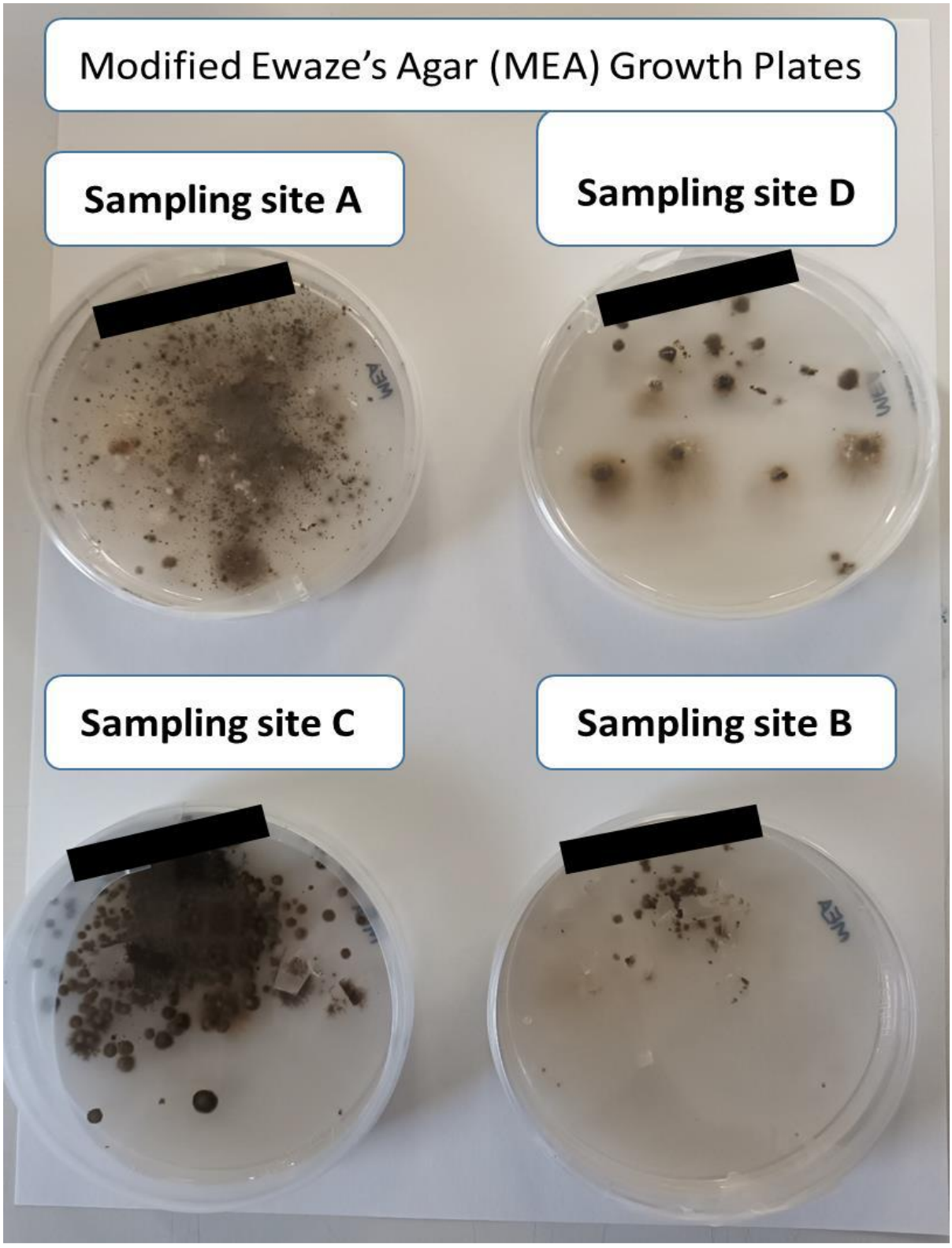
DNA Analysis

DNA was then extracted from pure fungal colonies for subsequent PCR based analysis. DNA was also extracted from *Baudoinia compniacensis* CBS 123031 which was obtained from the Westerdijk Fungal Biodiversity Institute (WFBI) in the Netherlands for validation of the PCR based identification approach being employed and to act as a reference type strain for the phylogenetic analysis.

