Penicillium glabrum cork colonising isolates - preliminary analysis of their genomic similarity

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Summary The cork stopper manufacturing process includes an operation, known as stabilisation, by which humid cork slabs are extensively colonised by fungi. The effects of fungal growth on cork are not completely understood although they are considered to be involved in the so-called "cork taint" of wine. It is essential to (a) identify environmental constraints which define the appearance of the colonising fungal species and (b) trace their origin to the forest and/or the manufacturing space. The present article correlates two sets of data, from consecutive years and the same season, of systematic sampling of two manufacturing units, located in the North and South of Portugal. *Chrysonilia sitophila* dominance was confirmed, followed by a high diversity of *Penicillium* species. *Penicillium glabrum*, which was found in all samples, was the most frequently isolated species. *P. glabrum* intra-species variability was investigated using DNA fingerprinting techniques revealing highly discriminative polymorphic markers in the genome. Cluster analysis of *P. glabrum* data was discussed in relation to the geographical location of strains, and results suggest that *P. glabrum* arise from predominantly the manufacturing space, although cork specific fungi can contribute.

Key words Cork, *Penicillium glabrum*, DNA fingerprint, Fungal genomic similarity

Cork is derived from the bark of *Quercus suber* L., an extremely important product for Portugal, Spain and some other Mediterranean countries. It is a light-weight and extremely useful material, with good thermal insulating properties and is elastic, compressible and impermeable to gas or liquid [1,2]. The ability of cork to sustain high compression levels [3] is why it is the most suitable material for sealing wine bottles. Portugal is the world's leading producer of cork with 54% of the market [3]. The global economic importance of cork is in direct relation to the sales of wines for obvious reasons.

Cork stoppers manufacturing includes an operation, known as stabilisation [3], during which the humid boiled cork slabs are extensively colonised by fungi [4,5]. Cork boiling is necessary since it produces a material with the adequate humidity and flexibility to enable the stoppers to be punched from the raw material [6]. The "traditional" dominant fungus was *Chrysonilia sitophila* (Mont.) Arx. However, this has been replaced by a more complex colonisation process, where several fungal genera, especially *Penicillium*, coexist with *C. sitophila* [4,5].

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Fungal growth may interfere in the stoppers (a) sensorial quality (e.g. off-flavours from fungal metabolism [7,8]), (b) safeness (e.g. mycotoxins production [9]) and (c) mechanical performance (e.g. degradation of cork tissues [10]). All these process are strongly influenced by the species diversity and growth conditions [7,11,12]. However, the overall implications of fungi growth over cork are difficult to assess, mostly because of the high diversity of the species which occur.

It was observed that viable fungal spores were in deep cork cellular layers, which may have originated from the source forest. Similar, spores survived the boiling treatment involved in cork manufacturing and germinated to form mycelium (Silva Pereira, unpublished results). However, the environmental constraints which determine the appearance of the colonising fungal species and the geographical origin of such consortia are not known. Several factors require to be determined the: (a) diversity of species, (b) dominant species, (c) stability of the consortium under different stresses, and (d) regional similarity of species. To identify the regional similarity of the fungal isolates systematic sampling was carried out inside two manufacturing units located in North and South Portugal.

Industry has explored various manufacturing practices that may influence the diversity of the cork colonising species. In the present case, North and South units represent a synergistic productive system. The South unit is an intense cork slab boiling unit, which includes a short stabilisation period (less than one week). The North unit is a non-boiling unit, where mature slabs of ca. four days are received, already boiled, from the South unit.

The diversity among *Penicillium* isolates was investigated by standard morphological assessments. The

regional similarity of the *Penicillium glabrum* isolates was determined using DNA fingerprint methods. This is the first report concerning the dynamics of cork colonising fungal consortia.

Methods

Microorganisms. Fungi were collected inside two Portuguese industrial units situated in Santa Maria da Feira (North unit) and in Ponte de Sôr (South unit). The South unit is a cork slab boiling unit and North unit is an integrated cork stopper industry. Fungal species were isolated and preserved as reported previously [5]. *Penicillium* type strains from ATCC (American Type Culture Collection), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), FRR (Food Science Australia, Ryde) and MUM (Micoteca da Universidade do Minho), were used as reference specimens for the morphological and molecular analysis.

