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Chestnut rots: disease incidence and molecular identification of causal agents

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Abstract

Chestnut fruits are popular fruits commercialized as fresh or processed ready to be used as products. Because of its high moisture and suitable nutrient content, the chestnut kernel has the potential to support the growth of a wide spectrum of spoilage fungi. The major post-harvest problems associated with stored chestnut fruits are rots, which result in major losses in fruit quality. Although the empirical knowledge determines a high level of rot in Portuguese chestnuts, there are no scientific studies on the incidence and severity of the problem, and the causal agents have never been identified. This knowledge is of the utmost importance and urgency for industry and retailers to correctly address the issue, in an effort to reduce yield loss due to rot.

The aims of this work were: i) to determine the incidence, abundance and diversity of rots in three chestnut varieties of Trás-os-Montes – Judia, Longal and Martaínha – at different post-harvest stages of storage and processing, and ii) to identify the main potential agents of rots.

For this purpose chestnuts were internally and externally inspected for presence of damages, infestation and infection.

Samples from variety Martaínha were identified as the most resistant to fungal growth, while samples from variety Longal were less resistant to fungal growth and infestation. A high diversity of species has been molecularly identified by sequencing the ITS region: 37 different species belonging to 16 genera. The dominant fungal species found with high frequency were *Mucor racemosus* f. *sphaerosporus* (24.2% of frequency), *Penicillium brevicompactum* (16.7%) and *Penicillium thomii* (causal agents of green rot; 13.9%), *Ciboria batschiana* (the causal agent of black rot; 10.6%) and *Botrytis cinerea* (the causal agent of grey rot10.6%). *Gnomoniopsis smithogilvyi*, the causal agent of brown rot, was also identified with a frequency of 6.4%.

The results show that the causal agents of various chestnut rots already identified in other countries are also present in Portuguese nuts. Studies must follow with the aim of developing control measures against the identified rot-causing fungi.

Keywords: black rot; brown rot; storage fungi; *Gnomoniopsis smithogilvyi; Ciboria batschiana; Penicillium* sp.

Resumo

A castanha é um fruto popular comercializado e consumido tanto em fresco como processado. Devido à sua elevada humidade e teor de nutrientes, a castanha tem o potencial de permitir o crescimento de um amplo espectro de fungos de deterioração. Os principais problemas pós-colheita associados à castanha armazenada são as podridões, que resultam em grandes perdas na qualidade dos frutos. Embora o conhecimento empírico evidencie um alto nível de podridão nas castanhas portuguesas, não existem estudos científicos sobre a incidência e gravidade do problema, e os agentes causais nunca foram identificados. Esse conhecimento é da maior importância e urgência para a indústria e os comerciantes resolverem o problema adequadamente, no sentido de reduzirem as perdas de rendimento devido à podridão.

Os objetivos deste trabalho foram: i) determinar a incidência, abundância e diversidade de podridões em três variedades de castanha de Trás-os-Montes - Judia, Longal e Martaínha - em diferentes estágios pós-colheita de armazenamento e processamento; e ii) identificar os principais agentes causais das podridões.

Para esse fim, as castanhas foram inspecionadas interna e externamente quanto à presença de danos, infestações e infeções. Amostras da variedade Martaínha foram identificadas como as mais resistentes ao crescimento de fungos, enquanto amostras da variedade Longal foram menos resistentes ao crescimento e infestação de fungos.

Uma alta diversidade de espécies de fungos foi identificada molecularmente através da região ITS: 37 espécies diferentes pertencentes a 16 géneros. As espécies dominantes encontradas com alta frequência foram *Mucor racemosus* f. *sphaerosporus* (24.2% de frequência), *Penicillium brevicompactum* (16.7%) e *Penicillium thomii* (13.9%) (agentes causais de podridão verde), *Ciboria batschiana* (agente causal da podridão negra; 10.6%) e *Botrytis cinerea* (agente causal da podridão cinzenta; 10.6%). *Gnomoniopsis smithogilvyi*, agente causal da podridão castanha, também foi identificado, com frequência de 6.4%.

Estes resultados mostram que os principais agentes causais das podridões da castanha se encontram também nas castanhas portuguesas. Estão em curso estudos para desenvolvimento de métodos de controlo des fungos nas castanhas.

Palavras-chave: podridão negra; podridão castanha; fungos de conservação; *Gnomoniopsis smithogilvyi; Ciboria batschiana; Penicillium* sp.

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1. INTRODUCTION

1.1. Framework

Chestnuts (*Castanea sativa* Mill.) play a very important role in human nutrition owing to their nutrient composition and their potentially beneficial effects on health. They are recommended as part of a gluten-free diet in case of celiac disease, and reduction of coronary heart disease and cancer (Gonçalves et al., 2010). Chestnuts have a very low-fat content, because of their high content of unsaturated fatty acids and especially because they are cholesterol-free. They are rich in fiber, vitamin and starch, so they have been considered as a good source of energy. The fruits also have a high content of polyphenols, with gallic acid and ellagic acid predominating among the hydrolyzable and condensed tannins (Gonçalves et al., 2010).

In Portugal, chestnut tree has an economic, social and landscape importance. Trás-os-Montes, in the Northeast of the country, is the first Portuguese chestnuts producer region, with 80% of the national production. The chestnuts produced in this region are of high quality, and that has been recognized by the European Union with Protected Denomination of Origin "Castanha da Terra Fria" (Rodrigues, 2010).

Chestnut fruit is a seasonal product, commercialized as fresh or processed ready to use products. The high moisture and the rich nutrient content of the chestnuts represent storage problems because these conditions are supportive of infestation by insects and infection by a wide spectrum of spoilage fungi, resulting in major losses in fruit quality. These conditions make it necessary for chestnuts to be controlled at both pre- and post-harvest stages. The major post-harvest problems associated with these fruits are rots, which can be caused by several different fungal agents. Depending on the causal agent, rots can be of different types: brown, black, pink, white or green, and can develop at different stages: pre-harvest, post-harvest, and storage.

1.2. Aims of the work

The storage of chestnut represents the most important problem on an industrial scale because of the growth of a wide spoilage spectrum of fungi which conducts to the appearance of rots. As a result, chestnuts lose their fruit quality and commercial value.

In this work, we intended to:

i) Determine the incidence, abundance, and diversity and fungi and rot in three chestnut varieties of Trás-os-Montes: Longal, Judia and Martaínha;

ii) Identify the main potential agents of chestnut rot in the chestnut varieties;

iii) Identify the stages of storage and processing where rots become more significant;

The general aim of the work was to obtain information necessary for the future development of strategies for reduction of rot and associated chestnut losses in storage.

2. LITERATURE REVIEW

2.1. Chestnuts

Chestnuts are produced by a variety of species of the genus *Castanea*, family Fagaceae. There are four main economic chestnut species depending on the geographical area: *Castanea crenata* and *Castanea mollissima* are predominant in Asia and produce Asian chestnuts (Japanese and Chinese), *Castanea dentata* is found in North America and North America, and *Castanea sativa* is the European chestnut, also known as sweet chestnut. *C. sativa* is suitable for humid and cold climates and does not support long, hot, dry periods (Serrano et al., 2001).

Chestnut trees are a valuable resource for many Mediterranean mountainous areas, due to its edible fruits and the good quality timber that makes it one of the most important forest and agronomic species in the Mediterranean basin. Also, natural chestnut forests contribute to reducing soil water erosion and in preserving very complex natural ecosystems. Nowadays, there is a renewed demand for high-quality chestnut fruit, which is also important for the economy of several mountain communities. *C. sativa* was traditionally an important source of food for human populations, particularly in rural areas, and was already cultivated during Roman times (Baryshev et al., 2014).

European chestnuts are highly consumed because of their interesting nutritional characteristics. They are rich in carbohydrates (around 40%), mostly starch, and present minerals, vitamins and appreciable levels of fiber, but low amounts of protein (2−4%) and low amounts of fat (1.5−5%). They are also an interesting source of essential fatty acids (Barreira et al., 2009). Nutritionally, chestnuts differ from other nuts for their low-fat content which makes them ideally suited for low-fat diets (Gentile et al., 2010) They are a rich source of minerals, antioxidants, vitamins E and C, and phenolics (gallic and ellagic acids), which are beneficial to health (Vasconcelos et al., 2010).

2.2. Chestnuts in Portugal

In Portugal, European chestnut is mostly located in the central and northern region of Portugal, where they assume an important role for the environment and the local economy, due to the harvest of fruit, wood, and mushrooms (Bragança et al., 2009). Portugal is the fourth largest producer of chestnut in Europe and seventh in the world, with an annual

production of 24.7 thousand tons and orchard area of 35 thousand hectares (FAO, 2015). As of 2013, chestnut culture in Trás-os-Montes occupied 88% of the national area devoted to chestnut production and corresponded to 84% of the national production (INE, 2014). The region integrates three chestnut Denominations of Protected Origin (DOP) - Terra Fria, Padrela and Soutos da Lapa. It is the fruit with major significance in the Portuguese import/export balance. Roughly 70-75% of Portuguese chestnuts are intended for exports, essentially to Italy, Spain and traditional markets of Portuguese emigration (France and Brazil). There are no known statistics for processed chestnuts, but it is known that most of them are exported already processed (Rodrigues, 2010).

2.3. Production stages of chestnut

Chestnuts are typically harvested from October to November and are one of the easiest nut varieties to harvest. There are many methods to harvest chestnuts: hand-harvest, sweep, vacuum, or a combination of all, and the preferred method will depend on a number of factors, for instance, quantity, aim, productivity, orchard size and localization, and slopes in the orchard (Sieber et al., 2007).

In traditional orchards from the Portuguese Northeast, fruits are usually harvested from the ground, at first by hand into 50 kg bags, on a weekly basis. When all fruits have fallen from the trees, the collection is made mechanically with a suction machine, cleaned from soil, leaves and other dirt, sacked, and transported to the warehouse. The nuts are separated into groups by size (calibre) and quality and are then stored in warehouses, usually without controlled temperature and atmosphere until processing. For high-quality fruits, long storage is made in chambers under controlled temperature (Rodrigues, 2010).

While high calibre and high-quality chestnuts are usually directed for the fresh market, fruits with small calibre or showing any defects are diverted to an array of different products. In fact, chestnuts can assume various industrial forms, such as frozen at -40 °C, sterilized in aluminium sacks (116 °C for 30 to 35 min after vacuum sealing), tinned (with a preservative liquid), stored in vials (with a preservative liquid), or dried. These forms, which are mainly used in gastronomy, are an alternative and economically profitable way of increasing the value of chestnut products and reducing the amount of waste resulting from the industrial processing of low calibre, polyspermic and broken fruits.

2.4. Chestnut rots as major problems of chestnut production

The production of nuts may be compromised to variable extents as a consequence of abiotic stresses, pathogens, and pests, whose presence can reduce nuts yield and quality in pre-harvest and/or post-harvest conditions. Some of the most damaging threats of chestnut affect tree health by significantly reducing its vitality and by a substantial decline of the production (Lione et al., 2019). There are two main diseases, chestnut blight caused by the ascomycete fungus *Cryphonectria parasitica*, and ink disease caused by the Oomycete *Phytophthora* spp., that have caused the decline of chestnut areas in Portugal from 80 000 ha in the years 1950 to 45 000 ha in 2013 (Lione et al., 2019).

Other pests and pathogens, however, act directly at the fruit level. Chestnut is very attractive to fungi and insects larval attack due to the high level of sugars and the high water activity. These can cause serious damage and therefore pose a considerable problem for chestnut cultivation. Weevils attack chestnut tissues creating holes. Those holes, with 1 to 2 mm in diameter on the outer shell, represent tunnels of the insect attack (such as *Cydia fagiglandana* Zell and *Curculio elephans* Gyll.) (Pedrazzoli et al., 2012), and damage increases concomitantly with the development of the larvae. Fungal infections often start in the larval galleries of insects (Wells and Payne, 1980).