Following isolation of DNA from the samples, PCR reactions targeting the large subunit rRNA subunit gene (LSU) were performed using a primer set that can amplify fungal rDNA from a wide range of taxonomic groups (White et al., 1990). The PCR conditions employed are provided in Appendix 1. Products of the expected size were then purified and sequenced by Sanger sequencing at Eurofins Genomics.
When PCR product sequencing showed mixed species samples, these PCR products were cloned and the cloned genes were subsequently amplified by M13 PCR and re-sequenced.

Results

As previously mentioned, growth was observed on MEM in the samples taken from all four locations (Figure 1).



The PCR product sequences were analysed and the closest related sequences were identified using the Basic Local Alignment Search Tool (BLAST) which is hosted at https://blast.ncbi.nlm.nih.gov/Blast.cgi (NCBI)

These sequences were then aligned to generate phylogenetic trees.

Results from the phylogenetic analyses obtained for sampling sites A and B are shown in Figures 2 and 3 respectively, while results obtained from Irish Distillers sampling site D (D29) together with those obtained from the A, B and C samples are shown in Figure 4.

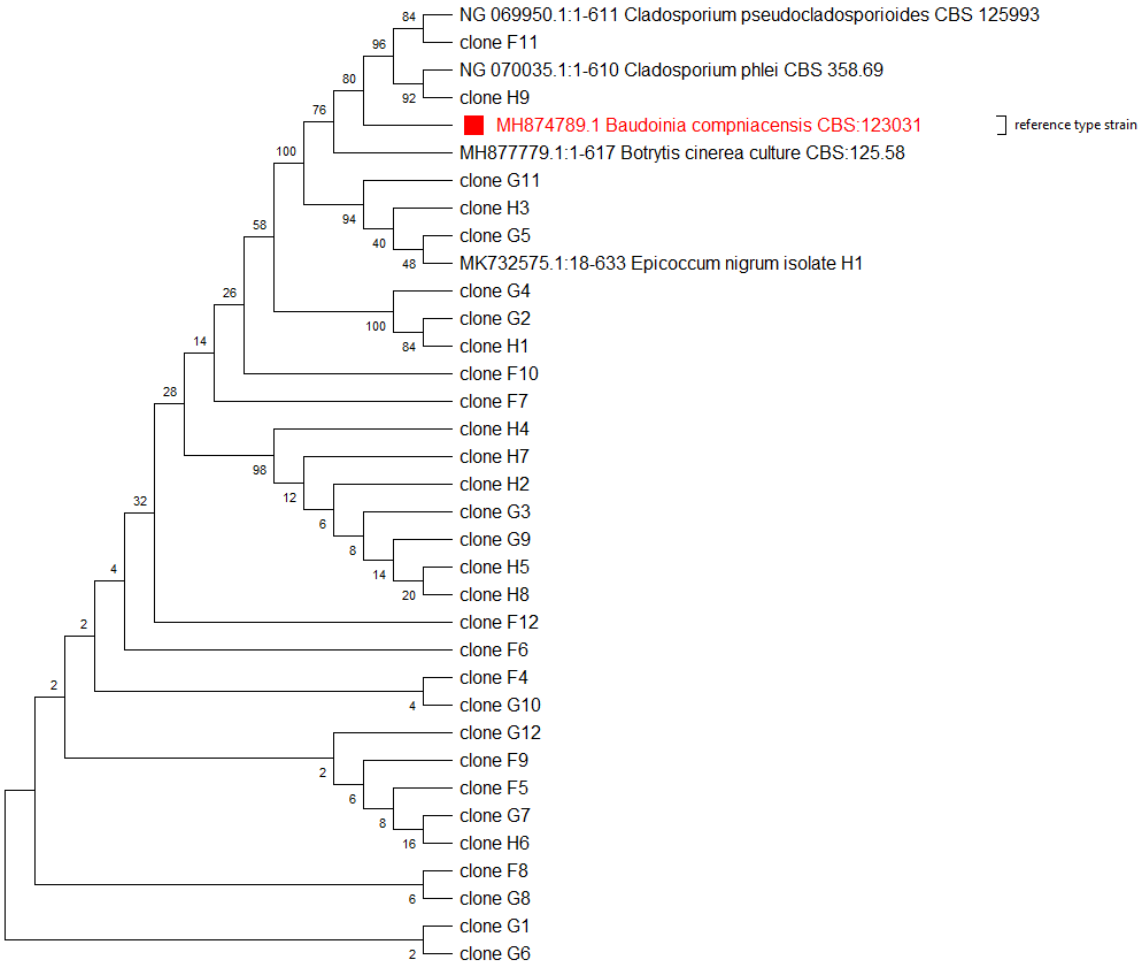


Figure 2: Neighbor-Joining phylogenetic tree of cloned partial LSU rRNA genes from samples taken from **Sampling Site A**, and of the closest related sequences from BLAST searches. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 50 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (50 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 35 nucleotide sequences in total. There were a total of 1104 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

