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Multilocus sequence identification of *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers

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Abstract

Despite several studies reporting *Penicillium* as one of the most frequent fungal genera in cork planks, the isolates were rarely identified to species level. We conducted a detailed study to identify *Penicillium* species from the field to the factory environment prior to and after boiling the cork planks. A total of 84 samples were analyzed. Of the 486 *Penicillium* isolates phenotypically identified, 32 representative or unusual strains were selected for identification by multilocus DNA sequence type. Cork proved to be a rich source of *Penicillium* biodiversity. A total of 30 taxa were recognized from cork including rarely seen species and 6 phylogenetically unique groups. Spores of some species lodged deep in cork can survive the boiling process. *P. glabrum*, *P. glandicola* and *P. toxicarium*, species with high CFU numbers in the field, are still frequently present in cork after boiling. Other species are killed by the boiling treatment and replaced by *Penicillium* species originating from the factory environment. Species known to contribute to cork taint were isolated at all stages. Good manufacturing practices are necessary at all stages in the preparation of cork planks to minimize the load of *Penicillium* species that produce cork taint.

Keywords: *Penicillium*; Mycology; Industrial microbiology; Cork; Biodiversity; Taxonomy

1. Introduction

Cork is produced from the bark of *Quercus suber* L. (cork oak tree). Cork oak grows mainly in western Mediterranean countries, with Portugal being the world leader in cork production, with more than 720 thousand hectares of cork forest. Cork trees can live as long as 350 years and the bark is harvested as thick 'planks' every 9–12 years during the productive tree life. Because of its cellular structure and suberization, cork is light, compressible, elastic, impermeable both to liquid and gases, resistant to wear and tear, and has excellent thermal and acoustic insulating properties, among other highly valued characteristics. This natural product is used for various purposes, but its most traditional and valuable industrial application is that of wine bottle stoppers [6,27].

The microbial diversity associated with cork is highly relevant to food quality and safety. Filamentous fungi in cork can cause moldy off-odors in 0.5–2% of cork stoppers, a defect in bottled wine known as cork taint, mostly due to 2,4,6-trichloroanisole (TCA) [12]. *Penicillium* is reported to be the most frequent fungal genus isolated from cork at all stages [23,24]. Nevertheless, reports with species identification are rare; moreover, identification is mainly based on phenotypic characteristics. *Penicillium* is a complex and species-rich genus with more than 225 species conforming

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to certain morphological criteria [18,19]. Identification based on phenotype is useful for species with easily recognized distinctions from other species, but identification of closely related species requires taxonomic expertise and is ultimately subjective. Species identification is important because distinct TCA levels [2], volatile organic compounds [8] and secondary metabolites [4] are produced according to the species. The lack of well-established species identification methods is a major drawback for microbial studies of cork, as the changes in *Penicillium* species composition during the stopper manufacturing process are not known.

A single locus phylogenetic study of *Penicillium* [14] using DNA sequences from the ITS and ca. 650 nucleotides of the large subunit rDNA (ID region) was performed using the type isolates of most *Penicillium* species from the subgenera *Aspergilloides*, *Furcatum* and *Penicillium* along with *Eupenicillium* species. This provides a reference database to tentatively assign new isolates to known species using genetic similarity. By using multilocus DNA sequences and the genealogical concordance theory, the boundaries of genetic isolation between species can be found [31], and even closely related taxa whose disposition by phenotype is unclear can be relegated to synonymy or distinct species status.

We detected and identified *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers, so as to evaluate changes in mycobiotal composition through processing steps, to identify probable environmental sources of the species and to assess any effect these species might have on the quality and safety of wine.

2. Materials and methods

2.1. Bark processing and sampling

Cork planks (18) were taken during the summer of 2005 from trees grown in southern Portugal at two distinct sites, A (12 planks) and B (6 planks), and analyzed for changes in their mycobiota at 4 distinct stages. Cork planks were marked, stacked so as not to contact the soil and left to stabilize in the field for some weeks (stage 1; field stabilization of planks). Planks of low quality were chosen to evaluate microbial changes during processing. Later, planks were transported to cork preparation units at Santa Maria de Lamas or the CTCOR, where they finished stabilizing (stage 2; factory stabilization of planks). After a minimum of 6 months of stabilization, planks were boiled in water for 1 h. Boiled planks from sites A and B were stacked (stage 3; planks up to 3 days post-boiling) and flattened in separate storerooms for 2–4 weeks (stage 4; prepared cork planks). Natural cork stoppers were extracted from best quality matured planks and agglomerate stoppers were produced from the remainder, according to the process described in http://www.celiege.com.