Nuts can also contain spores before harvest (Jermini et al., 2006). Some moulds are considered endophytes that colonize the fruits at various stages during their development but do not cause any symptoms of the disease until after fruit fall (Dennert et al., 2015). Expansion of fungal mycelia in the fruits and degradation of the cotyledons occur mainly during storage. At early infection stages, it is not easy to differentiate slightly mouldy or parasitized nuts from the good ones until they are processed or consumed (Rutter et al., 1990).

Some surveys revealed several fungal species associated with chestnut fruits, including *Penicillium*, *Aspergillus* and *Alternaria* (Wells and Payne, 1975; Rodrigues et al., 2013), but these fungi are natural inhabitants of the skin surface and are not necessarily associated with internal fruit damage. In fact, significant inconsistencies between the fungal infection capabilities on intact chestnut fruit and on its kernel have been reported (Chen and Zhou, 2011). In a recent review, Lione et al. (2019) report a long list of fungi that have been associated with chestnut spoilage, specifically rot. *Ciboria batschiana* (Zopf) N.F. Buchw., (syn. *Sclerotinia pseudotuberosa*), *Phoma castanea* Peck, and *Phomopsis endogena* (Speg.) Cif. have traditionally been considered the most important fungi responsible for chestnut rots,

while others have rarely been reported: *Acrospeira mirabilis* Berk. & Broome, *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea* Pers., *Colletotrichum acutatum* J.H. Simmonds, *Coniophora puteana* (Schumach.) P. Karst., *Cryptodiaporthe Castanea* (Tul. & C. Tul.) Wehm. Buchw., *Cytodiplospora castanea* Oudem., *Discula campestris* (Pass.) Arx, *Dothiorella* spp., *Fusarium* spp., *Mucor* spp., *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Penicillium* spp., *Pestalotia* spp., *Phomopsis viterbensis* Camici, Rhizopus spp., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Trichoderma* spp., and *Truncatella* spp.

In the last decade, however, a steep rise in the incidence of rotten nuts has been extensively observed by chestnut growers in some regions of Europe and Australasia (Smith and Agri, 2008; Smith and Ogilvy, 2008; Gentile et al., 2010; Visentin et al., 2012), and the fungus *Gnomoniopsis smithogilvyi* L.A. Shuttlew., E.C.Y. Liew & D.I. Guest (2012) (syn. *Gnomoniopsis castaneae* Tamietti 2012) has been identified as a significant rot-causing agent (Visentin et al. 2012; Maresi et al., 2013; Shuttleworth et al., 2013; Dar and Rai, 2015; Dennert et al., 2015; Shuttleworth and Guest, 2017; Lione et al., 2019).

A summary of the fungi considered as the most important rot-causing agents in chestnuts is given in Table 1.

Table 1. Summary of the most important fungi causing chestnut rots.

Type of rot	**Fungus / causal agent**	**Country**	**Reference**
Black rot	*C. batschiana*	Greece	Donis-González et al., 2016 Tziros and Diamandis, 2018
Brown rot	*G.smithogilvyi* (*G. castaneae*)	Asia (India), Europe (Italy, France, Switzerland) Australia, New Zealand	Smith and Agri, 2008 Gentile et al., 2010 Visentin et al. 2012 Maresi et al., 2013 Shuttleworth et al., 2013 Dar and Rai, 2015 Dennert et al., 2015 Donis-González et al., 2016 Shuttleworth and Guest, 2017 Lione et al., 2019
	Phoma endogena *Phomopsis endogena* *Phomopsis castanea*	Italy and other European countries	Ferreira-Cardoso and Vasconcelos, 2009 Donis-González et al., 2016
Pink rot	*Colletotrichum acutatum*	Italy	Gaffuri et al., 2015

2.4.1. Black rot

Black rot is a cause of significant yield losses, reported as a post-harvest disease (Vettraino et al., 2011). Chestnuts can appear healthy from the outside but show black rot on the inside (Tziros and Diamandis, 2018). The shell of chestnuts that present black rot at an advanced level appears darker and a little blackness can be covered with small raised structures of cream color that are the fructifications of the causal fungus (Figure 1). *C. batschiana* has been identified among the most important fungi that cause black rot. In 2014, this fungus was accounted responsible for 50% yield loss in chestnut orchards in Northern Greece (Tziros and Diamandis, 2018).

Figure 1. Example of chestnuts infected by black rots

2.4.2. Brown rot

Brown rot affects the kernel of the chestnut, resulting in browning and necrosis of the endosperm (Figure 2). The causative agent of brown rot was previously identified as *Phoma endogena* or *Phomopsis endogena*, but more recently, *G. smithogilvy* in Australia (Shuttleworth et al., 2013) and *G. castanea* in Piedmont (Northern Italy) (Gentile et al., 2010) have also been identified. In Australia, Shuttleworth et al. (2013) found the *G. smithogilvyi* anamorph as mainly associated with chestnut rot, as an endophyte isolated from asymptomatic chestnut flowers, leaves, stems and developing fruit, and the *G. smithogilvyi* teleomorph as a saprobe overwintered on dead burrs on the orchard floor. Symptoms mainly

occur at the post-harvest stages, however, observations by Australian growers indicate chestnuts can be affected while still attached to the tree (Shuttleworth et al., 2013).

In Italy, *Discula pascoe* sp. nov., the anamorphic state of *Gnomonia pascoe* sp. nov., later identified as *G. castaneae,* (name proposed by Visentin et al. (2012), the syn. *G. smithogilvyi*), was isolated sporadically from the nuts just after the fruit set, and with frequency increasing 25-80% in 2007 and 4-84% in 2008 in completely ripened nuts. In the diseased nuts, small chalky areas originated at the ripening time from the endosperm outer layers, and developed towards the centre of the kernel; progressively their colour turned brown and the endosperm mummified. This fungus, being able to colonize asymptomatically the branches and the immature nuts, was shown to have an endophytic lifestyle with a true pathological behaviour only on the ripened nuts (Gentile et al., 2010). In Switzerland, *G. castaneae* was also reported as the major causal agent of brown rot, and it was found in both symptomatic and asymptomatic fruits (Dennert et al., 2015).

In 2018, Shuttleworth published a correspondence letter discussing and clarifying the name of the brown rot causal agent (Shuttelworth, 2018). The author considered that *G. smithogilvyi* and *G. castaneae* are the same fungus, and that the correct name of the main causal agent of chestnut rot in Australia, New Zealand, and Europe is *G. smithogilvyi*.

Figure 2. Example of chestnuts infected by brown rot.

2.4.3. Pink rot

Pink rot presents an intense pink coloration of the endosperm (Figure 3). The mycelia appear first in white, then in grey and pale orange or pink. It is sometimes associated with brown rot symptoms (Maresi et al., 2013). *Colletotrichum acutatum* has been reported as the causal agent of pink rot, even though a very low level of infection with this pathogen was recorded up to present in the chestnut trees studied (Gaffuri et al., 2015).

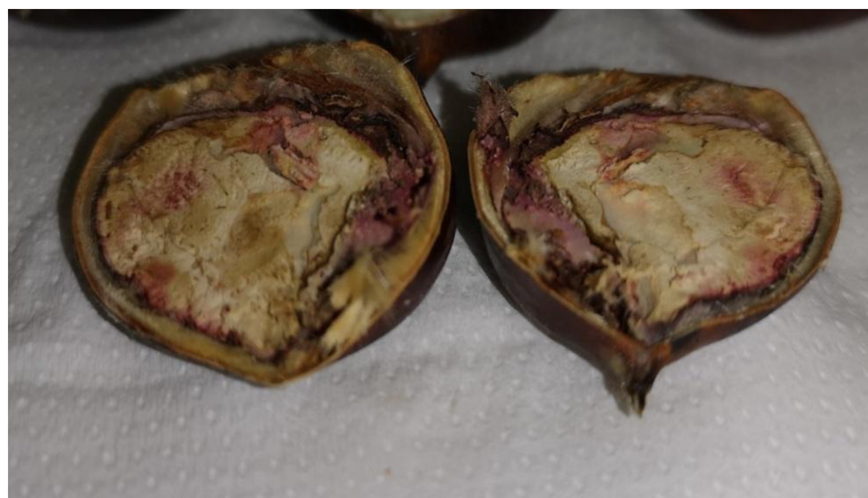

Figure 3. Example of chestnuts infected by pink rot

2.5. Processes of post-harvest preservation

Several methods have been developed for chestnut pasteurization, including methyl bromide (MeBr) fumigation (Ahmed, 2001), hot water bath (Kwon et al., 2001), low temperature and controlled atmosphere storage, irradiation and submerging in icy water for peeled fruit and Radiofrequency (RF) (Hou et al., 2018).

Chestnuts treated by MeBr fumigation have been banned in many countries in 2010 following an international regulation, the Montreal Protocol (UNEP, 2006), because of its harmful effects not only to human health but also to the environment (Antonio et al., 2012). The development of environmentally friendly non-chemical alternative methods to control fungi in postharvest chestnuts is thus a necessity.

Controlled atmospheres, namely storage under ultralow oxygen associated with refrigeration, have been demonstrated to reduce fungal growth on fruits (Barkai-Golan, 1990), but the technology is too expensive for industrial applications. Hot water dip treatment

is generalized in the chestnut industry and is recognized as a valid treatment against larvae infestation, but it can compromise the fruit quality (Aegerter and Folwell, 2000) and is not fully efficient against fungi. Water curing tests using specific microorganisms have also been carried out on chestnuts (Migliorini et al., 2010). After water curing, lower fungi growth was observed than the in controls, but due to a long period of treatment, water curing is difficult to be used for industrial scale applications. Gamma radiation has been extensively tested on chestnuts, and the survival of yeasts and *Aspergillus parasiticus* was strongly compromised (Antonio et al., 2012). Nonetheless, the treated products are not well accepted by consumers and by some international markets.

Radio frequency (RF) heating holds potential for pathogen control in chestnuts, since it can rapidly raise the temperature of fruits volumetrically and significantly reduce heating time to avoid the quality loss caused by the slower heating rate in conventional thermal treatments (Hou et al., 2018). The study by Hou et al. (2018) demonstrated that RF treatments have clearly increased heating speed in chestnut samples compared to warm air heating. The heating time needed only 5.4 min to heat the 2.5 kg chestnuts from 20 °C to 55 °C using RF energy, and 170 min for chestnuts to reach 52.5 °C using hot air at 55 °C. Also, the quality of the chestnut was not significantly affected by the RF treatments (Hou et al., 2018).

3. MATERIALS AND METHODS

3.1. Collection of samples

The study was performed on European chestnut (*C. sativa*) from the industry Sortegel in Braganca, Trás-os-Montes, Portugal, during the growing season 2018-2019. Samples were collected in different processing stages, as detailed in Table 2, from three varieties (Longal, Judia and Martaínha), in a total of 33 samples. The samples (of approximately 1 kg of chestnuts) were collected from the palox pallets from the storage room (stages of storage/process P2, P3, P6, P7, P8 and P9) or from the rejection palox (P10), and packaged separately in a paper envelope. The envelope was immediately sealed, labelled appropriately with an identification code, stage of processing and collection date, then transported to the laboratory. Chestnut samples were stored in cold chamber at 4 °C for immediate analysis or at maximum 2 days.

Table 2. Description of sampling points, storage period and sampled varieties.