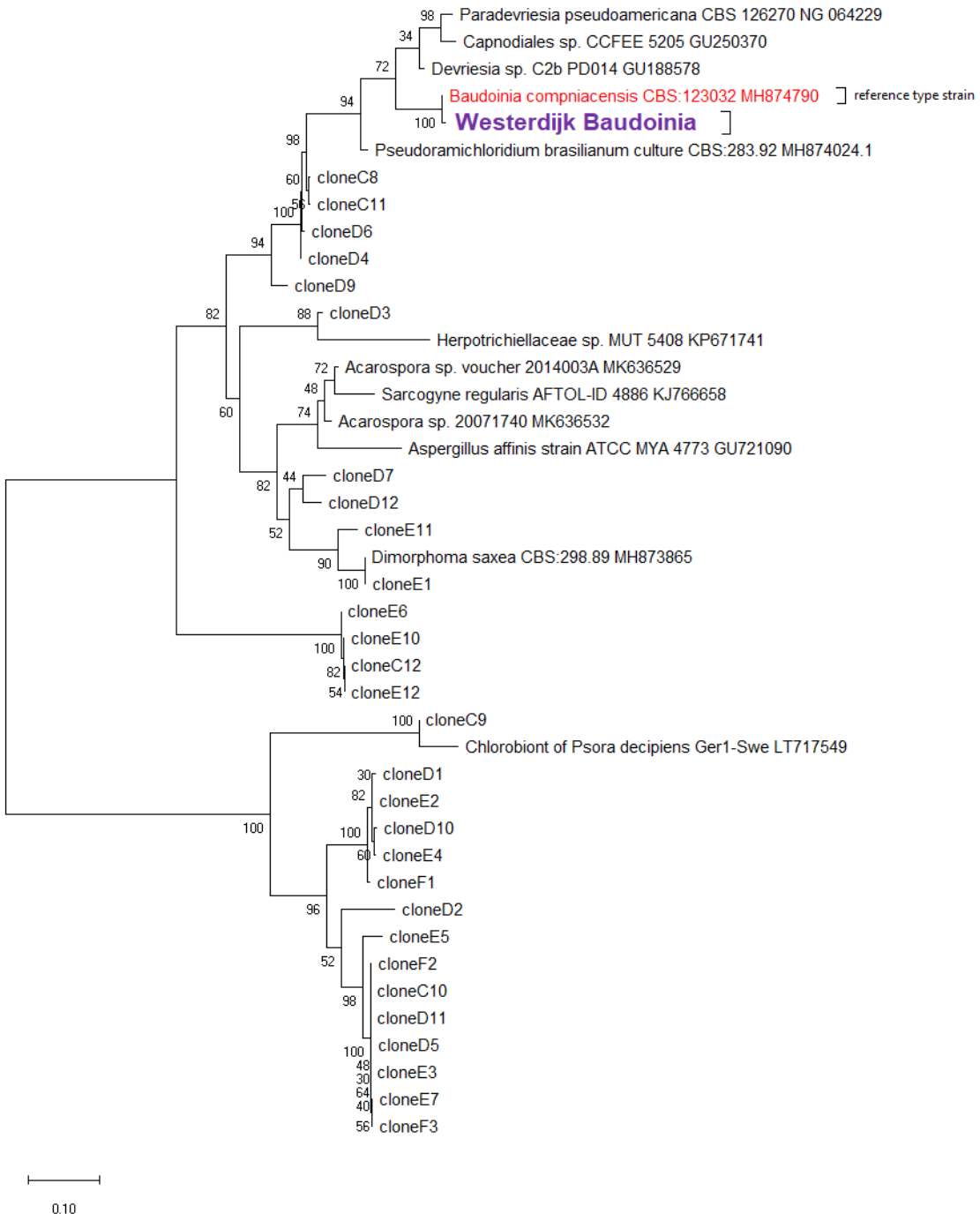


Figure 3: Maximum-Likelihood phylogenetic tree of cloned LSU rRNA genes PCR amplified from samples taken from **Sampling Site B**, and from the closest related sequences in BLAST searches. Also added are *Baudoinia compniacensis* (red font) as a reference and the sequenced PCR product from *Baudoinia compniacensis* obtained from the Westerdijk Fungal Biodiversity Institute, (WFBI) in the Netherlands (purple font). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the

heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 42 nucleotide sequences. There were a total of 2149 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

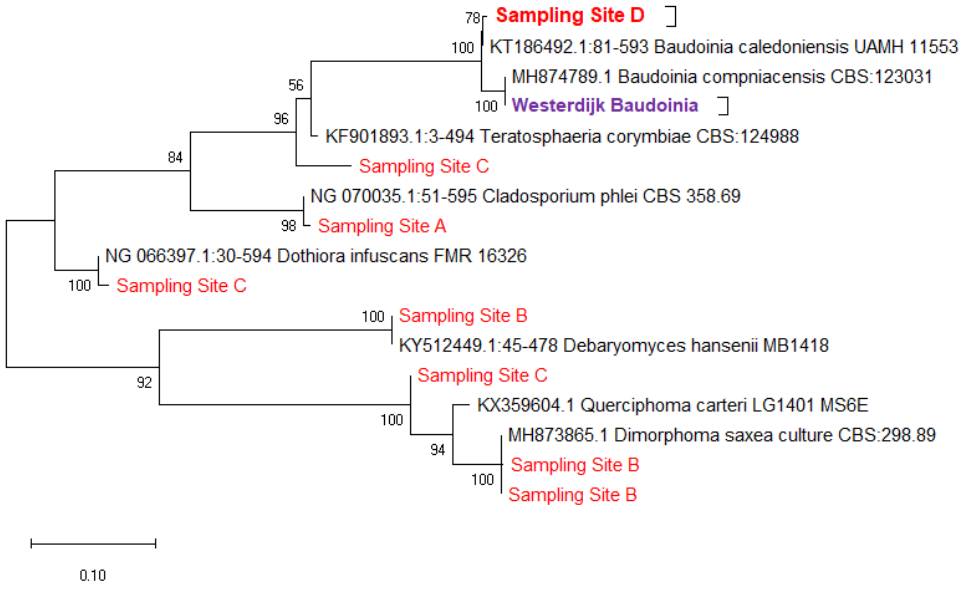


Figure 4: Maximum-Likelihood phylogenetic tree of sequenced LSU rRNA fungal genes from various sampling locations (red font). The sequence amplified in-house from the WFBI strain is in purple font ('Westerdijk Baudoinia'). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 17 nucleotide sequences. There were a total of 1775 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Outcome of the Investigation

From the **Sampling Site A** samples (Figure 2) it is clear that none of the PCR products obtained could be identified as *Baudoinia compniacensis,* with clone H9 being located in the same clade as a *Cladosporium phlei* strain and clone F1 being located in the same clade as *Cladosporium pseudocladosporiodes*. *Cladosporium* spp*.* are dematiaceous moulds that are widely prevalent in outdoor air and which are found in many different types of soil as well as on all types of senescing and dead plant matter; with a few species also been known to grow on moist building materials.

From the **Sampling Site B** samples (Figure 3) it is also clear that none of the PCR products obtained could be identified as *Baudoinia compniacensis.* Included in this analysis is the PCR product obtained from the *Baudoinia compniacensis* type strain obtained from the Westerdijk Fungal Biodiversity Institute (WFBI) in the Netherlands, which appears in the same clade as the reference type strain *Baudoinia compniacensis* CBS:123032. This validates the PCR based identification approach being employed.

