Food Control 144 (2023) 109346

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

It's what's inside that counts: DNA-barcoding of porcini (*Boletus* sp., Basidiomycota) commercial products reveals product mislabelling

Mauro Rivas-Ferreiro [a], Alberto Otero [a], Paloma Morán [a,*]

[a] Centro de Investigación Mariña (CIM), Departamento de Bioquímica, Genética e Inmunología, Facultade de Bioloxía, Universidade de Vigo, 36310, Vigo, Spain

ARTICLE INFO

Keywords:
DNA-barcoding
Boletus edulis
Mislabelling
Dehydrated porcini
Phylogenetics
Health risks

ABSTRACT

In the edible mushroom market, porcini (*Boletus edulis* Bull., and related species) are a very economically valuable product, with 20–100 tons of these mushrooms being sold each year worldwide. However, products sold as "*Boletus edulis*" are often a mix of different species, some of them of less value than "*Boletus edulis*" but sold at the same price. DNA-Barcoding is a powerful tool that can help uncover this kind of fraud in a rapid and accurate way.

In this study, we combine visual inspection with DNA barcoding and phylogenetic analyses to assess the quality and provide accurate identifications of nine different dehydrated porcini commercial products destined for human consumption. DNA was extracted and the internal transcribed spacer (ITS) region, the fungal DNA barcode, was amplified and sequenced from a total of 108 samples (twelve per product) and five reference *Boletus* sp. sporocarps from the UVigo-FUNGI fungarium. Five products contained mushrooms in various states of decay, worm infestation and pathogen contamination, which would not be suitable for sale as fresh sporocarps following the current legislation. *B. edulis* was only found in three of the nine products, being *B. reticulatus* the most abundant species. Two Asian species of porcini, *B. bainiugan* and *B. meiweiniuganjun*, were also found in one product during the phylogenetic analyses. Three non-porcini Boletaceae were found, but only one (*Imleria badia*) could be identified to species level. Lastly, four samples were identified as the fungal pathogen *Hypomyces chlorinigenus*, indicating decay of the sporocarps.

Overall, our results show that DNA-barcoding is a powerful tool for the identification of mislabelling and fraud in porcini products in a rapid and accurate way.

1. Introduction

Porcini are among the most sought-after edible mushroom species (Ciesla, 2002). They are also among the most collected species worldwide (Gelardi, 2020; Leonardi et al., 2005; Sitta & Davoli, 2012), with 20–100 tons of these mushrooms being consumed every year (Hall et al., 2003; Wang & Chen, 2014). The name "Porcini" refers to a group of more than 60 species within the genus *Boletus*, that occur naturally in temperate forests of the northern hemisphere, with a great species diversity in North America (24 species) and Southeast Asia (29 species) (Gelardi, 2020). The four European species of *porcini*, oftentimes referred to as "*Boletus* group *edulis*", are perhaps the most charismatic of the genus: *B. edulis* Bull.: Fr. *sensu stricto*, *B. aereus* Bull.: Fr., *B. pinophilus* Pilat et Dermek y *B. aestivalis* Fr. (Peintner et al., 2007). While distinct, the European porcini species's morphology is very variable with climate, interactions with other species, etc. (Noordeloos & van der Linde, 2018), which can result in misidentifications within *Boletus* and even with other genera.

In nature, porcini are ectomycorrhizal; that is, they form a symbiosis with plants from the families Fagaceae, Betulaceae, Malvaceae, Cistaceae, Salicaceae, Ericaceae and Pinaceae (Águeda et al., 2008). This complex relationship, forged throughout the evolutionary history of both partners, has depleted the ectomycorrhizal porcini of most of its saprotrophic abilities, which is the reason why these species cannot be cultivated in the same way white button mushrooms (*Agaricus bisporus*) or oyster mushrooms (*Pleurotus* spp.) are. While attempts to achieve *in vitro* cultivation of porcini mushrooms have been made (Endo et al., 2014; Mediavilla et al., 2016; Rivas-Ferreiro et al., 2021; Águeda et al., 2008), the commercialization of porcini currently depends on sporocarp picking from their natural habitat (Hall et al., 1998).