Code	Processing stage	Storage period	Sampled varieties (# replicas)
P2	Chestnuts sterilized by hydrothermal bath and immediately sampled	0 days	Judia (3) Longal (2)
P3	Chestnuts sterilized by hydrothermal bath and sampled after 15 days of storage	15 days	Judia (1)
P6	Chestnuts sampled immediately after reception, without sterilization	0 days	Judia (3) Longal (3) Martaínha (2)
P7	Chestnuts stored without sterilization and sampled after 15 days of storage	15 days	Judia (3) Longal (1) Martaínha (1)
P8	Chestnuts stored without sterilization and sampled after 30 days of storage	30 days	Judia (1) Longal (3) Martaínha (1)
P9	Chestnuts stored without sterilization and sampled after 45 days of storage	45 days	Judia (2)
P10	Chestnuts rejected after manual selection	0 days	Judia (3) Longal (4)

3.2. External inspection of chestnut samples

Fifty chestnuts per sample (in a total of 1650 chestnuts) were randomly selected for external inspection. The external quality of fruits was evaluated by checking for visual defects, i.e., visible signs of pre- and post-harvest damage: 1) cracks; 2) signs of infestation (presence of exit holes caused by the chestnut weevil); 3) signs of fungal infection (visible mould growth), as described by Overy et al. (2003). The observed symptoms were described for each chestnut to determine the percentage of external infection and infestation per sample (Figure 4).

Figure 4. External inspection of the chestnuts.

3.3. Internal inspection

After external inspection, the 50 fruits were cleaned using running tap water, surface-disinfected by immersing in 10% commercial bleach for 2 minutes, washed three times with sterile distilled water, and blot-dried in sterile absorbent paper. Chestnuts were aseptically bisected longitudinally from stylar end to hilum with a sterile knife, and then visually inspected for the presence of internal moulds and insect larvae (Figure 5). Whenever signs of rot were present, the rot was identified. The percentage of rotten surface of each chestnut was visually estimated to determine the level of infection: Level 0 (L0): no visible mould infection; Level 1 (L1): 1-25% of chestnut internal surface covered by moulds, Level 2 (L2): 26-50%, Level 3 (L3): 51-75%, Level 4 (L4): 76-100%, as proposed by Donis-González et al. (2016). The infested and infected chestnuts were counted and expressed as a percentage of the total number of analysed chestnuts.

Figure 5. Internal inspection of the chestnuts.

3.4. Isolation of fungi from chestnuts

From each sample, 10 chestnuts with symptoms of kernel infection were sorted for isolation and identification of contaminating fungi. Five explants of the rotten parts were cut aseptically and plated on solidified potato dextrose agar (PDA; Biolife, Milan, Italy) in 9 cm Petri dishes (Figure 6). For samples with less than 10 nuts showing visible signs of fungal infection, non-infected chestnuts were used to complete the 10 chestnuts per sample. Plates with the explants were sealed with parafilm and incubated at 25°C for 6 days.

After the incubation period, each different growing colony was isolated and transferred the number of times necessary to obtain pure cultures into 6 cm Petri dishes containing 10 mL of Malt Extract Agar (MEA: Malt 20 g/L, Glucose 20 g/L, peptone 1 g/L, agar 20 g/L, autoclaved for 15 min at 120 ºC) for morphological and molecular identification. Plates were incubated using the same conditions as before. A total of 557 fungi was isolated from the samples. Fungal isolates were morphologically identified on the basis of their cultural characteristics such as colony size, shape, opacity, colour, form, surface growth, elevation, margin consistency, and were grouped by morphotypes. From these, 220 isolates representative of all the morphotypes were selected for molecular identification. All pure isolates (spores and mycelium) were preserved in 2.0 mL cryovials containing 1.5 mL of 30% glycerol with 0.05% of tween 80. Cryovials were left one hour at room temperature and at 4 °C overnight to allow glycerol to diffuse into the cells, and then at -20 °C until further analysis (Rodrigues et al., 2013).

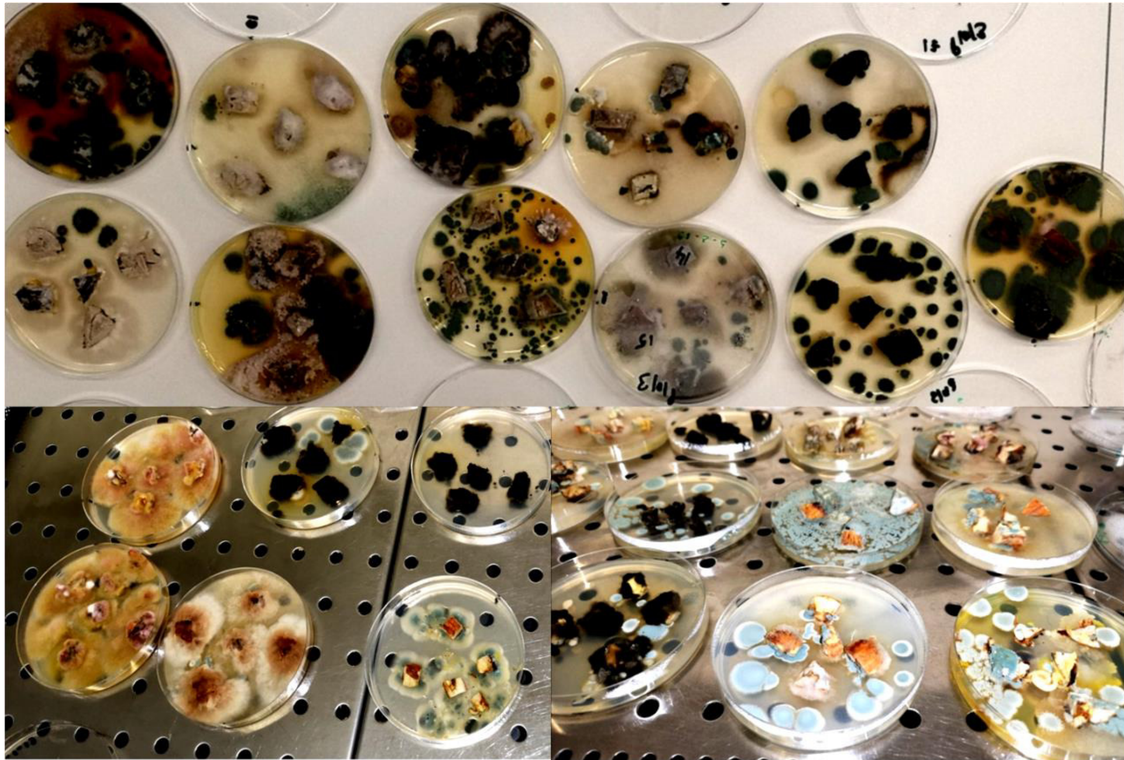

Figure 6. Inoculation of chestnut explants for fungal isolation: fungal growth after 6 days of incubation at 25 ºC.

3.5. Molecular identification of fungal isolates

3.5.1. DNA extraction

The total genomic DNA was extracted according to the SDS protocol described by Rodrigues et al. (2018). A loop full of spores or mycelium of fresh fungi was transferred to an eppendorf tube containing 300 µL of 0.5% SDS lysis buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 0.5 g of sterile glass beads with a diameter of 0.4 to 0.6 mm (Sigma, St. Louis, Missouri, USA). Mechanical disruption of cell walls achieved by vortexing for 5 min at maximum speed. Polysaccharides and proteins were precipitated by adding 500 µL of cold 3M sodium acetate (pH 5.5) and gently mixed by inversion and stored at -20 °C for 30 min. Thereafter the solution was centrifuged at 10,000 rpm for 10 min (4 °C) for phase separation. The clean supernatant was recovered and centrifuged again at 10,000 rpm for 10 min (4 °C). Finally, the supernatant was mixed with one volume of cold isopropanol stored at (-20 °C) to precipitate the DNA. The mixture was well mixed by inversion for a few minutes and incubated at -20 °C for one hour and centrifuged at 10,000 rpm for 10 minutes (4 °C). The DNA pellet was washed twice with 500

µL of 70% cold ethanol and centrifuged at 10,000 rpm for 7 min (4 °C) and air-dried. The DNA was dissolved in 30 to 50 µL of ultrapure water depending on the yield and stored at -20 ° C.

3.5.2. Electrophoresis of genomic DNA

Electrophoretic analysis was carried out to determine the quality and concentration of the genomic DNA in 0.8% agarose gels with Tris-Acetate-EDTA buffer (TAE: 40 mM Tris-HCl; 40 mM acetic acid; 1.0 mM EDTA, pH 8.0) stained with GelRed. Runs were made in TAE buffer with electrophoresis Mini-Sub® Cell (Bio-Rad), at constant voltage of 90 V for 45 minutes. A mix of 3 µL of genomic DNA and 1 µL of Blue Loading Buffer were loaded on the gel. In the end of the separation, the DNA was visualized under UV light and digital image was obtained using ChemiDoc™ XR+ System with Image Lab™ Software (Bio-Rad).

Considering the electrophoretic analyses, genomic DNA with good quality were selected for PCR amplification of the 18S rRNA and 28S rRNA gene using the universal primers ITS1 (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (Rodrigues et al., 2018), which amplify a 600 bp segment. PCRs were run on 25 µL reaction mixtures in a BioRad My cycler thermalcycler, following the conditions described in Table 3.

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Table 3. PCR conditions for the amplification

Amplification programme	Conditions
Initial denaturation	94 ºC, 3 min
Denaturation	94 ºC, 30 sec
Annealing	55 ºC, 30 sec
Extension	72 ºC, 2 min
Final extension	72 ºC, 10 min

PCR products obtained were separated on a 1.2% agarose TAE gel compared to a DNA size marker 100 bp DNA Ladder (BIORON). Electrophoretic runs and image acquisition were as previously described.

3.5.3. Sequencing and Sequence analysis

Sequence data were obtained by Sanger sequencing. PCR products were sequenced in one direction, using the primer ITS1. The sequences were manually corrected by comparison with the chromatogram and aligned with the NCBI (National Centre for Biotechnology Information) GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm to identify the fungi.

3.6. Statistical analysis

For the comparison of means of quantitative variables, samples were tested for homogeneity of variances by Levene's test. Since samples failed this criteria, variances were analysed by one-way ANOVA, and Multiple Comparisons between samples pairs were computed using the Tamhane's T2 test. The mean differences were significant at $P < 0.05$.

The fungal diversity of the samples was determined by the indices *Richness* and *Simpson Diversity Index (SDI)*, calculated based on Rodrigues et al. (2013). *Richness* corresponds to the number of species identified in each sample. *SDI* can be taken as the number of species which effectively contribute to diversity, and it takes into consideration the number of species present in the sample, as well as the abundance of each species. This index was calculated as the reciprocal form of Simpson's Index (1/D), as follows:

$$SDI = 1 / \Sigma(P_i/P_n)$$

Where:

$\Sigma(P_i/P_n)$ corresponds to D (Simpson's Index)

P_i is number of individuals of a given species

P_n is the total number of individuals

4. RESULTS AND DISCUSSION

4.1. External and internal inspection of chestnuts

Chestnut samples were obtained from three different varieties (Judia, Longal and Martaínha) and seven different processing stages (P2, P3, P6, P7, P8, P9, P10) as explained in Table 2. The external and internal quality of fruits was evaluated by checking for visual defects. The observed symptoms were described and counted to determine the percentage of damage, infection and infestation.

4.1.1. Damage

The results of damage observed in chestnut samples after external inspection are presented in Figure 7.