Morphological characterisation. *Penicillium* species were identified using the methodology of Pitt [13] that includes morphological analysis on different solid media and microscopic observation of the fungal reproduction structures.

DNA analysis. Five day old fungal colonies grown at 25 °C on Malt Extract Agar (MEA, Merck), were used from which to extract the DNA. Small pieces of ca. 1 cm^2 area were cut from the surface of the fungal colony and the mycelium was immediately added to and mixed in (2 min, vortex) an Eppendorf tube containing ca. 200 µl volume of Glass beads (425-600 microns SIGMA) in 500 µl of lysis buffer (50 mM Tris, pH 8.0; 250 mM NaCl; 50 mM EDTA; 0.3 % (w/v) SDS). The suspension was incubated for 1 h at 65 °C, homogenised again for 2 min in the vortex, and centrifuged at 4 °C to recover the supernatant (14000 *g*, 10 min). The mixture was incubated for 30 min at 37 °C with 1.2 µl of RNAse (SIGMA) solution (25 mg/ml), extracted with 500 µl of a mixture of chloroform:isoamyl alcohol (24:1) and recovered by centrifugation (8000 *g*, 10 min) The DNA was precipitated with a mixture of 3 M potassium acetate (pH 5.2) and cold ethanol (-20 °C), 1/10 and 2.5 volumes, respectively and the pellet recovered after centrifugation (14000 *g* for 10 min). The DNA sample was then washed in ethanol:water (7:3), recovered by centrifugation (14000 g, 5 min) and re-equilibrated in 100 µl of TE buffer. To qualitatively evaluate the yield of the extraction, DNA was visualised in agarose gel (1%, w/v) stained with ethidium bromide.

PCR fingerprinting. Four single primers were used. The oligonucleotide sequences were the core region of the phage M13: GAG GGT GGC GGT TCT and 3 SSRs (simple sequence repeats): $(GTGC)_4$ $(GA)_8YG$ and $HVH(GTG)_5$. Amplification reaction mixture contained: fungal DNA, sterile ultra pure water, dNTP Mix (10 mM), $MgCl_2$ (25 mM), PCR buffer (10x), immolase (BIOLINE) (5 u/µl) and primer (50 pmol/µl) (1:17.3:0.5:2.5:2.5:0.2:1). PCR conditions were: 1 cycle at 94 °C for 5 min, 40 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min and a final amplification cycle at 72 °C for 6 min. The PCR amplified product was resolved by agarose gel electrophoresis (1.2 % w/v; 75V, 11 h) and DNA bands were visualised as described above.

PCR profile analysis. The restriction profiles of each isolate were analysed using BioNumerics software, version 4 (Applied Maths, USA). The gels were normalized in order to avoid inter- and intra-gel variation and aligned by associating bands of the internal molecular weight standards on each gel with stored reference positions. Dendrograms were constructed according to the DICE coefficient similarity.

Results

The quick growth and dominance of *C. sitophila* was confirmed, followed by the growth of several *Penicillium* species (Table 1). A smaller incidence of other genera, such as *Trichoderma*, *Mucor*, *Aspergillus* and *Cladosporium* was also observed (data not shown). In terms of species dominance and diversity the cork colonisation profile reported was similar to that previously observed [5].

Thirty two isolates of *Penicillium* were collected, which were classified into 13 species and one unidentified isolate (Table 1 and figure 1). *Penicillium* species diversity was variable between different units and in the same unit in different years (Table 1) although some species were consistent.

P. glabrum was the most frequent species (13/32) and was used to assess genomic similarity and relate to geographical location. Discrimination between different strains of *P. glabrum* was obtained by DNA fingerprint analysis, exploiting the difference between high polymorphic regions of the fungal genome [14]. DNA fingerprint data was clustered together combining the results of single primer DNA amplification, at different discriminatory levels: low (two primers sets: sequence of M13 and $(GTGC)_4$) or high (four primers sets: sequence of M13, $(GTGC)_4$), $(GA)_8YG$ and $HVH(GTG)_5$) (Figures 2 and 3, respectively).