Commercialization of porcini mushrooms in Europe must comply with Regulation (EU) Nº 1169/2011 of the European Parliament and of

* Corresponding author.
E-mail address: paloma@uvigo.es (P. Morán).

https://doi.org/10.1016/j.foodcont.2022.109346
Received 21 June 2022; Received in revised form 10 August 2022; Accepted 27 August 2022
Available online 1 September 2022

Fig. 1. Dehydrated commercial porcini products used in this study. Samples B1–B8 were purchased as commercial products, sample B9 was acquired in a street market.

the Council of 25 October 2011, on the Provision of Food Information to Consumers. This regulation establishes that labels must include the name of the food product, the list of ingredients, net quantity, expiration date and country of origin. Some European countries have specific laws about mushroom picking and commercialization, including lists of marketable mushroom species. In Spain, the commercialization of wild mushrooms is regulated by the Royal Decree 30/2009; this decree includes a list of species that can be sold fresh, including the four European porcini species. It also establishes the requirements that commercial products have to meet, such as the correct identification of the species (with genus and species name being indicated in the label), being well preserved without signs of rot or arthropod, worm of mollusk contamination, without foreign matter on the surface and without signs of pathogenic microbial agents. In Italy, the legislation establishes the term "*Boletus edulis* and group" on labels, referring to all species that belong to the genus *Boletus* sect. *Boletus* (Sitta & Floriani, 2008); those include the European species mentioned above, plus North African and Asian species that fall within this clade. Countries like the United Kingdom, although they include mushrooms in their general food and environmental legislation, do not have specific regulations regarding mushroom picking or commercialization (Peintner et al., 2013).

The high demand for porcini species in Europe has led distributors to source their *Boletus* spp. from other continents, which leads to products being potentially mislabelled as "*Boletus edulis*" (Cutler et al., 2021; Dentinger & Suz, 2014; Raja et al., 2017). Sequencing the Internal Transcribed Spacer (ITS) region, selected as the universal Fungi barcode (Schoch et al., 2012), would help in the assessment of compliance with the regulations, specifically with regards to proper species identification. Research has shown the importance of DNA barcoding in the unequivocal identification of fungal species such as the ones present in food products containing mushrooms (Cutler et al., 2021; Dentinger & Suz, 2014; Raja et al., 2017), while also highlighting the gaps in the barcoding databases caused by many species not having yet been assigned a barcode (Osmundson et al., 2013; Y. Zhang et al., 2021).

In this study, a combination of visual inspection, DNA barcoding and phylogenetics was used to 1) revise the compliance of dried porcini products with European and local regulations, 2) confirm the identity of the mushrooms present in the products being sold as *Boletus edulis* through DNA barcoding and 3) assess the use of phylogenetic tools to further examine the identity of the species present in said products.

Table 1
Information on the commercial porcini products analysed in this study. Information on products B1–B7 was obtained from the label; information on B8 was obtained through communications with the distributor; information on B9 was obtained from the seller.

Product	Country of origin	Species in label	Price €/Kg
B1	Poland	*Boletus edulis*	75,00
B2	Turkey	*Boletus edulis*	223,00
B3	EU	*Boletus edulis*	132,67
B4	Spain/EU	*Boletus edulis*	250,00
B5	Bulgaria	*Boletus edulis*	147,00
B6	Europe	*Boletus edulis* and group	295,00
B7	EU/outside of EU	*Boletus* spp. *edulis*, *reticulatus* and *aestivalis*	137,50
B8	China	*Boletus edulis*	95,00
B9	Montenegro	*Boletus edulis*	30,00

2. Materials and methods

2.1. Sample collection and visual inspection

Nine dehydrated porcini (*Boletus* spp.) commercial products were selected as a source for samples for this study (Fig. 1). The products were purchased both online and from local distributors. Information on the content of each product is provided in Table 1.

For the analyses, twelve hymenium-bearing sporocarp pieces were randomly selected from each product, for a total of 108 samples. Visual inspection was carried out in all products to identify signs of decay, rot, or presence of foreign matter, arthropods, worms, molluscs or microbial pathogens, to check their compliance with European and local regulations.

Five specimens identified as *Boletus edulis*, *B. reticulatus*, *B. aereus* and *B. pinophilus* from the UVIGO-Fungi fungarium and collected in Galicia (NW Spain) were also sequenced and included in the study as references for said species.