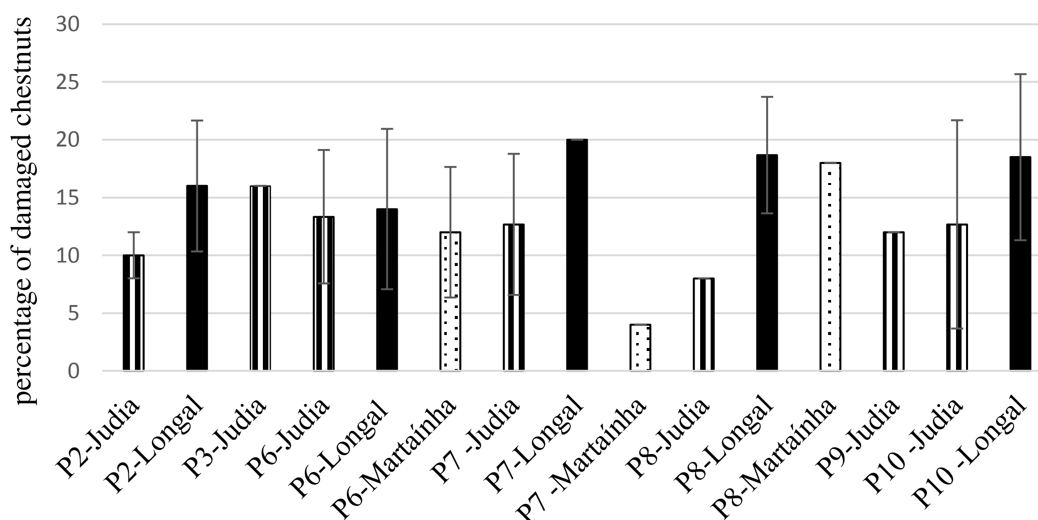

Figure 7. External inspection of chestnuts: Percentage of damaged chestnuts at different processing stages for the varieties Longal, Judia and Martaínha.

Damage was present in all samples from the three varieties with different percentages. The highest percentages of damaged chestnuts were detected in variety Longal for all the analysed stages. But, when varieties where considered in bulk (Longal, Judia and Martaínha, independently of the processing stage), differences between them were not significant ($p > 0.05$). The lowest percentage of damages was observed in Martaínha, stage P7. When comparing damage between stages of processing (independently of varieties), no significant

differences were observed ($p > 0.998$). This is due to the big differences between varieties for the same processing stage.

4.1.2. Infestation

Infestation was externally inspected by checking the presence of exit cracks or holes caused by chestnut pests. After making the external inspection, infestation was also evaluated internally. The observed symptoms of infestation from the outside and inside were described and counted to determine the percentage of infestation, as summarized in Figure 8. Attention is drawn to the fact that these results were only analysed as trends; they were not statistically computed, given the low and different number of replicas between processing stages.

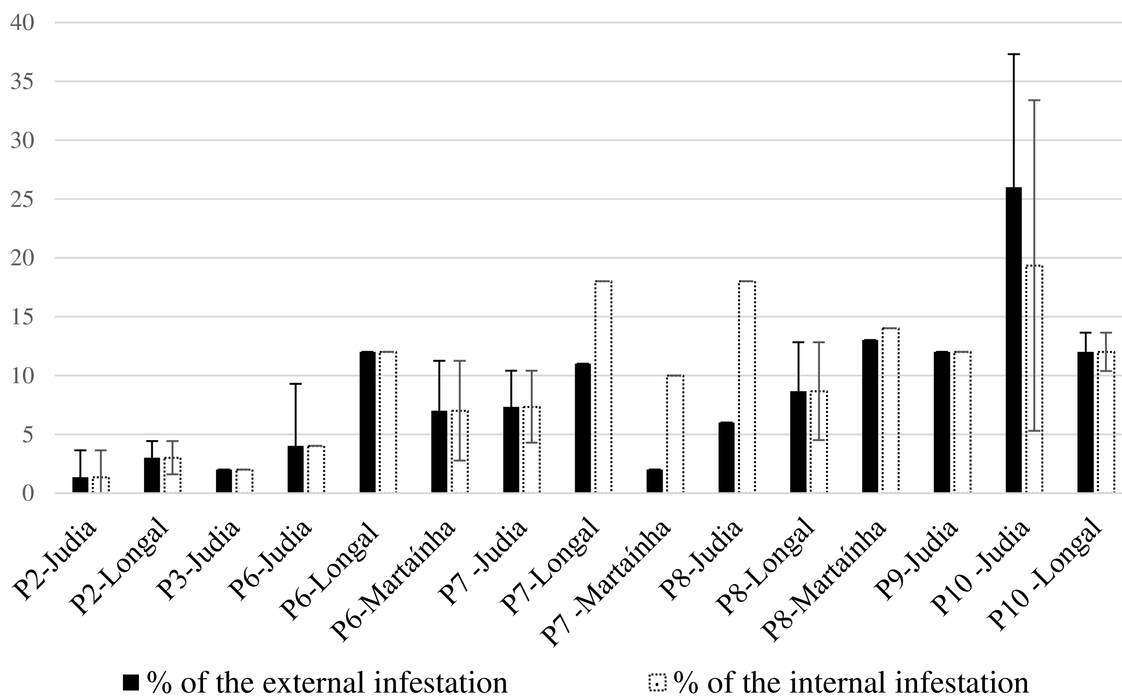

Figure 8. Percentage of external and internal infestation at different processing stages for the varieties Longal, Judia and Martaínha.

The highest percentage of internal infestation was registered in samples from variety Judia from processing stage P10. This result was expected, because the processing stage P10 corresponds to the chestnuts rejected based on visual defects. The sterilized chestnuts from processing stages P2 and P3 (0 and 15 days of storage) presented the lowest percentage of chestnut infestation. On the other hand, chestnuts that were stored immediately after

reception without going through the sterilization process (P6 to P9) showed a higher percentage of internal infestation than sterilized chestnuts (P2 and P3). Also, storage time showed to have a negative impact on infestation trend in non-sterilized chestnuts, since internal infestation increased concomitantly with the augmentation of the period of storage.

The higher average of infestation was detected from variety Longal comparing to Judia, in all processing stages except for P10.

4.1.3. Fungal Infection

The percentage of chestnuts showing external visible mould growth is shown in Figure 9. No visible moulds were detected in samples from variety Judia in processing stage P3 and P6 and samples from variety Martaínha in P7. The highest percentage of infection was detected in samples from processing stage P8 for the variety Martaínha. When varieties are compared (independently of the processing stage), no significant difference is detected between samples ($p > 0.336$). However, variety Longal is generally more heavily infected during all the processing stages than Judia.

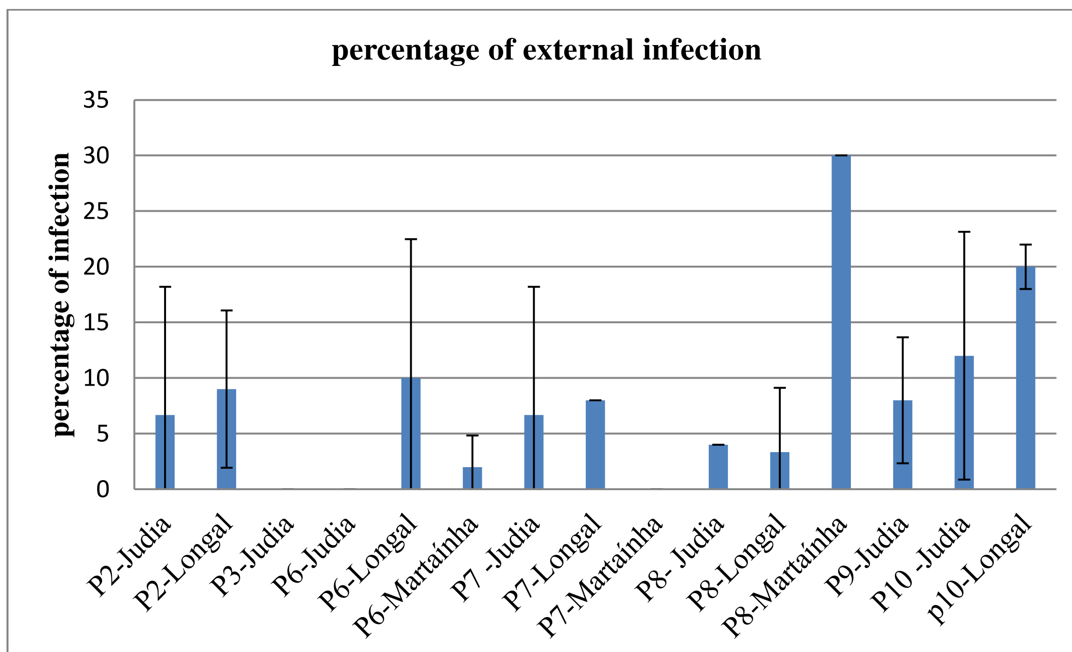

Figure 9. Percentage of external fungal infection at different processing stages for the varieties Longal, Judia and Martaínha.

The internal infection of chestnuts was also determined, and the level of infection was estimated by determining the percentage of rotten chestnut. These results are presented in Figure 10. Samples stored without sterilization generally presented more infection of level 4 (76 to 100% of internal surface covered by moulds). Comparing samples among variety, the variety Judia showed the lowest percentage of infection during processing stages P2, P6, P7 and P10, while Longal presented a higher percentage of infection for Level 2 or more. Comparing Judia for P2 and P3 (sterilized) and for P6, P7 and P8 (not sterilized), there is a clear increase in the level of infection throughout time of storage, showing that storage conditions allow for the proliferation of fungi and rot. For Longal, the trend is not evident, since a decrease in visual rot (higher % L0 and L1) is observed from P7 to P8.

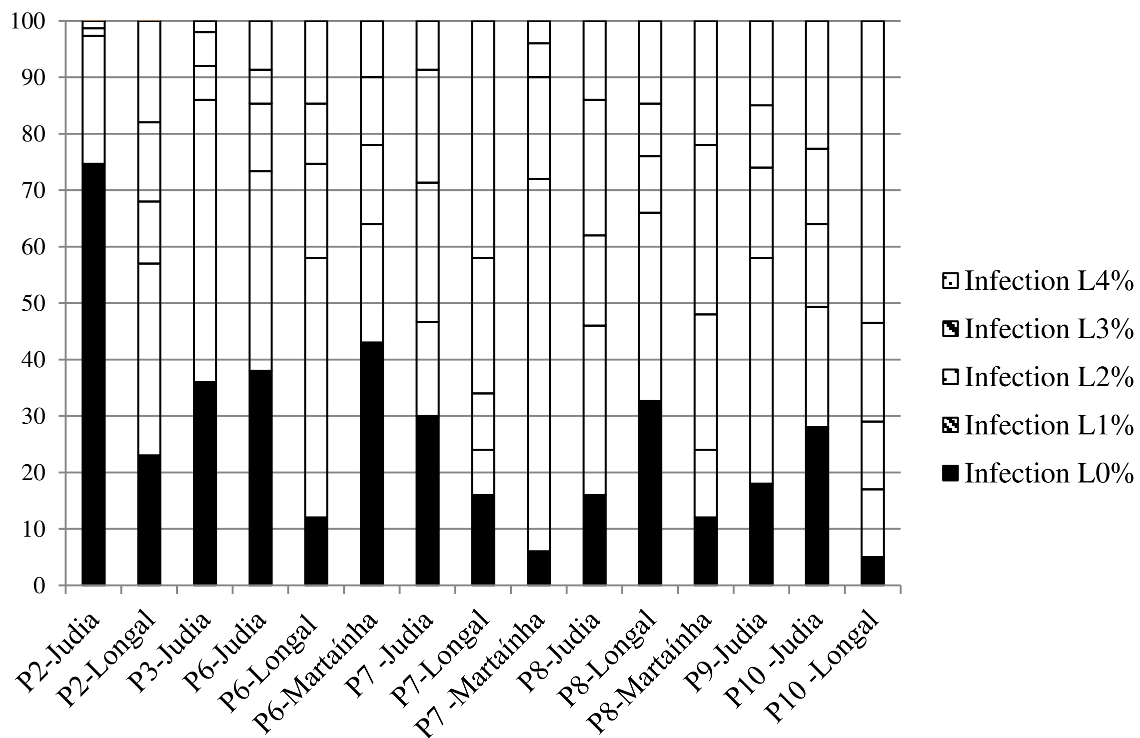

Figure 10. Percentage of internal fungal infection at different processing stages for the varieties Longal, Judia and Martaínha.