From the **Sampling site C** samples, it is clear that none of the PCR products that were obtained could be identified as *Baudoinia compniacensis.* Following initial growth only 3 morphotypes were observed growing on the isolation plates (Figure 1) and after DNA extractions and genetic analyses it was clear that the picked isolates were pure axenic cultures. Following analysis these fungal species were identified as strains that were closely related to *Teratosphaeria corymbiae*, *Dothiora infuscans* and *Debaryomyces hansenii*. (Further details on the characteristics of these three fungi are outlined in Appendix 3). None of these were identified from the Sampling Site D.

It is clear from Figure 4 that the sample taken from **Sampling Site D** (Irish Distillers facility in Midleton) contained a *Baudoinia* species being in the same clade as *Baudoinia caledoniensis*, *Baudoinia compniacensis* and the PCR product obtained from the *Baudoinia compniacensis* type strain obtained from the WFBI, previously shown in Figure 3.

This further validates the PCR based approach employed and provides definitive proof of the presence of *Baudoinia* spp. in the sample taken from the Irish Distillers site. While we cannot definitively say that *Baudoinia* spp. are not present in the other three locations sampled, despite extensive efforts we have only found it to be present in the Sampling Site D sample taken from the Irish Distillery site.

Conclusions

Analysis of samples taken from the Irish Distillers facility in Midleton (Sample D) and neighbouring properties (Samples A, B and C) located in The Park, Midleton (P25TD28), was undertaken to identify a fungus potentially causing a discoloration problem. This initially involved growing samples taken from these four locations on MEM growth medium, a semi-selective media for the growth of *Baudoinia* spp. The analysis focused on *Baudoinia* spp as this fungus is known to be responsible for the staining of areas that it colonizes, which as a result are typically characterised by a highly conspicuous black colour. In particular the fungus grows in areas that are periodically exposed to very low levels of ethanol vapour, such as those that are present in the vicinity of

distillery aging warehouses and of commercial bakeries. (Further details on the characteristics of the fungus are outlined in Appendix 3).

Fungal growth was observed in all the samples taken from the four locations. Following PCR analysis involving the construction of phylogenetic trees, which included sequences from known *Baudoinia* spp for comparative purposes, only Samples D from the Irish Distillers facility in Midleton, was found to contain a 16s rRNA gene sequence from a *Baudoinia* species which was in the same clade as *Baudoinia caledoniensis*, *Baudoinia compniacensis* and the PCR product obtained from the *Baudoinia compniacensis* type strain obtained from the WFBI. We can therefore conclude that Sample D contained a *Baudoinia* spp. and that no *Baudoinia* spp were present in the other samples that were analysed. Thus while we cannot definitively say that *Baudoinia* spp. are not present in the Samples taken from properties (A, B and C) located in The Park, Midleton; we can conclude that we have been unable to identify *Baudoinia* spp. to be present in these samples.

References

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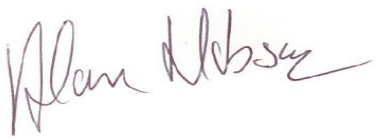

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Appendix 1: Details of PCR reactions undertaken.

Primers D1F (5' GCA TAT CAA TAA GCG GAG GA 3') and D2R (5' TTG GTC CGT GTT TCA AGA CG 3') were employed. The PCR cycle conditions comprised of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. with a final extension step at 72°C for 5 min. PCR products were visualized by agarose gel electrophoresis. Where multiple or non-specific bands were observed, bands of the expected size were excised from the gels and purified using Thermo Fisher GeneJet Gel Extraction kit according to the manufacturer's instructions. Products of the expected size were then purified using Thermo Fisher GeneJet PCR Purification kit according to the manufacturer's instructions. Purified products were sequenced by Sanger sequencing at Eurofins Genomics.

Appendix 2: Current information on *Baudoinia compniacensis* from the scientific literature.