2.2. Isolation and morphological identification of fungi

A total of 84 cork pieces were analyzed. Cork pieces of 200–300 g were ground to 2–3 mm granules. At stage 1, the outer bark was ground separately from the inner bark. One g of granulated cork per sample was aseptically placed in 100 ml Erlenmeyer flasks with 50 ml of sterile 0.1% peptone solution, and agitated with an orbital incubator at 25 °C and 220 rpm for 2 h [2]. Aliquots of 0.1 ml were diluted and spread in dichloran Rose Bengal with chloramphenicol agar (DRBC, Oxoid), incubated for one week at 25 °C. In addition, some cork granules were directly placed on malt extract agar (MEA) for detecting fungi with rapid growth. Representative isolates grown in the plates were transferred to MEA for preliminary identification and examined phenotypically using the methodology of Pitt [18]. Representative isolates of the *Penicillium* species found were preserved locally on MEA slants at −4 °C and in two culture collections, MUM and NRRL (Table 1).

2.3. SEM observation

Granules (50–100) were screened under the stereomicroscope from samples of all stages for conspicuous *Penicillium* reproductive structures. Selected cork granules were coated with gold and examined directly by SEM.

2.4. DNA sequence analysis

A subset of strains representative of the species found or those of problematic identification was selected for molecular analysis. The complete list of strains sequenced and their GenBank accession numbers are shown in Table 1.

DNA was isolated from mycelium using a variation of the method of Peterson et al. [17]. Instead of vortexing mycelium and glass beads in 15 ml disposable tubes for 45–60 s, the process was scaled down so that vortexing could be accomplished in a 2 ml screw-cap microcentrifuge tube.

DNA amplification details for the ITS and lsu-rDNA (ID), β-tubulin (BT2), calmodulin (CF) and RNA polymerase beta (RPB2) loci are provided in Table 2.

DNA sequence similarity searches were conducted on the GenBank BLAST server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). A BLAST server was also implemented locally using a database of unpublished *Penicillium* sequences from type cultures [Peterson unpublished]. If an unknown sequence was a perfect match for the sequence from a type culture and the phenotype matched that of the type, the unknown sequence was identified as that species. For isolates for which the sequence differed from types by more than 2–3 nucleotides, sequences were aligned with type sequences from the database using ClustalW [32], fine-aligned using a text editor and subjected to parsimony analysis. Species most closely related to the unknown sequence were selected on the basis of ID locus parsimony analysis and were sequenced at the BT2, CF and RPB2 loci for concordance analysis. PAUP* [30] was used for weighted and unweighted parsimony analysis, and MrBayes 3.1 [21] was used for Bayesian analysis. Analytical criteria for concordance analyses are detailed by Dettman and associates [5].

Table 1
List of *Aspergillus*, *Geosmithia* and *Penicillium* strains isolated in this study from cork planks identified by multilocus DNA sequence typing and phenotypic methods (total number of strains: 32)