2.2. DNA extraction

A small piece of hymenium was collected from all 108 samples and the five reference specimens and stored in 1.5 ml tubes. In the pieces where contamination was visible to the naked eye, the sample was carefully scraped using a scalpel and collected in an 1.5 ml tube with ethanol for subsequent DNA extraction. Total DNA extraction was

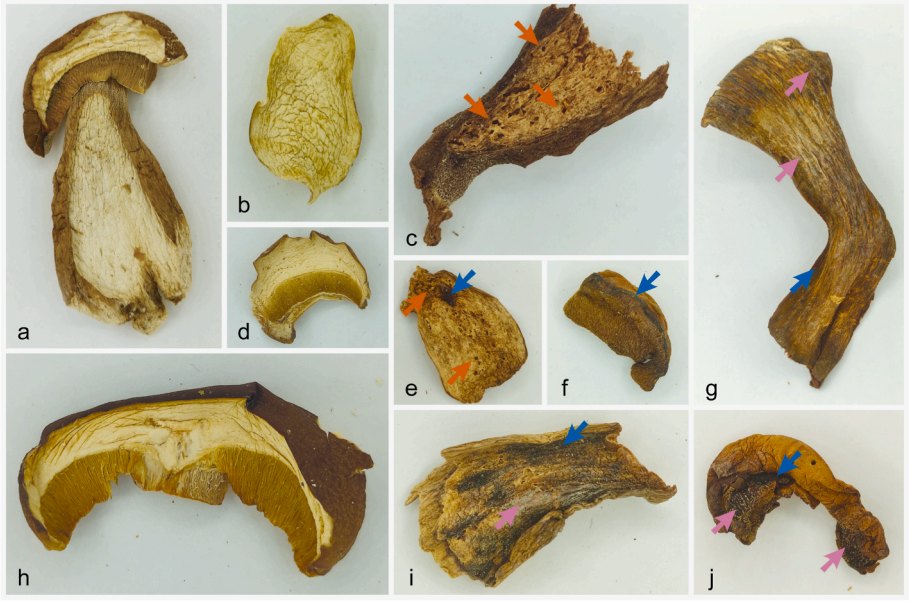

Fig. 2. Some dried porcini from the commercial products used in this study showing good condition (a and b from product B6, d from B1, h from B9), and clear signs of poor condition (c from product B3, e from B8, f from B4, g from B5, i and j from B7), such as: worm infestation (orange arrows), decay (blue arrows) and pathogenic microbial activity (pink arrows).

performed using the NZY PLANT/FUNGI gDNA ISOLATION KIT (NZYTech, Portugal) following the manufacturer's instructions for fungal gDNA extraction. The resulting DNA was stored at −20 °C until PCR amplification.

2.3. PCR amplification and sequencing

For amplification of the rDNA ITS region, the ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers were used (White et al., 1990). In a reaction tube, 1 μl of DNA sample was added to 10 μl of NZYTaq II 2x Colourless Master Mix (NZYtech, Portugal), 1 μl of 10 nM ITS1F/ITS4 primer mix and 8 μl of ddH2O, for a total reaction volume of 20 μl. Cycling parameters were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and primer extension at 72 °C for 40 s; and a final extension of 72 °C for 7 min. To confirm PCR amplification, gel electrophoresis was carried out in 2% agarose, TBE 1X buffer gels with GreenSafe Premium (NZYTech, Portugal) for DNA visualisation, and ran 25 min at 100 V.

2.4. Sequence cleanup and identification

The amplified DNA was sent to the Genomics Service in the Scientific-Techological Support Center for Research (University of Vigo) for purification with ExoSap (ThermoFisher) and for bi-directional sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a capillary sequencer SeqStudio Genetic Analyzer (Applied Biosystems).

The raw trace files were edited and the consensus sequences were aligned using the built-in Geneious Global Alignment algorithm (Geneious, Biomatters). A first identification was assigned to each sample using the nucleotide Basic Local Alignment Search Tool (BLAST) search to verify identity. BLASTn search was employed using nucleotide collection (nr/nt). Threshold identity was set up at 97%.

2.5. Phylogenetic analysis of porcini samples

As shown in previous studies (Giusti et al., 2021), sequences deposited in the databases could be incorrectly identified, potentially leading to misidentifications of other sequences through BLAST. Therefore, a phylogenetic analysis was carried out to confirm identifications within the true porcini species.