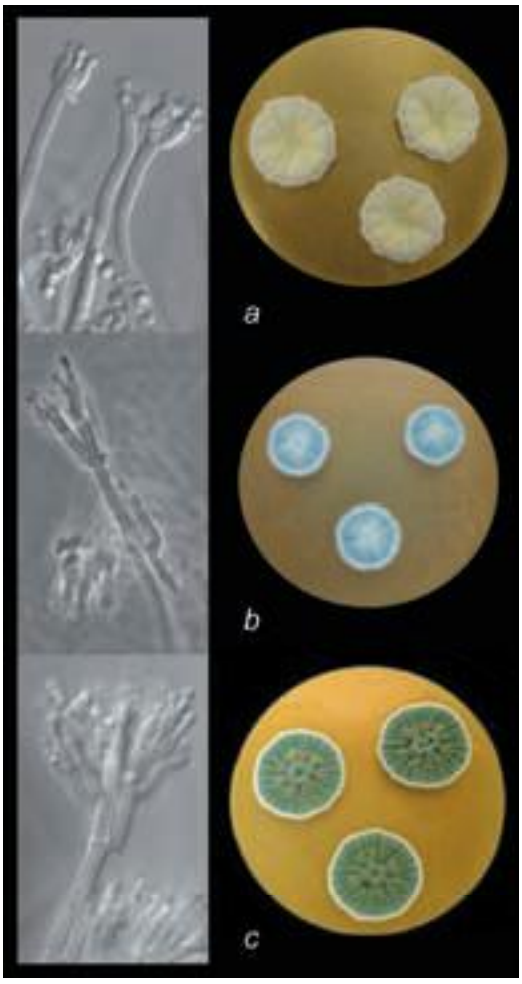

Figure 1. Macroscopic aspect of selected fungi colonies and the characterisation of their reproductive structures (X1000) observed under Nomarski phase contrasts microscopy. a, *Penicillium hirayamae* (Monoverticillate); b, *Penicillium fenneliae* (Biverticillate) and c, *Penicillium olsonii* (Terverticillate).

Table 1. Species identification of *Penicillium* taxa.

1st year	2nd year
North Unit	
Penicillium glabrum (N a1-7)	*Penicillium glabrum (*N b1)
Eupenicillium hirayamae (N a8)	*Penicillium decumbens* (N b2)
Penicillium variabile (N a9)	*Penicillium* sp. (N b3)
Penicillium glandicola (N a10)	*Penicillium glandicola* (N b4)
Penicillium olsonii (N a11)	*Penicillium brevicompactum* (N b5)
South Unit	
Penicillium glabrum (S a1-3)	*Penicillium glabrum* (S b1-2)
Penicillium decumbens (S 4-5)	*Penicillium decumbens* (S b3)
Penicillium variabile (S a6)	*Penicillium adametzii* (S b4)
Penicillium fenneliae (S a7)	*Penicillium restrictum* (S b5)
	Eupenicillium hirayamae (S b6)
	Penicillium janczewskii (S b7)
	Penicillium diversum (S b8)
	Penicillium corylophilum (S b9)

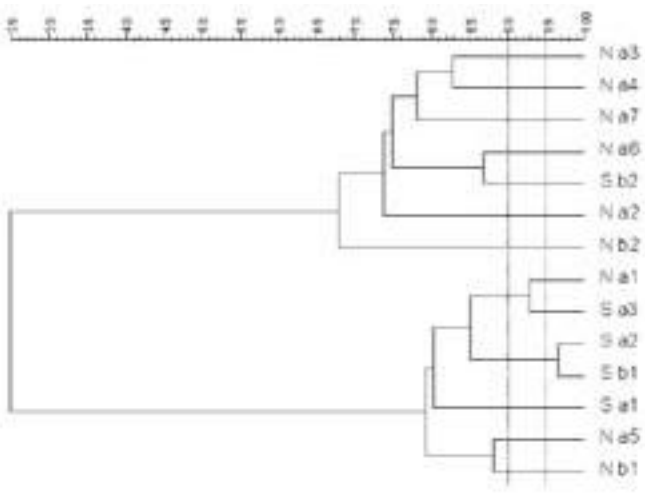

Figure 2. Cluster analysis of *Penicillium glabrum* genomic similarity using DNA fingerprint data (two single primers were used: the sequence of core zone of the phage M13 and $(GTGC)_4$)).