Species	Strain no.		Source[a]	ID difference from type strain/length	GenBank accession no.		
	NRRL[b]	MUM[c]			ID region	Calmodulin	β-tubulin
Aspergillus bridgeri	35624		Cork bark	3 gaps/1179	EF200084	EF198578	EF198550
Penicillium species1 (near *E. tularense*)	35620		Cork bark	23/1150	EF200080	EF198574	EF198546
Penicillium species 2	35616	06.55	Cork bark	93/1115	EF200076	EF198570	EF198543
	35677	06.57	Cork bark	93/1115	EF200093	EF198588	EF198560
	35678	06.143	Cork bark	93/1115	EF200094	EF198589	EF198561
P. angulare	35630		Cork bark	1/1141	EF200087	EF198582	EF198554
	35633		Cork bark	1/1141	EF200088	EF198583	EF198555
	35683		Boiled cork	1/1141	EF200096	EF198591	EF198563
P. chrysogenum	35635		Cork bark	0/1132	EF200090	EF198585	EF198557
	35688		Dried boiled cork	1/1132	EF200101	EF198591	EF198563
P. citreonigrum	35629		Cork bark	0/1128	EF198645	EF198581	EF198553
P. commune	35686		Boiled cork	1/1143	EF200099	EF198584	EF198566
P. decaturense	35636	06.96	Cork bark	1/1134	EF200091	EF198558	EF198586
Penicillium species 3 (near *P. diversum*)	35613	06.04	Cork bark	24/1157	EF200075	EF198569	EF198542
	35623		Cork bark	13/1135	EF200083	EF198577	EF198549
P. fellutanum	35622	06.17	Cork bark	5/1128	EF200082	EF198576	EF198548
	35619		Cork bark	5/1128	EF200079	EF198573	EF198545
Penicillium species 4 (near *P. glabrum*)	35682		Boiled cork	14/897	EF200095	EF198590	EF198562
P. glabrum	35621	06.07	Cork bark	0/1125	EF200081	EF198575	EF198547
	35626	06.64	Cork bark	1/1089	EF200086	EF198580	EF198552
	35684		Boiled cork	0/1135	EF200097	EF198592	EF198564
P. glandicola	35685		Boiled cork	1/1141	EF200098	EF198593	EF198565
P. janthinellum	35634		Cork bark	0/1135	EF200089	EF198584	EF198556
P. novae-zeelandiae	35618		Cork bark		EF200078	EF198572	EF198544
P. olsonii	35687		Boiled cork	0/1140	EF200100	EF198595	EF198567
Penicillium species 5 (near *P. purpurogenum*)	35637	06.56	Cork bark	38/1142	EF200092	EF198587	EF198559
P. steckii	35625	06.78	Cork bark	3/1142	EF200085	EF198579	EF198551
"*P. meleagrinum var. viridiflavum*"	35627	06.11	Cork bark	0/1141	EF198531	EF198518	EF198510
	35638	06.61	Cork bark	1/1142	EF198528	EF198519	EF198511
P. toxicarium	35628	06.16	Cork bark	2/1128	EF198662	EF198643	EF198615
	35614	06.03	Cork bark	1/1128	EF198661	EF198642	EF198619
Penicillium species 6 (near *P. variabile*)	35617	06.109	Cork bark	8/1147	EF200077	EF198571	

[a] Cork bark = stage 1; boiled cork = stage 3; dried boiled cork = stage 4.
[b] NRRL: Agricultural Research Service Culture Collection, Peoria, IL, USA.
[c] MUM: Micoteca da Universidade do Minho, Braga, Portugal.

3. Results

3.1. Prevalence of different fungal species in cork

Penicillium was the genus most frequently present in cork bark at all stages. The number of *Penicillium* CFUs in cork samples varied from less than 5×10^2 CFUs to more than 10^7 CFUs per g of cork (Table 3). The CFU numbers of *Penicillium* from inner bark were significantly higher than in the outer bark ($P < 0.01$, using the Kruskal–Wallis test). The highest counts were detected in samples from site A in the field (stage 1) in the inner cork bark and from site A after boiling (stage 3). Occasionally, other fungi were isolated, such as *Aspergillus* (*A. bridgieri*, *A. fumigatus* and *A. niger*), *Chrysonilia sitophila*, *Cladosporium* spp., *Mucor* spp., *Paecilomyces* spp., *Trichoderma* spp., unidentified dematiaceous fungi and coelomycetes.

Using SEM, fungal hyphae and reproductive structures typical of the genus *Penicillium* were detected in samples from stages 1 (site A, inner bark), 3 and 4, visibly attached to the substrate (Fig. 1a). Reproductive structures were detected under SEM (Figs. 1–4), as well as a high number of spores (Fig. 5). Structures of other fungal genera were also recorded in the different stages, possibly of dematiaceous fungi and *Chrysonilia*.

3.2. Isolation and identification of Penicillium species

A total of 486 *Penicillium* strains were isolated and identified phenotypically using the methodology of Pitt [18]. Among these, 32 strains were analyzed by DNA sequence similarity of the ID locus or by multilocus DNA sequence concordance analysis (Table 2). When identification based on phenotype was dubious due to atypical morphology or intermediate forms between closely related species, it was corrected using DNA sequence information. A total of 30 taxa (Table 4) were identified.