4.2. Morphological and molecular identification of fungal isolates

The main objective of this study was to identify the main potential agents of chestnut rot in Trás-os-Montes, Portugal. For this, 557 fungal isolates were obtained from chestnuts at storage and during processing stages from the varieties Judia, Longal and Martaínha. Fungi were firstly identified based on morphological characteristics and grouped by morphological similarity (morphotypes) as shown in Figure 11, and representative isolates of each morphotype were selected for molecular identification (Figure 12).

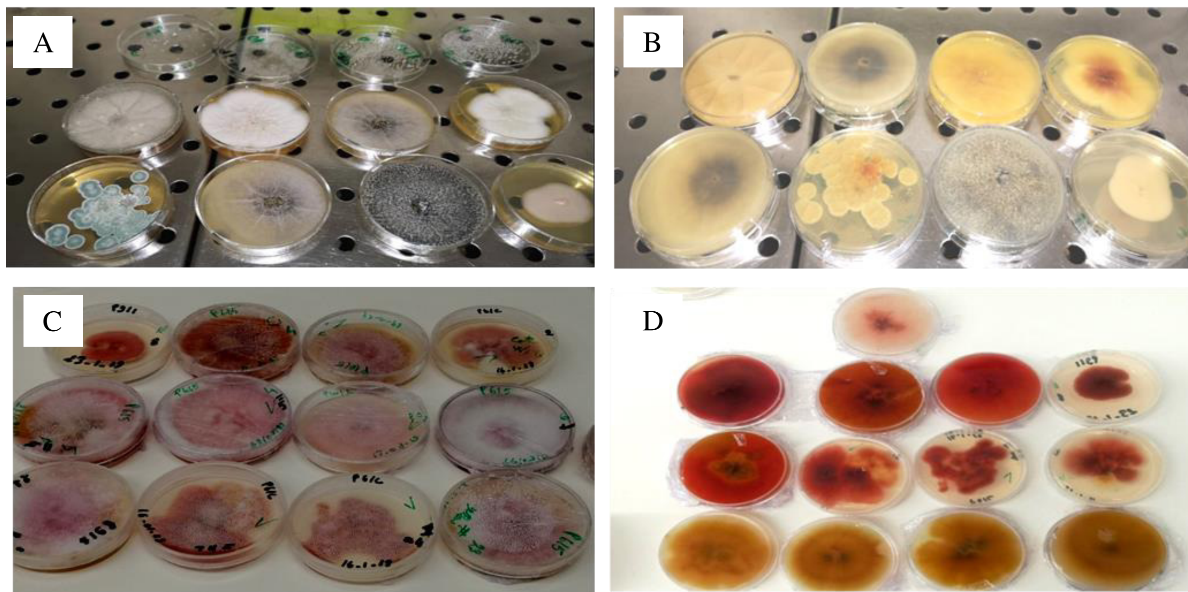

Figure 11. Examples of isolated fungi: A) and B) Pure cultures of fungi isolated from chestnut explants (obverse and reverse, respectively); C) and D) Morphotype grouping of fungi (obverse and reverse, respectively).

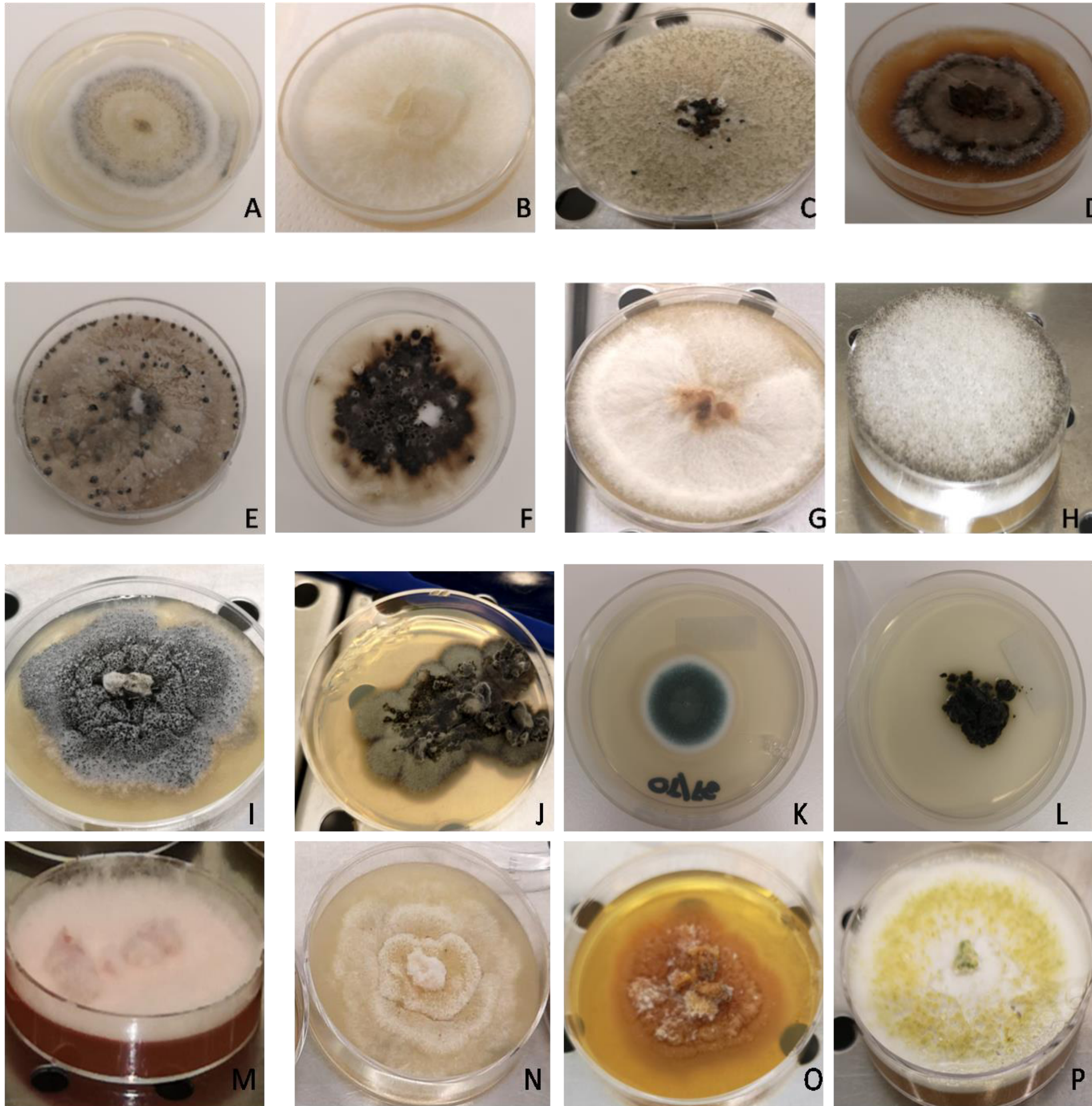

Figure 12. Examples of isolated fungi in MEA: A) *G. smithogilvyi;* B) *T. viridescens*; C) *Coniella fragariae;* D) *Rustroemia echinophila;* E) *B. cinerea;* F) *Ciboria americana*; G) *Peniophora meridionalis*; H) *M. racemosus* f. *sphaerosporus*; I) *Phacidium mollerianum;* J) *Coleophoma paracylindrospora;* K) *Pen. brevicompactum*; L) *C. batschiana*; M) *F. oxysporum;* N*) Phacidium fennicum*; O) *Xylaria* sp; P) *Pen. thomii.*

The total genomic DNA was extracted from the pure culture of 220 fresh fungi using the SDS protocol. The results of agarose gel electrophoresis (examples in Figure 13) showed that the genomic DNA was generally of good quality and enough amounts for PCR purposes. Figure 14 illustrates the results of the PCR amplifications of the ITS region. The amplification showed PCR products with the expected size of approximately 600 pb.

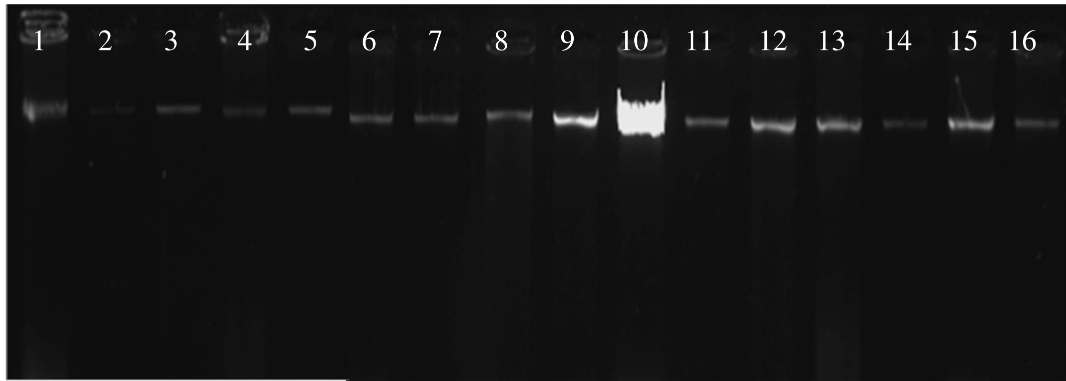

Figure 13. Agarose gel electrophoresis of genomic DNA. 1. *B. cinerea*, 2. *Pen. thomii*, 3. *Cladosporium cladosporioides*, 4. *Pen. glabrum*, 5. *Pen. brevicompactum*, 6. *B. tulipae*, 7. *Pen. bialowiezense*, 8. *C americana*, 9. *Didymella americana*, 10. *C. batschiana*, 11. *Pen thomii*, 12. *C.batschiana*, 13. *P. mollerianum*, 14. *P. fennicum*, 15. *F anguioides*, 16. *F. acuminatum.*

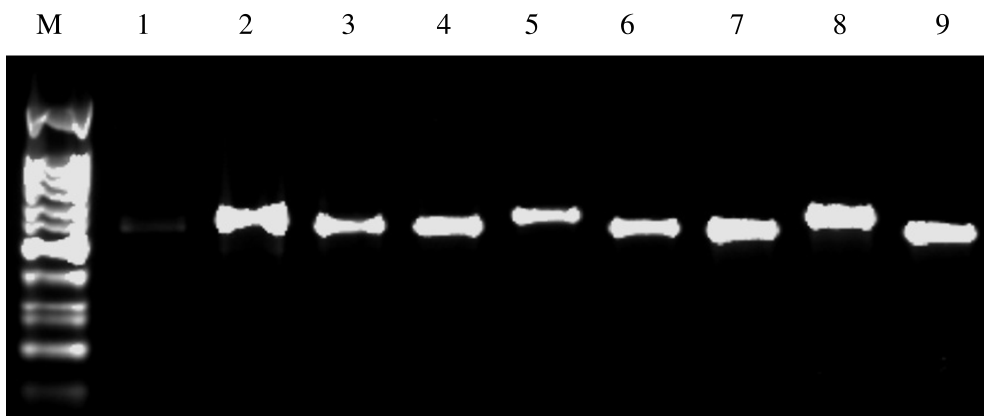

Figure 14. PCR product using primers ITS1-F and ITS4. M. molecular size marker (100 bp ladder). 1. Failed reaction, 2. *Pen. polonicum*, 3. *P. mollerianum*, 3. *F. acuminatum*, 4. *B. cinerea*, 5. *Co. paracylindrospora*, 6. *P. fennicum*, 7. *Curvibasidium cygneicollum,* 8. *Pen. brevicompactum.*

Eighty isolates were sequenced for molecular identification by the ITS region. From the eighty fungi identified molecularly and compared with the NCBI database, 37 different species were identified belonging to 16 genera: *Alternaria, Botryotinia, Botrytis, Ciboria, Cladosporium, Coleophoma, Coniella, Cytospora, Fusarium*, *Gnomoniopsis, Mucor, Penicillium, Phacidium, Rustrtoemia, Trichorderma* and *Xylaria*. The list of identified species is shown in Table 4.