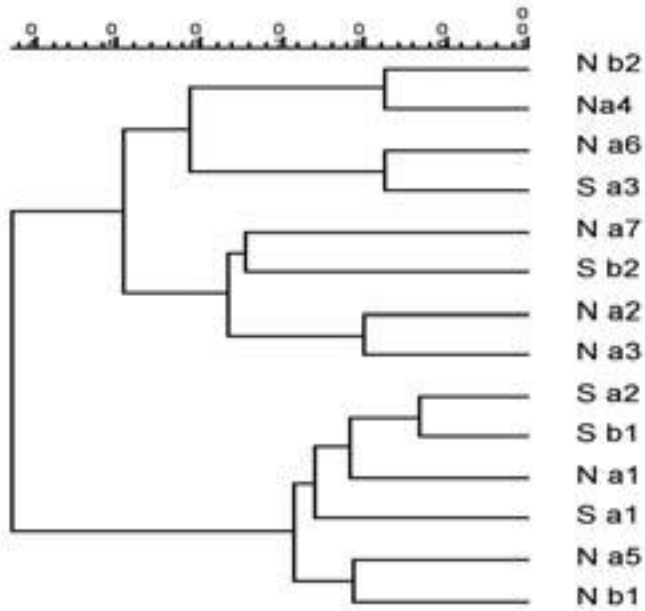

Figure 3. Cluster analysis of *Penicillium glabrum* genomic similarity using DNA fingerprint data (four single primers were used: the sequence of core zone of the phage M13, $(GTGC)_4$), $(GA)_8YG$ and $HVH(GTG)_5$).

The lower discriminatory dendrogram (Figure 2) show that *P. glabrum* isolates belonging to the same unit tended to be clustered together at least from the northern unit. In the second year there is an evident decrease in *P. glabrum* diversity in both units (Table 1), yet the genomic similarity of the South unit isolates tended to be apparently higher for the same unit at consecutive years than for different units (Figure 2). The dendrogram show a well defined North cluster, grouping six isolates from the North (total of nine). Only one South unit isolate (S b2) appear in the North unit cluster (Figure 2). In addition, four isolates from the South unit (total of five) grouped with three isolates from the North unit (N a1, N a5 and N b1) suggesting that the South cluster has a rather weak identity. This seems to suggest that cork endogenous species are, at least to certain extent, involved in the colonisation process. Only two *P. glabrum* isolated from the South unit (S a2 and S b1) and two isolated from the North unit (N a5 and N b1) in different samplings sets, show similarity near 95% and 90%, respectively. Moreover, only S a3 and N a1 isolates, originated from different units, show more than 90-95% similarity, possibly indicating a shared geographical origin. On the other hand, when increasing the discriminative power of the fingerprint analysis, the similarity level between the isolates decreases (Figure 3). The North unit cluster contains exactly the same six isolates previously clustered together. In this dendrogram the other cluster assembles the same number of isolates from each unit, losing a regional identity. In both analysis S a2 and S b1 and N a5 and N b1 clustered alone in the same branch. S a3 and N a1 reported, relative to the previous analysis, a much lower level of similarity weakening the hypothesis of a share origin.

Discussion

The preliminary analysis of the genomic similarity of the fungal species found to colonise cork, indicated that the transference of cork from the South to the North had not resulted in the same fungal species profiles (Table 1). This was further indicated by the low genomic similarity among the *P. glabrum* isolates (Figures 2 and 3). Additionally, *P. glabrum* isolates belonging to the same unit show a tendency to cluster together, suggesting that the genetic structure of cork colonising *P. glabrum* population is, to a certain degree, correlated to the population spatial structure.

These findings suggest that cork colonisation is rapidly dominated by the resident *P. glabrum* species in the manufacturing space, rather than through an active germination of viable fungi inside the cork structure. However, this is contradicted by possible horizontal transference of at least one *P. glabrum* Sb 2 strain (Figures 2 and 3) indicating that cork endogenous species also influence, to a certain extent, the resident population. The identification of the geographic origin of the colonising fungal species could help in controlling during the maturation process.

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