Table 2
Primers and regions amplified for multilocus concordance analysis

Locus	Primer	Sequence	Annealing temperature (°C)	Amplicon length (nucleotides)	Reference
BT2	BT2a BT2b	GTT AAC CAA ATC GGT GCT GCT TTC ACC CTC AGT GTA ACC CTT GGC	58	ca 500	[7]
CF	CF1 CF4	AGG CGG AYT CTY TGA CYG A TTT YTG CAT CAT RAG YTG GAC	54	ca 650	[17]
ID	ITS-5 D2R	GGA AGT AAA AGT CGT AAC AAG G GGT CCG TGT TTC AAG ACG	52	ca 1150	[17]
RPB2	5F 7CR	GAY GAY MGW GAT CAY TTY GG CCC ATR GCT TGY TTR CCC AT	57	ca 1050	[9]

Using the phylogenetic species concept [31], each of the *Penicillium* taxa indicated by numbers 1–6 was distinct from previously known species. Apart from taxa in the *Biverticillium* section, all other taxa showed evident differences in cultural and morphological characters from the most closely related species and were unsatisfactorily identified using the manuals. *Penicillium* species 4 is closely related to *P. glabrum* but is readily distinguishable by its slower growth rate and echinulate spores. Two undescribed taxa of very slowly growing fungi were isolated in this study, i.e. *Penicillium* species 1 (near *Eupenicillium tularense*), with no detectable sexual state and characteristic penicilli with heavily inflated metulae, and *Penicillium* species 2 with yellow-brown round spores, not closely related to any known *Penicillium* species. Detailed descriptions of the taxa and their phylogenetic placement will be the subject of a separate publication.

Concerning the *Biverticillum* section, *Penicillium* species 3 (NRRL 35613, NRRL 35623) closely resembled *P. diversum* and differed from that species in ITS1 and ITS2 DNA sequence. A thorough study with additional isolates and loci will be required to determine its identification and to verify that it is undescribed. *Penicillium* species 5 resembled *P. purpurogenum*, but differed markedly from the type isolate in its ITS and lsu rDNA sequence. *Penicillium* species 6 (NRRL 35617) resembled *P. variable* but differed from that species at nearly 1% of ID locus nucleotide positions and may represent an undescribed species. While certainty does not yet exist concerning the taxonomic status of these isolates, they differ genetically from the type strains of the species that they resemble; they are from a unique habitat and are noteworthy for both reasons.

Penicillium toxicarium has been regarded as a synonym for *P. citreonigrum* [18,19] but is considered here to be a distinct species. Separate phylograms based on each of the four loci studied (not shown) are congruent in the central branch separating the two species, and according to established criteria [5,31], these are distinct species. Sequences are deposited as GenBank numbers EF198486–EF198502; EF198605–EF198624; EF198625–EF198644; EF198645–EF198663 and AF033456. "*Penicillium meleagrinum* var. *viridiflavum*" [1] was described by Abe, but lacked a Latin description making the description invalid. It has been relegated to synonymy with *P. janthinellum* [19], but we found that it is genetically close to the *P. sumatrense* type strain. Congruence of the phylograms from each of four loci (not shown) established this to be a separate species that will be validated elsewhere. Sequences for the analysis have been deposited as GenBank numbers EF198503–EF198513; EF198514–EF198522; EF198523–EF198531; EF198532–EF198541 and AF033424.