The ITS sequences from the samples identified as porcini were combined with the five sequences generated from the Galician collections; additionally, 52 sequences from various *Boletus* spp. used in previous studies (Burke et al., 2009; Dentinger et al., 2010; Dentinger & Suz, 2014; Feng et al., 2012; Giusti et al., 2021; Kjøller & Clemmensen, 2009; Leonardi et al., 2005; Morris et al., 2008; Obase et al., 2011) were retrieved from GenBank and added to the dataset. The sequence from sample b1_s12, identified as *Imleria badia*, was used as the outgroup for the phylogenetic analysis.

Sequences were aligned using the algorithm L–INS–i in MAFFT version 7 (Katoh et al., 2019); the alignment was then explored and edited in Geneious Prime. A maximum likelihood analysis was carried out in the online IQ-TREE server (Trifinopoulos et al., 2016). Partitions were manually determined for the ITS1, 5.8 S and ITS2 subregions, and a substitution model was automatically assigned to each one of them by IQ-TREE; the analysis was carried out with the ultrafast bootstrap option, exploring 1000 alignments. The consensus tree was explored using FigTree v1.4.4 (Rambaut, 2010). and edited using Adobe Illustrator 2021 (Adobe Inc., United States).

3. Results

3.1. Visual inspection of commercial samples

Products B1, B2, B6 and B9 were in good condition, with few visible worm galleries in the dried mushroom pieces, mostly at the bottom of the stipes, and no clear signs of decaying sporocarps (Fig. 2). Products B3, B4, B5, B7 and B8 did have worm galleries on all surfaces of the mushrooms, with many pieces showing signs of decay such as darker areas, gelatinized hymenium, and a white powdery structure covering the mushroom surface belonging to a parasitic fungus (Fig. 2). Products B5 and B7 did also show signs of rehydration at the time of opening, made apparent by the soft texture of the mushroom pieces.

Most products were labelled as containing only "*Boletus edulis*". Product B6 was labelled as containing "*Boletus edulis* and group"; product B7's label indicated "*Boletus* spp. *edulis*, reticulatus and

Table 2
Summary of the product sequencing results and comparison between label information, BLAST identification and phylogenetic results.

Product	Label ID	BLAST ID	Phylogenetic ID of *Boletus* sp.
B1	*Boletus edulis*	*Boletus edulis* (6 samples) *Boletus reticulatus* (5 samples) *Imleria badia* (1 sample)	*Boletus edulis* (6 samples) *Boletus reticulatus* (5 samples) *Imleria badia* (1 sample)
B2	*Boletus edulis*	*Boletus aereus* (8 samples) *Boletus reticulatus* (4 samples)	*Boletus aereus* (8 samples) *Boletus reticulatus* (4 samples)
B3	*Boletus edulis*	*Boletus edulis* (3 samples) *Boletus reticulatus* (8 samples) *Boletus aereus* (1 samples)	*Boletus edulis* (3 samples) *Boletus reticulatus* (8 samples) *Boletus aereus* (1 samples)
B4	*Boletus edulis*	*Boletus reticulatus* (6 samples) *Boletus aereus* (5 samples) *Hypomyces chlorinigenus* (1 samples)	*Boletus reticulatus* (6 samples) *Boletus aereus* (5 samples) *Hypomyces chlorinigenus* (1 samples)
B5	*Boletus edulis*	*Boletus aereus* (9 samples) *Boletus reticulatus* (2 samples) *Hypomyces chlorinigenus* (1 samples)	*Boletus aereus* (9 samples) *Boletus reticulatus* (2 samples) *Hypomyces chlorinigenus* (1 samples)
B6	*Boletus edulis* and group	*Boletus pinophilus* (7 samples) *Boletus reticulatus* (5 samples)	*Boletus pinophilus* (7 samples) *Boletus reticulatus* (5 samples)
B7	*Boletus edulis* *Boletus reticulatus*	*Boletus aereus* (7 samples) *Boletus reticulatus* (3 samples) *Hypomyces chlorinigenus* (2 samples)	*Boletus aereus* (7 samples) *Boletus reticulatus* (3 samples) *Hypomyces chlorinigenus* (2 samples)
B8	*Boletus edulis*	*Boletus edulis* (3 samples) *Boletus reticulatus* (7 samples) *Tylopilus* sp. (1 sample) *Sutorius* sp. (1 sample)	*Boletus bainiugan* (3 samples) *Boletus meiweiniuganjun* (7 samples) *Tylopilus* sp. (1 sample) *Sutorius* sp. (1 sample)
B9	*Boletus edulis*	*Boletus edulis* (11 samples) *Boletus reticulatus* (1 samples)	*Boletus edulis* (11 samples) *Boletus reticulatus* (1 samples)

aestivalis" [sic], being the last one a synonym for *Boletus reticulatus* and, thus, redundant.