The fungus *Baudoinia compniacensis,* also known as Whiskey fungus, Warehouse staining fungus and Distillery fungus is a black fungus with a velvety or crust-like morphology. It can grow on a variety of different surfaces including brick, metal, stainless steel, concrete, plastic and on plant vegetation; in regions that are subject to periodic exposure to low levels of ethanol vapour, such as those in the vicinity of distillery aging warehouses and commercial bakeries (Craig et al., 2021; Ewaze et al., 2007). The fungus is responsible for the staining of areas that it colonizes which is characterised by a highly conspicuous black colour. It is believed that the fungus can metabolize carbon sources such as ethanol, and can grow in exterior settings when exposed to low levels of ethanol vapours (5-10 parts per million) in the atmosphere. In the presence of ethanol, even at such low levels; the fungus is believed to use the enzymes alcohol dehydrogenase and acetaldehyde dehydrogenase which together with enzymes from the glyoxylate cycle allow it to generate sufficient energy to grow via the Tricarboxylic Acid Cycle (Ewaze et al., 2008a).
It is a member of the order *Capnodiales* and like other members of this order is a saprobe which lives on dead or decaying organic material and which are commonly found in plant litter, or in more extreme ecological niches. For example *Zasmidium cellare* another member of the order can grow in wine cellars when exposed to alcohol vapours in places that are cool and damp. Like with *Baudoinia*, ethanol vapours stimulates growth of the fungus, which grows in thick brown mats of brown mycelium that coat the cellar walls (Videira et al., 2017). In addition to being able to act as a nutrient to support growth of *B. compniacensis*, ethanol has also been reported to act as a germination activator and an inducer of cellular responses in the fungus, providing further evidence to explain its prevalence near warehouses that store whiskey (Scott et al., 2016).

With respect to any potential human and animal health risks, relating to *Baudoinia* spp; there are no reports to date in the scientific literature of health risks resulting from either short or long term exposure to *B. compniacensis* (Indiana State Department, 2019). In addition recent whole genome wide analysis of 101 different Dothideomycete genomes including *Baudoinia* species (Haridas et al., 2020), have shown no evidence of the presence of genes encoding proteins or metabolites that could potentially be detrimental to either humans or animals.

Appendix 3: **Further details on the characteristics of the three fungi identified from Sample C.**

Teratosphaeria corymbiae

Little is known about *Teratosphaeria corymbiae*, with the only known reports in the scientific literature describing it as an endophyte of eucalyptus (Crous et al., 2019). There are no reports of *T. corymbiae* growing in locations related to distilleries and the metabolic potential of this species has not been explored.

Dothoria infuscans

This fungus was first described in 2018 and there are only two reported occurrences of the species to date in the scientific literature. The species was reported to be present on the surface of a wall in Spain but no further information about the nature or location of the wall is available. http://www.indexfungorum.org/Names/NamesRecord.asp?RecordID=824999

Debaryomyces hansenii

This is a yeast species that can grow at low temperatures, low pH and high salt concentrations and has been identified in dairy products (Breuer and Harms, 2006). *D. hansenii* produces ethanol by fermentation and thus can tolerate environmental ethanol vapours but to our knowledge there are no reports of the species consuming ethanol as a carbon source nor of it growing on inert substrates such as walls.

FOLLOW-UP ACTIONS

The licensee is required to complete the actions outlined in this site visit report within the specified timeframes. Where required, the licensee shall also respond to actions specified in Compliance Investigations and/or submit a response to this site visit report via the EDEN system. The licensee shall maintain a documentary evidence, for review by the Agency, that the prescribed actions were completed within the required timeframe.

(i) Compliance Investigations

The Agency may generate a Compliance Investigation through the EDEN system and issue instructions and actions to the licensee. The licensee will receive notification when an instruction or action is issued and the licensee must respond to the actions within the Compliance Investigation within the specified timeframe.

(ii) Response to Site Visit Report

Where the licensee is requested to (or wishes to) respond to the Agency in relation to this site visit report, the licensee may select the 'Make a Response' link on the actions taken by the licensee to address the issues raised in this site visit report and the target completion dates. This Licensee Public Response provides the licensee with an opportunity to inform both the Agency and the public about the implementing of actions set out in the Agency site visit report. The response must be submitted **within 30 calendar days** of the issue date of this site visit report.

(iii) Publication of Reports

The site visit report will be made available for public viewing via the Agency's Licence Enforcement Access Portal (LEAP), within one day of the issue date. The Site Visit Report and the Licensee Public Response will also be published on the Agency's website, www.epa.ie, 60 calendar days after the site visit report issue date (on the Licence Details Page for the relevant licence).

Please note that licensees are required to comply with the conditions of the licence at all times, and where non-compliance occurs, compliance must be restored within the shortest possible time. These actions will be verified during subsequent Agency visits. Please quote the above Inspection Reference Number in any correspondence in relation this Report.