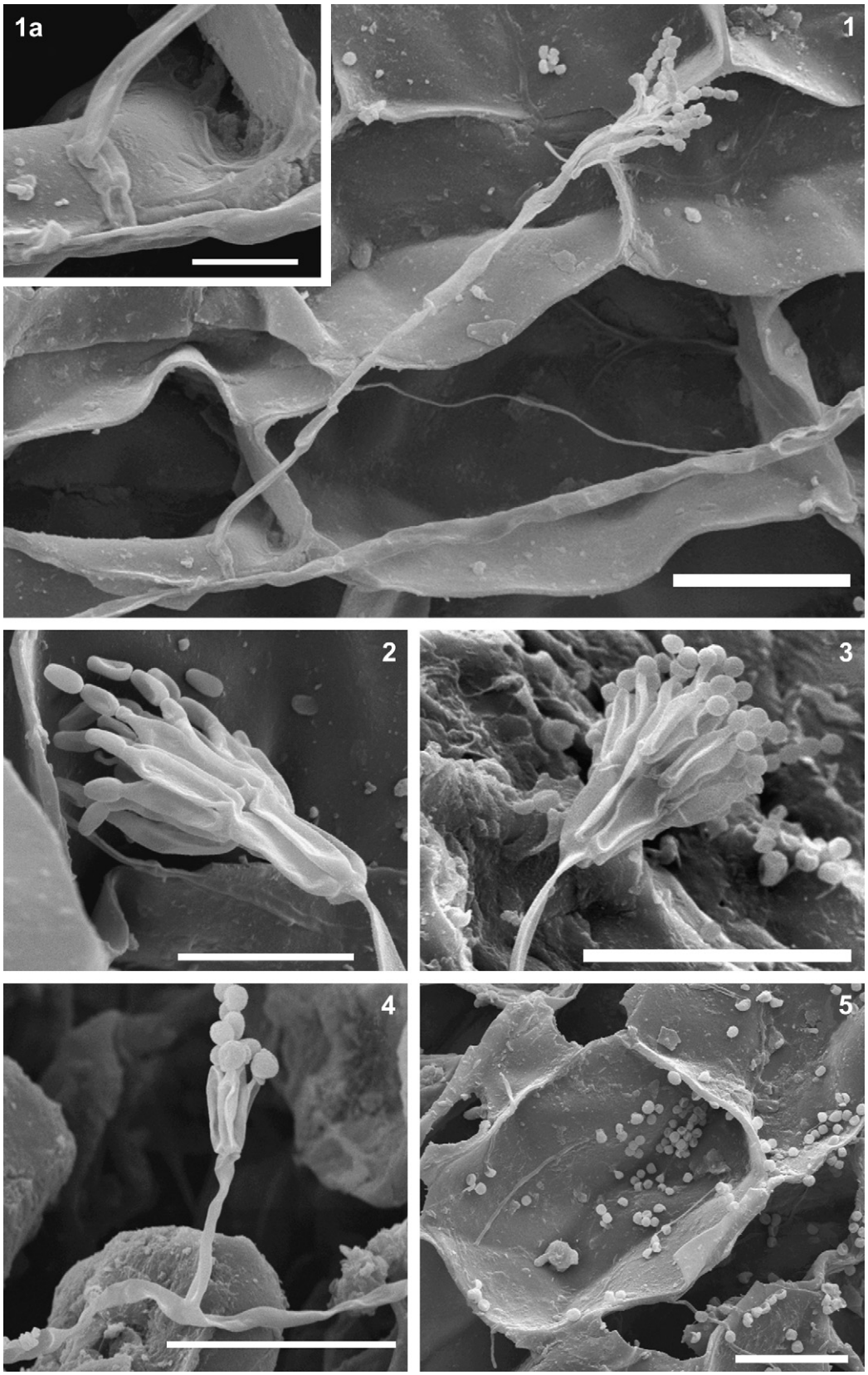
Table 3
Penicillium CFU number in cork samples at different stages of cork manufacturing

Stage[a]	Site[b]	Frequency (no. of samples) with *Penicillium* CFUs/g in the range of:					Total no. samples
		$>10^7$	10^6–10^7	10^5–10^4	$\leq 10^3$	$\leq 5 \times 10^2$	
1	A In	6	1	5	0	0	12
	A Out	0	0	12	0	0	12
	B	0	0	6	0	0	6
2	A	0	0	11	1	0	12
	B	0	0	3	3	0	6
3	A	8	4	0	0	0	12
	B	0	0	0	4	2	6
4	A	1	5	6	0	0	12
	B	0	0	5	1	0	6

[a] Stage 1: raw cork in the field; stage 2: raw cork in the factory; stage 3: after boiling; stage 4: after drying.
[b] In: inner bark layer; out: outer bark layer.

3.3. Changes in the mycobiota during cork plank processing

Penicillium diversity and species frequency found in the cork bark varied at the 4 stages (Table 4). CFU number and *Penicillium* species richness was highest in the field, but was greatly reduced at the end of stabilization in the unit (stage 2). The CFU number of *Penicillium* increased in site A samples following boiling (stage 3), reaching similar levels as found in stage 1. On the other hand, the CFU number of *Penicillium* decreased in site B samples to the lowest point, with 100% of samples exhibiting less than 10^3 CFU per g of cork and 33% of samples having no detectable fungal colonies at all. Nevertheless, the CFU number of *Penicillium* at the end of the drying stage (stage 4) was similar in samples from both sites.