Among the identified species, some were present with high incidence, as shown in Figure 15. From the dominant fungal species *M. racemosus* f. *sphaerosporus* was the most predominant one, followed by *Pen. brevicompactum, Pen. thomii, C. batschiana*, *B. cinerea, Pen.polinicum, C. americana, P. mollerianum, R. echinophila, T. viridescens* and *G. smithogilvyi,* all with more than 20% of incidence. The other fungi were found rarely.

Table 4. Fungal species identified in this study, with reference to the most similar strain used for identification, GenBank accession numbers of the comparison strains, percentage of similarity and the number of isolates of each species molecularly identified.

Molecular identification	GenBank accession number	% Similarity with reference strains	Number of isolates
Alternaria alstroemeriae CBS 118809	NR_163686.1	100.0%	2
Alternaria leptinellae CBS 477.90	NR_111866.1	100.0%	2
Botryotinia ficariarum CBS 177.63	MH858257.1	100.0%	1
B. cinerea CBS 261.71	MH860108.1	100.0%	9
B. tulipae CBS 286.71	MH860126.1	100.0%	3
C. americana CBS 117.24	KF859925.1	100.0%	2
C. batschiana CBS 331.35	MH855694.1	100.0%	2
Cladosporium cladosporioides CBS 129108	MH865207.1	100.0%	3
Co. paracylindrospora CBS 115328	KU728492.1	100.0%	3
Coniella fragariae CBS:180.48	KX833566.1	99.8%	1
Cu. cygneicollum CBS:7951	KY102978.1	99.8%	1
Cytospora sp. CBS 116856	KY051826.1	100.0%	3
Didymella americana CBS 185.85	FJ426972.1	100.0%	1
F. acuminatum CBS 131258	MH865933.1	99.8%	2
F. anguioides CBS 172.32	MH855263.1	99.8%	1
F. oxysporum CBS 132476	MH866024.1	100.0%	2
G. smithogilvyi CBS 130190	MH865607.1	100.0%	6
Kabatiella microsticta CBS 342.66	MH858817.1	98.4%	1
Mollisia sp.	DQ008233.1	99.0%	1
M. racemosus f. *sphaerosporus* CBS 258.39	MH856006.1	100.0%	2
Pen. bialowiezense CBS 227.28	MH854996.1	100.0%	1
Pen. brevicompactum CBS 126334	MH863910.1	100.0%	4
Pen. cyclopium CBS 129875	MH865559.1	100.0%	1
Pen. expansum ATCC 7861	NR_077154.1	100.0%	1
Pen. glabrum CBS 125543	NR_163530.1	100.0%	1
Pen. glandicola CBS 498.75	MH860946.1	99.8%	1
Pen. polonicum CBS 110332	MH862860.1	100.0%	2
Pen. thomii CBS 132168	MH865966.1	100.0%	2
Peniophora meridionalis CBS 289.58	MH857789.1	99.7%	2
P. fennicum CBS 457.83	KJ663840.2	100.0%	1
P. mollerianum CBS 138856	KR873247.1	100.0%	6
Pilidium acerinum CBS 403.71B	MH860187.1	100.0%	1
R. echinophila CBS 111549	KF545333.1	100.0%	3
Sporothrix variecibatus CBS 121961	KP017070.1	100.0%	1
Stromatinia narcissi CBS 354.47	MH856286.1	99.2%	2
T. viridescens CBS 433.34	NR_138429.1	100.0%	1
Xylaria sp.	KU683978.1	100.0%	1

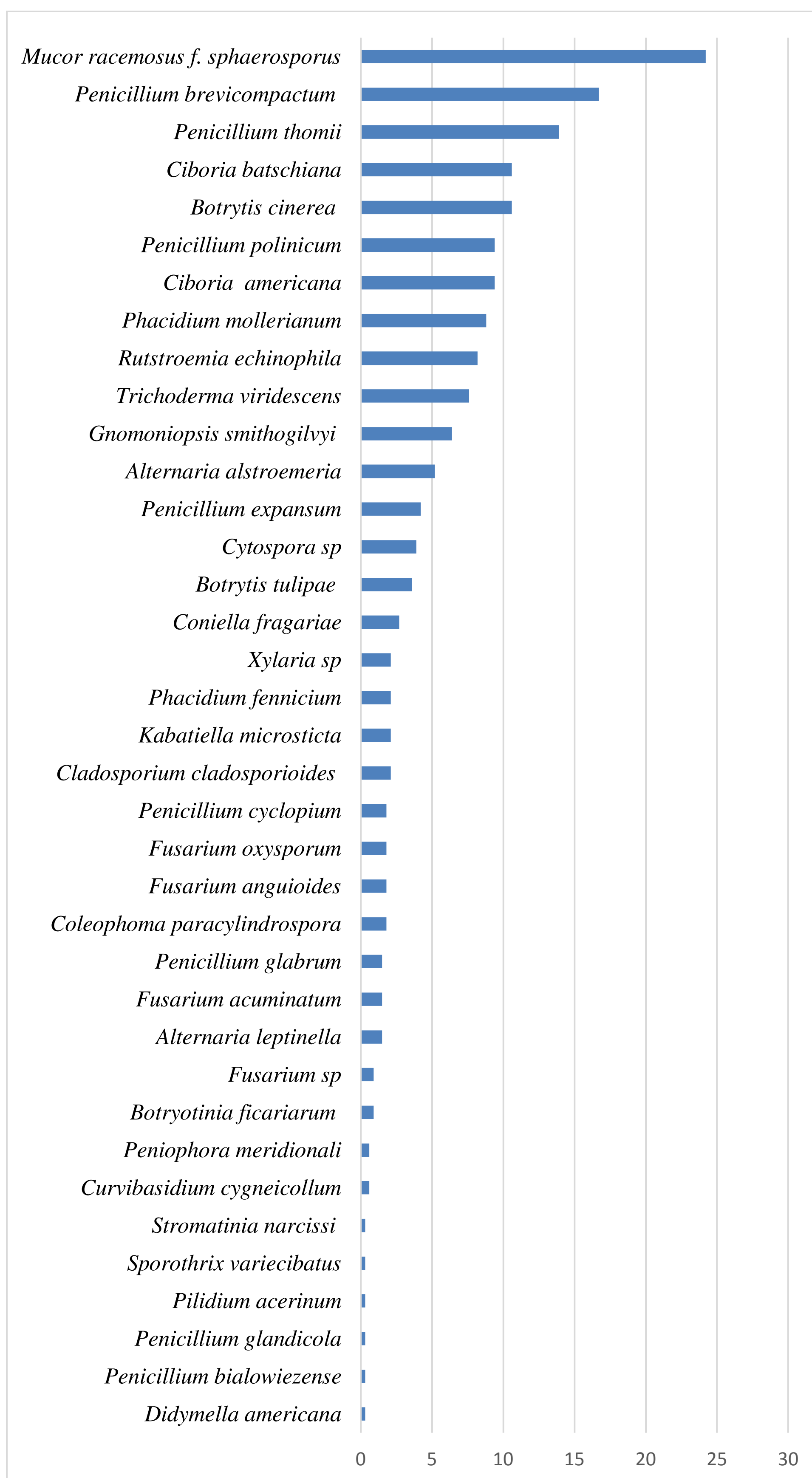

Figure 15. Frequency of the different species (in percentage of analysed chestnuts).

Some of the dominant fungi found in our chestnuts have been previously associated with chestnut rot or decay agents, and they are referred in Table 5. The most dominant fungus found in chestnuts was *M. racemosus*, and *B. cinerea* was also frequently isolated. These are cosmopolitan fungi widely distributed in nature, and are generally associated with fruit decay. *B. cinerea* is considered the causal agent of grey rot in chestnuts (Sieber et al., 2007; Donis-González et al., 2016).

G. smithogilvyi was a frequently isolated species in our study and is currently considered the major causal agent of chestnut brown rot in several countries in Europe, and also in New Zealand and Australia. It has been found in different climatic regions in Switzerland (Dennert et al., 2015), and has been reported to be a ubiquitous pathogen in Australia (Smith et al., 2008; Shuttleworth et al., 2012), in Italy (Visentin et al., 2012; Maresi et al., 2013), in Switzerland (Dennert et al. 2015) and in the United Kingdom (Lewis et al., 2017).

C. batschiana has been identified as agent of chestnut black rot and responsible for causing severe postharvest losses by disease. It has been reported in Greece (Tziros & Diamandis, 2018), Italy (Vettraino et al., 2005; Maresi et al 2013) and USA (Donis-González et al., 2016).

Penicillium was one of the most significant genera isolated from chestnuts. From the eight species identified, *Pen. brevicompactum*, *Pen. thomii* and *Pen. polonicum* were the dominant ones. Similar results had been reported in a survey on commercial chestnuts, where Overy et al. (2003) detected high frequencies of *Penicillium.* Also Sieber et al. (2007) found that chestnuts from Switzerland orchards were colonised predominantly by *Penicillium* spp. and *Mucor* spp. Furthermore, Prencipe et al. (2018) have recently identified *Pen. bialowiezense, Pen. brevicompactum, Pen. glandicola, Pen. polonicum* as chestnut contaminants. These species of *Penicillium* are not only considered pathogens capable of causing green rot in fruits, but they are also associated with the production of several mycotoxins in chestnuts, as it has already been reported in Italy (Prencipe et al., 2018).

Table 5. Most significant fungi isolated from stored chestnut and their previous association with chestnut rot or decay

Species	Comments and references
B. cinerea (syn. *Botryotinia fuckeliana*)	Genus has been reported to cause grey rot and decay in chestnuts in USA (Sieber et al., 2007; Donis-González et al., 2016).
G. smithogilvyi syn. *G. castaneae)*	The species has been reported as a causal agent of chestnut rot in Australia (Smith and Ogylvi, 2008; Shuttleworth et al., 2012), Italy (Visentin et al., 2012; Maresi et al., 2013), Switzerland (Dennert et al., 2015), the United Kingdom (Lewis et al., 2017), and as the cause of canker of *C. sativa* in India (Dar and Rai 2013, 2015), Switzerland (Shuttleworth et al., 2012; Visentin et al., 2012; Pasche et al., 2015) and New Zealand (Shuttleworth et al., 2012; Visentin et al., 2012). It has also been associated with necrosis of leaves and galls induced by *Dryocosmus kuriphilus* (chestnut gall wasp) on *Castanea* spp. in Italy (Magro et al., 2010; Tamietti et al., 2010; Vinale et al., 2014) and as an endophyte (Visentin et al., 2012).
C. batschiana (syn. *Sclerotinia pseudotuberosa*)	It has been reported as the cause of black rot of chestnuts in Greece (Tziros and Diamandis, 2018) and in Italy (Maresi et al., 2013). It was reported as a ubiquitous endophyte (Vettraino et al., 2005) in chestnuts, and the cause of decay in chestnuts stored without water-curing treatments (Migliorini et al., 2010).
C. americana (syn. *Rutstroemia americana*)	It has been reported in the mycobiota of chestnut in Greece, as a saprophyte (Diamandis and Perlerou, 2001).
Lanzia. echinophila (syn. *Rutstroemia echinophila*)	Has been identified growing on *C. sativa* in Europe as a saprophyte. It has been reported in Greece, Macedonia, Romania and Turkey (Road and Weaver, 1993)
Fusarium. sp.	Genus has been reported to cause mould and decay in chestnuts, especially in storage (Sieber et al., 2007; Donis-González et al., 2016).
Mucor. sp.	It has been associated with the spoilage of nuts (Overy et al., 2003; Panagou et al., 2005; Rodrigues et al., 2012; Visentin et al., 2012; Gaffuri et al., 2017).
Alternaria. sp.	Has been associated with the spoilage of chestnut fruits (Wells and Payne, 1975; Rodrigues et al., 2013).
Penicillium. sp.	Genus has been recorded as the cause of green chestnut rots (Overy et al., 2003; Rodrigues et al., 2013; Donis-González et al., 2016; Prencipe et al., 2018). Several species, like *Pen. bialowiezense, Pen. brevicompactum*, *Pen. expansum, Pen. glandicola* and *Pen. polonicum* were found to cause rot and produce mycotoxins in chestnuts or chestnut products (Prencipe et al., 2018)
*P. mollerianum (*Syn*: Phoma molleriana)*	*P. mollerianum* has been isolated from *Eucalyptus* sp. leaves in Italy (Crous et al., 2019). *Phacidium* sp. has been identified as new postharvest agent of rot in apple and pear (Wiseman et al., 2016)
T. viridescens	It has been associated with chestnut spoilage in Canada (Overy et al., 2003). Also identified as biocontrol of chestnut blight (Rigling & Heiniger, 1994) and antagonist against *G. smithogilvyi* (Pasche et al., 2016).