3.2. PCR amplification and DNA sequencing

The ITS regions from all 108 samples plus the 5 reference sequences were successfully amplified and sequenced. All sequences retrieved in this study are available in GenBank (GB accession numbers: ON790669-ON790781); voucher material was deposited in the fungarium collection at the University of Vigo (UVIGO-Fungi, Pontevedra, Spain).

3.3. Species-level identification using BLAST

Of the 108 samples to be analysed, all but 7 correspond to *Boletus* spp. Of these seven, 3 samples were identified as other Boletaceae: one *Imleria badia* sample; one *Sutorius* sp. that could not be identified to species level; and one *Tylopilus* sp. that could not be identified to species level either. The remaining four samples were identified as the fungal parasite *Hypomyces chrysospermus*.

The 101 remaining samples were identified as *Boletus edulis* (23 samples), *Boletus reticulatus* (41 samples), *Boletus aereus* (30 samples) and *Boletus pinophilus* (7 samples) (Table 2).

3.4. Phylogenetic analysis of porcini species

Species delimitation through phylogenetic analysis of the *Boletus* spp. samples was successful; all species were well supported (<95% bootstrap values) in the ITS maximum likelihood consensus tree.

Samples from product B8, identified through BLAST as *Boletus edulis* or *B. reticulatus,* fell within the *B. bainiugan* and *B. meiweiniuganjun* clades in the phylogeny, respectively (Fig. 3). Those clades were closely related to *B. reticulatus*, and together with *B. aereus, B. shiyong, B. nobilissimus and B. quercophilus,* formed a well-supported clade (97% bootstrap value).

With the exception of the porcini samples from product B8, all other samples were correctly identified by BLAST.

4. Discussion

The visual inspection of the porcini products showed that their condition was, in many cases, not adequate. Clear signs of worm infestation, decay and pathogenic activity, which would prevent mushrooms from being sold fresh, were detected in five of the nine dehydrated products; this shows a lack of pest and pathogen control during the selection, dehydration and packaging of the mushrooms.

Fungal pathogens were detected through barcoding in three products, with all pathogens being identified as *Hypomyces chlorinigenus*, a very common bolete pathogen that degrades the cellular walls of the mushrooms (Touze-Soulet et al., 1980), changing its texture and thus lowering its marketability. To this day, the effects of this species and other parasites on human health upon consumption are unknown (Cutler et al., 2021).

While all products were labelled as containing *Boletus edulis*, this species was only found in three of them (20 samples in total). *Boletus reticulatus* was found in all products but one, and it was the most abundant species found in our sampling (34 samples). *Boletus aereus* was also quite abundant, being found in five out of the nine products (30 samples), while *Boletus pinophilus* was only found in one product, but it was abundant in it (7 samples).

While our BLAST analyses only found four species of porcini present among our samples, the phylogenetic analyses revealed the presence of two Asian *Boletus* species in product B8. This product, purchased in the UK, contained *B. bainiugan* and *B. meiweiniuganjun*. Upon contacting the distributor, it was revealed to us that the mushrooms were harvested in China. These findings are in accordance with those of Dentinger and Suz (2014), who described these two species from a commercial packet of dried porcini mushrooms picked in China and purchased from a retailer in the UK as well.

Of the other three Boletaceae found in our sampling, only *Imleria badia* was identified to species level. This mushroom, albeit edible, is widely regarded as of poorer quality than the true porcini (bib_Esteve_Raventós_et_al_2007bib_Esteve_Raventós_et_al_2007). It can be distinguished from porcini mushrooms for their yellowish flesh that bruises blue, their yellow-green angular pores that also bruise blue-grey, and their viscous pileus when wet (Noordeloos & van der Linde, 2018). It is a widely consumed mushroom, and it is one of the species allowed for commercialization in Poland (Polish Ministry for Health, 2020). However, some reports of allergic reactions caused by this mushroom led H. Li et al. (2021) to classify it as edible with conditions. This proves the importance of having a proper identification in the label of commercial dried mushroom products destined for consumption, as certain people can have adverse reactions to some edible species (Gawlikowski et al., 2015).