Figs. 1–5. SEM images of cork granules with visible fungal growth. 1, 2, 3: distinct biverticillate penicilli in the inner cork bark in the field (stage 1); 1a: detail showing attachment of penicilli to cork cells; 4: monoverticillate penicilli in boiled cork (stage 3); 5: spores deposited in cork cells (scale bars: 1a = 5 μm; 1 = 50 μm; 2 = 10 μm; 3–5 = 20 μm).

Table 4
Frequency (number of positive samples) of the *Penicillium* species identified from the 486 isolates from cork at each manufacturing process stage

Species[a]	Frequency as no. of positive samples								
	Site A					Site B			
	Stage 1[b]		Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4
	Inner[c]	Outer							
Near *P. diversum*	**11**[c]	**4**	**4**	–	2	**6**	**2**	1	1
P. glabrum	**10**	**11**	**7**	**12**	**12**	**5**	**4**	1	**3**
near *P. variabile*	**10**	**4**	2	2	1	–	–	–	–
"*P. meleagrinum var. viridiflavum*"	**9**	**3**	**3**	**3**	–	–	–	–	–
P. toxicarium	**9**	**4**	**6**	**5**	–	**2**	**5**	–	**3**
near *P. purpurogenum*	**5**	1	2	–	2	–	–	–	–
P. glandicola	**4**	–	–	**9**	**12**	–	–	–	–
P. citreonigrum	**3**	1	–	–	–	**2**	–	–	1
Geosmithia sp.	2	1	–	–	–	–	–	–	–
P. citrinum	2	2	2	2	–	–	–	–	–
P. fellutanum	2	–	–	–	–	–	–	–	–
P. steckii	2	–	1	–	–	–	–	–	**6**
P. brevicompactum	1	–	1	**4**	**7**	–	–	–	1
P. novae-zeelandiae	1	–	–	–	–	–	–	–	–
P. chrysogenum	–	1	–	–	–	–	–	–	**5**
P. angulare	–	1	1	1	–	–	–	–	–
near *E. tularense*	–	1	–	–	–	–	–	–	–
P. commune	–	–	–	**3**	**4**	–	–	–	–
near *P. glabrum*	–	–	–	**3**	–	–	–	–	–
P. corylophilum	1	–	–	–	–	–	–	–	–
P. decaturense	–	1	–	–	–	–	–	–	–
P. janthinellum	–	1	–	–	–	–	–	–	–
P. minioluteum	–	1	–	–	–	–	–	–	–
P. rugulosum	–	1	–	–	–	–	–	–	–
P. funiculosum	–	–	1	–	–	–	1	–	–
P. aurantiogriseum	–	–	–	–	1	–	–	–	–
P. olsonii	–	–	–	–	–	–	–	1	–
P. spinulosum	–	–	–	–	–	1	–	–	–
P. waksmannii	–	–	–	–	–	–	–	1	–
P. simplicissimum	–	–	–	1	–	–	–	–	–
Total no. of taxa	15	16	11	11	8	5	4	4	7
Total no. of samples	12	12	12	12	12	6	6	6	6

[a] Species detected in ≥25% of samples from each manufacturing stage and site are indicated in bold.
[b] Stage 1: raw cork in the field; stage 2: raw cork in the factory; stage 3: after boiling; stage 4: after drying.
[c] Inner: inner bark layer; outer: outer bark layer.

The mycobiotal composition of boiled cork was distinct from that of the previous stages. Few of the field species remained relevant components of the cork mycobiota. *P. glabrum* and *P. glandicola* were the most frequent field species isolated in site A planks, while *P. glabrum* and *P. toxicarium* were the most frequent field species in site B planks. On the other hand, species not particularly associated with cork in the field increased their population and also dominated the mycobiota at the end of maturation, namely *P. brevicompactum* and *P. commune* in site A and *P. chrysogenum* and *P. steckii* in site B.