4.3. Fungal frequency and diversity

The results on fungal frequency and diversity detected in the different samples and processing stages are detailed in Table 6. *Mucor* was the most prevalent species (between 24.2% and 100% frequency) and it was present in all stages of production. It might be due to their capacity to grow on all possible substrates and in a wide range of temperature and humidity. The second highest frequency percentage was detected on fungi belonging to genera *Penicillium*. These results were also found by Sieber et al (2007) and Wells and Payne (1980), where *Mucor* and *Penicillium* were also frequently found to colonize chestnuts. The highest frequency among *Penicillium* is of *Pen. brevicompactum*. This fungus was detected throughout the stages irregularly. In fact, this fungus was detected at P6 with 5%, P7 with 16%, P8 with 6%, P9 with 75% and P10 with 12.9%. The second highest total frequency percentage in the genus was by *Pen. thomii* that reached 28.6% for P10. Its lowest rate was detected in P2 (8%) and P6 (8.8%). Unlike *Pen. brevicompactum,* the results showed that the frequency increased from early stage to final stages. This fungus proliferates when it is stored for longer periods.

The third highest frequency was detected for *C. batschiana* and *B. cinerea*, with an average of 10.6%. Unlike the other two genera, these fungi appear in all the stages except the sterilized samples. But *C. batschiana* achieves the highest frequency in P10 (21.4%), which can mean that a significant level of black rotten chestnuts is eliminated by sorting.

The highest percentage of frequency of *G. smithogilvyi* was detected at the stages P7 and P10 with 10% of frequency. It lowest frequency percentage was 6% in P8. As for *C. batschiana*, it does not appear in sterilized samples in stages P2 and P3. The results showed an irregularity in the occurrence of this fungus in non-sterilized samples throughout storage.

The occurrence of the isolated fungi in different processing stages showed that the highest number of isolates (87) was recovered from the processing stage P10. This result was expected due to the nature of the samples in this stage. In fact this stage is composed by the rejected infested and infected chestnuts. The maximum percentage of fungal frequency was detected during this stage for *Pen. thomii* (28.6%), *T. viridescens* (22.9%), the causal agent of black rot *C. batchiana* (21.4%) and *B. tulipae* (17.1%). The causal agent of brown rot, *G. smithogilvyi*, although not highly frequent (10.0%), showed a relatively important position in samples contamination.

On the other hand P2 showed the lowest level of fungal contamination. In P2 only 4 genera were found: *Alternaria*, *Mucor*, *Penicillium* and *Didymella*, being that the latter was

detected only once. *Mucor*, *Penicillium and Alternaria* were detected with important percentages. It can be concluded that those fungi were probably more resistant to the methods of disinfection. The only P3 sample analysed (Judia, sterilized, 15 days of storage) shows an even lower level of contamination than the Judia P2 samples. Conclusions are not taken from this, given the low number of P3 samples under analysis.

P6 samples, which are non-sterilized samples analysed immediately after reception, showed a higher level of contamination than P2, in frequency, richness and diversity index. If P2 and P6 are compared, it can be concluded that the sterilisation process applied to P2 samples has an important effect of fungal contamination. The results show a high percentage of fungal frequency in non-sterilized samples (P6 to P9), although time of storage could not be clearly associated with increased level of contamination.

Chestnut samples from processing stages without sterilization (P6, P7, P8 and P9) were compared in order to determine the effect of the storage period. P9 samples which are stored for the longest period (45 days) showed higher total frequency of infection than the other samples (P6, P7 and P8) as shown in Table 8. So we conclude that the storage period helps the proliferation of fungi. As found by Washington et al. (1997), the microflora of the kernel increased during storage. At harvest nuts were already colonised by fungi which have the potential to cause rots in the kernel tissue and those fungi infect the kernel during storage by developing from the already colonised shell tissue (Washington et al., 1997). But storage time also conducted to the elimination of fungi less resistant to the storage condition. By comparing the richness, the diversity of fungi decrease with the storage period in P6 were the samples was analysed immediately after reception the richness was 27 and in P9 only 11 species were detected (Table 6).

Samples were also analysed among varieties as detailed in Table 7 and Table 8, in order to determine the susceptibility of each variety to the different fungal contaminants. Results showed that, for the stages where all varieties were sampled (P6, P7 and P8), Martaínha presented the lowest total frequency of infection (3.6%), while the variety Longal had the highest fungal frequency (4.8%). We notice that for some fungi we have almost the same percentage of frequency, some of them are either present in all varieties and/or others have the highest or lowest records in all varieties like *M. racemosus* f. *sphaerosporus* that have the highest percentage in all varieties with almost the same percentage. *Pen. brevicompactum* attacks more the varieties Judia and Martaínha with almost the same percentage (respectively 21.7% and 20%) and is less present in Longal (7.6%). *T. viridescens* attacks the varieties Judia and Longal with the same total percentage of frequency.

For variety Judia, the highest frequency of fungi affecting chestnuts were *M. racemosus* f. *sphaerosporus* and *Pen. brevicompactum* with respectively 21.9% and 21.6%. The following fungi were detected only in Judia variety also but with a very low (0.4%) percentage: *Pen. bialowiezense, Pen. glandicola, Pi. acerinum, S. variecibatus, A. leptinella*. For Longal, the following fungi were detected only in this variety: *Cu. cygneicollum*, *Pen. cyclopium* and *Pe. meridionali*, all at very low frequencies. *Pen. glabrum* was only detected in Martaínha, with quite high percentage.

Some of fungi seem to have preference for certain varieties like *C. americana* that is present in Longal with 10.8%, while in Judia is present with a frequency of only 3.8%, *Pen. thomii* this is present in Longal with percentage of 21.7% and 10.5% for Judia; *Pen. brevicompactum* was present in Judia and Martaínha with 21.7% and 20%, and 77% for Longal. *C. batschiana* seems to be more 11.4% for Judia and 6.3% for Longal and Martaínha.

Table 6. Number of isolates, percentage of frequency and diversity indices, Richness and Simpson Diversity Index (SDI)

Sample type	Variety	# chestnut	*A. alstroemeria*	*A. leptinella*	*Bo. ficariarum*	*B. cinerea*	*B. tulipae*	*C. americana*	*Cl. cladosporioides*	*Co. paracylindrospora*	*Coniella fragariae*	*Cu. cygneicollum*
P2	Judía	30	1 (3.3)	-	-	-	-	-	-	-	-	-
P2	Longal	20	8 (40.0)	-	-	-	-	-	-	-	-	-
Total		50	9 (18.0)	0	0	0	0	0	0	0	0	0
P3	Judía	10	-	-	-	-	-	-	-	-	-	-
Total		10	0	0	0	0	0	0	0	0	0	0
P6	Judía	30	4 (13.3)	-	-	8 (26.7)	-	5 (16.7)	-	-	-	-
P6	Longal	30	-	-	-	3 (10.0)	-	12 (26.7)	-	-	-	2 (6.7)
P6	Martaínha	20	-	-	1 (5.0)	2 (10.0)	-	4 (20.0)	3 (15.0)	1 (5.0)	-	-
Total		80	4 (5.0)	0	1 (1.3)	13 (16.3)	0	21 (26.3)	3 (3.8)	1 (1.3)	0	2 (2.5)
P7	Judía	30	-	2 (6.7)	-	8 (26.7)	-	2 (6.7)	1 (3.3)	1 (3.3)	3 (10.0)	-
p7	Longal	10	-	-	-	2 (20.0)	-	1 (10.0)	1 (10.0)	-	1 (10.0)	-
P7	Martaínha	10	-	-	-	1 (10.0)	-	-	-	-	-	-
Total		50	0	2 (4.0)	0	11 (22.0)	0	3 (6.0)	2 (4.0)	1 (2.0)	4 (8.0)	0
p8	Judía	10	-	-	-	-	-	-	-	-	-	-
p8	Longal	30	1 (3.3)	-	-	5 (16.7)	-	3 (10.0)	1 (3.3)	1 (3.3)	1 (3.3)	-
P8	Martaínha	10	-	-	-	-	-	-	-	1 (10.0)	-	-
Total		50	1 (2.0)	0	0	5 (10.0)	0	3 (6.0)	1 (2.0)	2 (4.0)	1 (2.0)	0
P9	Judía	20	2 (10.0)	-	-	4 (20.0)	-	-	-	-	2 (10.0)	-
Total		20	2 (10.0)	0	0	4 (20.0)	0	0	0	0	2 (10.0)	0
P10	Judía	30	-	3 (10.0)	-	1 (3.3)	1 (3.3)	1 (3.3)	1 (3.3)	1 (3.3)	1 (3.3)	-
P10	Longal	40	1 (2.5)	-	2 (5.0)	1 (2.5)	11 (27.5)	3 (7.5)	-	1 (2.5)	1 (2.5)	-
Total		70	1 (1.4)	3 (4.3)	2 (2.9)	2 (2.9)	12 (17.1)	4 (5.7)	1 (1.4)	2 (2.9)	2 (2.9)	0
			17 (5.2)	5 (1.5)	3 (0.9)	35 (10.6)	12 (3.6)	31 (9.4)	7 (2.1)	6 (1.8)	9 (2.7)	2 (0.6)