4. Discussion

4.1. Penicillium *species concept and identification*

Single locus phylogenetic studies (e.g. refs. [14,22]) have investigated relationships in *Penicillium* and are sometimes used to reject the hypotheses of synonymy or inclusion of species in genera or subgenera, but by themselves they are not informative concerning species limits. A number of methods exist for the recognition of species [28]. Samson and Frisvad [22] addressed this issue by applying a polyphasic approach to defining species limits, using primary and secondary metabolism and microscopic morphology to supplement single locus genetic data. We used the phylogenetic species concept with species recognition by genealogical concordance, as it provides a conservative estimate of genetic isolation between species [3,5], in which species boundaries reside at the boundary between reticulate and divergent genealogy. It is objective and enables evaluating speciation between closely related taxa. We can apply the phylogenetic species concept to asexual fungi such as *Penicillium* due to their capacity for genetic recombination by parasexual mechanisms. Organisms that are genetically isolated will accumulate differences in morphological, ecological, extrolite and/or physiological traits by genetic

drift and selection. When evident differences from closely related species accumulate, formal validation of the delimited taxa as a species can be justified to create a classification system in closer agreement with objective species recognition.

Seifert et al. [25] demonstrated the utility and limitations of using cytochrome oxidase I sequences for bar code identification of species in the subgenus *Penicillium*. This gene is quite useful, except in the case of species complexes [25]. The most widely populated database of sequences in *Penicillium* is for the ID locus. The ID locus is generally identical within species and variable between species, but a few species share the same ID genotype [16]. The BT2, CF and RPB2 loci are very discriminatory, but at this time the databases do not fully cover *Penicillium*. Also, the BT2 locus does not distinguish between all species in the subgenus *Penicillium*. Using a combination of loci, we have been able to identify most species directly with the ID locus and have used multilocus data to resolve the taxonomic status of two other species. Each of the loci used here can be used for identification employing the genetic similarity approach embodied in sequence-based identification methods [11,13,25]. A fundamental limitation of DNA-based identifications is the species concept used to name the species. Currently, in *Penicillium*, the majority of species have been defined through phenotype [18], and phenotypic species have not been reexamined using either DNA sequence concordance or polyphasic approaches to verify the accuracy of the species concept. At this time, close reexamination of existing species and synonyms using multilocus DNA sequence data and polyphasic information will be very useful for enabling future DNA-based identification methods.

Using multilocus sequence data and the congruence theory (exclusivity criterion, 4), we recognized *P. toxicarium* as a valid species closely related to *P. citreonigrum*; we recognized "*P. meleagrinum* var. *viridiflavum*" as a genetically valid species closely related to *P. sumatrense*; and we found six taxa genetically isolated from related species. Moreover, the application of multilocus DNA sequence data has greatly simplified correct identification of phenotypically abnormal isolates. At least 20% of the biodiversity in cork might have gone unrecognized without multilocus sequence analysis.

4.2. Penicillium *diversity in cork*

Cork has proven to be an interesting source of *Penicillium* biodiversity. Among the 30 taxa isolated in our study, 16 taxa are new occurrences for this substrate compared to the species previously described [24,29], or else they represent previously unrecognized phylogenetic groups. *P. angulare* and *P. decaturense* are two recently described species that were previously isolated only from the surface of wood-decay mushrooms collected in North America [15].

Penicillium species can grow and reproduce in the inner layers of cork (Figs. 1–5). This was also found to be true in stoppers [20]. Inner cork layers can retain high humidity levels and at the same time protect hypha and spores from desiccation, solar radiation and major temperature variation. Cork can act as a protective environment for fungi able to use the nutrients available at the surface of cork cells, probably in the form of water-soluble compounds. However, it is also likely that some species found in cork might grow as endophytes [34]. Cork bark may be a favorable environment for *Penicillium* species reported to grow either endophytically or saprophytically, such as *P. brevicompactum*, *P. citrinum*, *P. chrysogenum*, *P. glabrum*, *P. janthinellum*, *P. olsonii*, *P. purpurogenum*, *P. rugulosum*, *P. simplicissimum* and *P. steckii* [33].