Sample type	Variety	*Cytospora* sp.	*D. americana*	*F. acuminatum*	*F. anguioides*	*F. oxysporum*	*Fusarium sp.*	*G smithogilvyi*	*K. microsticta*	*M. racemosus f. sphaerosporus*	*Pen. bialowiezense*
P2	Judía	-	1 (3.3)	-	-	-	-	-	-	10 (33.3)	-
P2	Longal	-	-	-	-	-	-	-	-	14 (70.0)	-
Total		0	1 (2.0)	0	0	0	0	0	0	24 (48.0)	0
P3	Judía	1 (10.0)	-	-	-	-	-	-	-	10 (100.0)	-
Total		1 (10.0)	0	0	0	0	0	0	0	10 (100.0)	0
P6	Judía	-	-	1 (3.3)	-	1 (3.3)	-	2 (6.7)	2 (6.7)	-	1 (3.3)
P6	Longal	1 (3.3)	-	-	2 (6.7)	-	1 (3.3)	-	2 (6.7)	8 (26.7)	-
P6	Martaínha	-	-	3 (15.0)	-	-	-	4 (20.0)	1 (5.0)	2 (10.0)	-
Total		1 (1.3)	0	4 (5.0)	2 (2.5)	1 (1.3)	1 (1.3)	6 (7.5)	5 (6.3)	10 (12.5)	1 (1.3)
P7	Judía	1 (3.3)	-	-	-	-	2 (6.7)	1 (3.3)	-	3 (10.0)	-
p7	Longal	3 (30.0)	-	-	-	-	-	4 (40.0)	-	3 (30.0)	-
P7	Martaínha	-	-	-	-	-	-	-	-	-	-
Total		4 (8.0)	0	0	0	0	2 (4.0)	5 (10.0)	0	6 (12.0)	0
p8	Judía	-	-	-	-	-	-	3 (30.0)	-	S	-
p8	Longal	1 (3.3)	-	-	-	-	-	-	2 (6.7)	11 (36.7)	-
P8	Martaínha	3 (30.0)	-	-	-	-	-	-	-	5 (50.0)	-
Total		4 (8.0)	0	0	0	0	0	3 (6.0)	2 (4.0)	25 (50.0)	0
P9	Judía	-	-	-	4 (20.0)	2 (10.0)	-	-	-	2 (10.0)	-
Total		0	0	0	4 (20.0)	2 (10.0)	0	0	0	2 (10.0)	0
P10	Judía	1 (3.3)	-	-	-	1 (3.3)	-	2 (6.7)	-	-	-
P10	Longal	2 (5.0)	-	1 (2.5)	-	2 (5.0)	-	5 (12.5)	-	6 (15.0)	-
Total		3 (4.3)	0	1 (1.4)	0	3 (4.3)	0	7 (10.0)	0	6 (8.6)	0
		13 (3.9)	1 (0.3)	5 (1.5)	6 (1.8)	6 (1.8)	3 (0.9)	21 (6.4)	7 (2.1)	80 (24.2)	1 (0.3)

Sample type	Variety	*Pen. brevicompactum*	*Pen. cyclopium*	*Pen. expansum*	*Pen. glabrum*	*Pen. glandicola*	*Pen. polonicum*	*Pen. thomii*	*Peniophora meridionali*	*P. fennicium*	*P. mollerianum*
P2	Judía	13 (43.3)	-	-	-	-	-	4 (13.3)	-	-	-
P2	Longal	3 (15.0)	-	-	-	-	10 (50.0)	-	-	-	-
Total		16 (32.0)	0	0	0	0	10 (20.0)	4 (8.0)	0	0	0
P3	Judía	-	-	3 (30.0)	-	-	-	-	-	-	-
Total		0	0	3 (30.0)	0	0	0	0	0	0	0
P6	Judía	3 (10.0)	-	-	-	-	9 (30.0)	4 (13.3)	-	2 (6.7)	5 (16.7)
P6	Longal	1 (3.3)	-	-	-	-	2 (6.7)	3 (10.0)	-	-	5 (16.7)
P6	Martaínha	-	-	-	5 (25.0)	-	-	-	-	-	7 (35.0)
Total		4 (5.0)	0	0	5 (6.3)	0	11 (13.8)	7 (8.8)	0	2 (2.5)	17 (21.3)
P7	Judía	2 (6.7)	-	-	-	-	-	-	-	-	3 (10.0)
p7	Longal	-	-	-	-	-	-	6 (60.0)	-	-	-
P7	Martaínha	6 (60.0)	-	-	-	-	-	2 (20.0)	-	-	-
Total		8 (16.0)	0	0	0	0	0	8 (16.0)	0	0	3 (6.0)
p8	Judía	-	-	-	-	-	-	-	-	-	-
p8	Longal	3 (10.0)	-	-	-	-	-	7 (23.3)	2 (6.7)	1 (3.3)	6 (20.0)
P8	Martaínha	-	-	-	-	-	1 (10.0)	-	-	-	1 (10.0)
Total		3 (6.0)	0	0	0	0	1 (2.0)	7 (14.0)	2 (4.0)	1 (2.0)	7 (14.0)
P9	Judía	15 (75.0)	-	-	-	1 (5.0)	-	-	-	1 (5.0)	-
Total		15 (75.0)	0	0	0	1 (5.0)	0	0	0	1 (5.0)	0
P10	Judía	5 (16.7)	-	-	-	-	7 (23.3)	14 (46.7)	-	3 (10.0)	-
P10	Longal	4 (10.0)	6 (15.0)	11 (27.5)	-	-	2 (5.0)	6 (15.0)	-	-	2 (5.0)
Total		9 (12.9)	0	0	0	0	9 (12.9)	20 (28.6)	0	3 (4.3)	2 (2.9)
		55 (16.7)	6 (1.8)	14 (4.2)	5 (1.5)	1 (0.3)	31 (9.4)	46 (13.9)	2 (0.6)	7 (2.1)	29 (8.8)

Sample type	**Variety**	***Pi. acerinum***	***R. echinophila***	***C. batschiana***	***S. variecibatus***	***St. narcissi***	***T viridescens***	***Xylaria* sp.**	**Richness**	**SDI**
P2	Judía	-	-	-	-	-	-	-	5	2.9
P2	Longal	-	-	-	-	-	-	-	4	3.3
Total		0	0	0	0	0	0	0	6	
P3	Judía	-	-	-	-	-	-	-	3	1.8
Total		0	0	0	0	0	0	0	3	
P6	Judía	-	9 (30.0)	-	1 (3.3)	-	1 (3.3)	-	16	12.9
P6	Longal	-	1 (3.3)	7 (23.3)	-	-	4 (13.3)	-	15	8.7
P6	Martaínha	-	1 (5.0)	-	-	-	-	1 (5.0)	13	8.9
Total		0	11 (13.8)	7 (8.8)	1 (1.3)	0	5 (6.3)	1 (1.3)	27	
P7	Judía	1 (3.3)	-	8 (26.7)	-	-	-	-	14	8.2
p7	Longal	-	-	-	-	-	-	-	8	5.7
P7	Martaínha	-	-	-	-	-	-	-	3	2.0
Total		1 (2.0)	0	8 (16.0)	0	0	0	0	15	
p8	Judía	-	-	-	-	-	2 (20.0)	1 (10.0)	4	2.4
p8	Longal	-	8 (26.7)	1 (3.3)	-	-	1 (3.3)	4 (13.3)	18	10.9
P8	Martaínha	-	-	2 (20.0)	-	-	-	-	6	4.1
Total		0	8 (16.0)	3 (6.0)	0	0	3 (6.0)	5 (10.0)	20	
P9	Judía	-	-	2 (10.0)	-	-	1 (5.0)	-	11	4.6
Total		0	0	2 (10.0)	0	0	1 (5.0)	0		
P10	Judía	-	1 (3.3)	13 (43.3)	-	-	7 (23.3)	-	17	7.6
P10	Longal	-	7 (17.5)	2 (5.0)	-	1 (2.5)	9 (22.5)	1 (2.5)	22	13.5
Total		0	8 (11.4)	15 (21.4)	0	1 (1.4)	16 (22.9)	1 (1.4)	24	
		1 (0.3)	27 (8.2)	35 (10.6)	1 (0.3)	1 (0.3)	25 (7.6)	7 (2.1)		

Table 7. Total percentage of infection for the three the varieties Longal, Judia and Martaínha

total % of frequency	*B. cinerea*	*C. americana*	*Cytospora sp.*	*F. acuminatum*	*F. anguioides*	*F. oxysporum*	*F. sp.*	*G. smithogilvyi*	*M. racemosus f.sphaerosporus*	*Pen. bialowiezense*	*Pen. brevicompactum*
Judia	10.9	3.8	2.3	0.5	2.8	2.4	0.9	6.6	21.9	0.5	21.6
Longal	9.8	10.8	8.3	0.5	1.3	1	0.6	10.5	35.7	0	7.6
Martaínha	6.6	6.6	10	5	0	0	0	6.6	20	0	20

total % of frequency	*Pen. cyclopium*	*Pen. expansum*	*Pen. glabrum*	*Pen. glandicola*	*Pen. poloinicum*	*Pen. thomii*	*P. mollerianum*	*R. echinophila*	*C. batschiana*	*T. viridescens*
Judia	0	4.3	0	0.7	7.6	10.4	3.8	4.7	11.4	7.4
Longal	3	5.5	0	0	12.3	21.6	8.3	9.5	6.3	7.8
Martaínha	0	0	8.3	0	3.3	6.6	15	1.6	6.6	0

Table 8. Total percentage of infection during the different processing stages

Sample type	Variety	Average frequency of all percentages
P2	Judía	2.6
P2	Longal	4.7
Total P2		3.5
P3	Judía	3.8
Total P3		3.8
P6	Judía	5.2
P6	Longal	4.5
P6	Martaínha	4.7
Total P6		4
P7	Judia	3.4
p7	Longal	5.7
P7	Martaínha	2.4
Total P7		3.7
p8	Judía	1.6
p8	Longal	5.3
P8	Martaínha	3.5
Total P8		4.7
P9	Judía	4.9
Total P9		4.9
P10	Judía	5.7
P10	Longal	5.9
Total P10		5.8

5. CONCLUSIONS

The storage of chestnut represents the most important problem on an industrial scale because of the growth of a wide spectrum of spoilage fungi which conducts to the appearance of rots. As a result, chestnuts lose their fruit quality and commercial value.

This dissertation focused on the molecular identification of the potential agents of rot in chestnut varieties of Trás-os-Montes: Judia, Longal and Martaínha at different post-harvest stages of storage and processing. To our knowledge, this is the first study on the identification of the main potential agent causing chestnut rot in Portugal.

As result to the present study:

- A high diversity of species has been identified: 37 different species belonging to 16 genera.
- The dominant fungal species were *M. racemosus* f. *sphaerosporus*, *Pen. brevicompactum, Pen. thomii, C. batschiana*, *B. cinerea, Pen. polonicum, C. americana, P. mollerianum, R. echinophila, T. viridescens* and *G. smithogilvyi.*
- Among the dominant species identified in this study, some have been previously associated with chestnut rot or decay: *B. cinerea* is considered as the causal agent of grey rot and decay; *G. smithogilvyi* is the major causal agent of chestnut brown rot in several countries in Europe, and also in New Zealand and Australia; *C. batschiana* has been identified as agent of chestnut black rot and responsible for causing severe postharvest losses by disease; *Pen. bialowiezense, Pen. brevicompactum, Pen. glandicola* and *Pen. polonicum* causing green rot in fruits, and also associated with the production of several mycotoxins in chestnuts.

The frequency, abundance and diversity of rots in three chestnut varieties of Trás-os-Montes – Judia, Longal and Martaínha - at different post-harvest stages of storage and processing have been determined. The results showed that:

- Sterilization with hydrothermal bath was effective against the elimination and reduction of several fungi. During the processing stage P2 the sterilised samples only 3 genera were found: M*ucor*, *Penicillium and Alternaria* which were more resistant to the methods of disinfection. The causal agents of brown rot *G.*

smithogilvyi, black rot *C. batschiana* and grey rot *B. cinerea* do not appear in sterilized samples.

- Samples from variety Martaínha were identified as the most resistant to fungal growth. They presented the lowest total percentage of frequency of infection and samples from variety Longal were the least resistant to the infection, as they registered the highest percentage of infection.

The general goal of this study was to obtain information on the potential agents of rot or decay in chestnuts in Trás-os-Montes, for the future development of strategies for reduction of rot, reducing the incidence of the disease and associated chestnut losses in storage.

Further research work is needed to study possible applications of radio frequency (RF) treatments for pasteurization of chestnuts to replace chemical methods or the biological control method by the use of the isolated fungi such as T. *viridescens* to improve the effectiveness of biocontrols on reducing chestnut rot.

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