4.3. *Changes in the* Penicillium *species with cork plank processing*

The highest *Penicillium* diversity was found in the field. At that stage, the cork mycobiota is characterized by both ubiquitous species (e.g. *P. glabrum*) and uncommon species, namely *P.* species 1 near *P. diversum*, *P. citreonigrum* and *P. toxicarium*. However, we observed a decrease in *Penicillium* in the planks after stabilization. The fungi seemed latent in the factory environment, while they were actively reproducing in the field, judging from the high spore counts detected in field samples. Other authors reported opposite observations during the stabilization stages [23]. Planks stabilizing in factories were subjected to environmental factors, and it is likely that exposure to distinct factors, especially humidity levels, could be the cause of the discrepancies and might be determinant for *Penicillium* numbers at that stage.

Most of the species with high CFUs in cork were able to survive the boiling process (Table 4). Boiling is effective in eliminating many fungal propagules, but some spores lodged deep in cork layers can still survive. The *Penicillium* species found were not heat-resistant, but probably were sheltered by the surrounding cork layers from the boiling water. It must be highlighted that cork is an impermeable and excellent insulating material, and the planks are several centimeters thick. Conditions for mold growth are more favorable after boiling, as the water activity of the cork is high, heat treatment may make nutrients more readily available to the fungi, and the planks are slowly drying at room temperature.

The mycobiota at the end of the maturation stage is a mixture of species found at the field stage and of ubiquitous saprophytes. Our findings are in agreement with those of other authors who previously proposed the tree and field environment as a primary source of contamination [24], and with authors who suggested that the factory environment surrounding cork in the mold rooms is a determinate factor for cork recolonization [12]. Ubiquitous saphrophytes apparently originating from the factory environment, such as *P. brevicompactum*, *P. chrysogenum*, *P. commune* and *P. steckii*, are strong competitors that lead to the exclusion of most of the field species native to cork after boiling, and can still be isolated from cork stoppers [20,24]. It is expected that this component of the *Penicillium* mycobiota varies with the species present in mold rooms of the cork factories.

P. glabrum, *P. glandicola* and *P. toxicarium* were present in all stages of the manufacturing process. *P. glabrum* is well known to cork workers, especially during the maturation stage.

This mold growth is sufficient to make the cork planks green from conidia, and some workers suffer suberosis, an allergic response to massive doses of *P. glabrum* spores [10]. *P. glandicola* also was reported in boiled cork in other surveys [29] and was found to be widespread, together with *P. glabrum* inside the industry compartments [12]. In our study, the origin of *P. glandicola* in boiled cork seemed to be correlated with initial field contamination of the planks, as it was common in site A but was never isolated in site B samples.

P. citreonigrum and *P. toxicarium* are relatively slowly growing species capable of producing several bioactive metabolites [4]. Nevertheless, no *Penicillium* species isolated in this study is a known producer of ochratoxin A (OA), the only mycotoxin for which a maximum legal limit in wine is regulated in the EU (2 μg/kg). Vega and co-workers [33] reported weak production of OA at ppt levels by a *P. brevicompactum* and a *P. olsonii* strain, but the identity of the metabolite was not confirmed. We screened all *Aspergillus* and *Penicillium* strains for OA production (unpublished data) in yeast extract-sucrose medium by HPLC-FL [26], and no OA was detected, as would be expected for the *Penicillium* species identified. It is noteworthy that *Penicillium* species 2 strains gave false-positive results by producing a metabolite with a similar retention time to OA. Nevertheless, after sample cleaning by imunoaffinity columns, the putative OA peak was not detected.

Numerous authors have pointed out *Penicillium* species capable of producing off odors that negatively affect cork quality, namely *P. citrinum*, *P. glabrum*, *P. glandicola* and *P. purpurogenum* [24], but it would be useful to reidentify these odor-producing isolates using multilocus DNA sequence data. Alvarez-Rodriguez and co-workers [2] isolated and identified four *Penicillium* strains using phenotypic methods, and ITS-RFLP and reported that *P. citreonigrum* and *P. purpurogenum* could moderately produce TCA when the chemical precursor 2,4,6-trichlorophenol (TCP) was given, bioconverting 11–13% of TCP into TCA, while *P. chrysogenum* was only able to transform 8% of TCP when grown directly in cork. However, Prak et al. [20] reported *P. chrysogenum* to be one of the fungi capable of the best yield of TCP conversion (20%), but the strain identity was not confirmed. As potential TCA-producing fungi were isolated from cork planks at all stages analyzed, our data emphasize the need to apply good manufacturing practices at all preparation stages following cork removal so as to minimize off-odor production.

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