The other two species found in this study could not be identified at

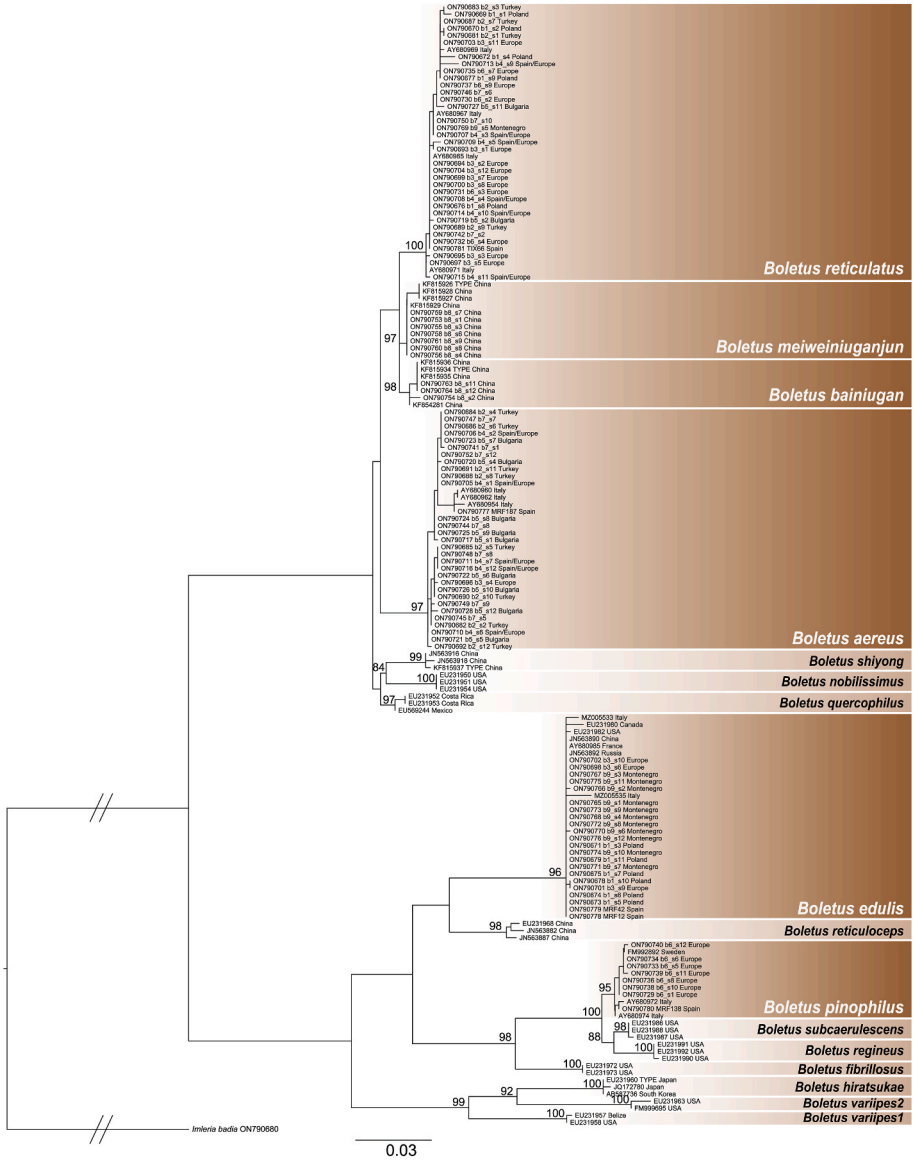

Fig. 3. Maximum Likelihood tree of all sequences identified as *Boletus* spp. (coded as productnumber_samplenumber), reference sequences for *B. edulis*, *B. reticulatus*, *B. pinophilus* and *B. aereus*, from MRF and TIX collections, and sequences recovered from GenBank for *B. bainiugan* (including TYPE), *B. meiweiniuganjun* (including TYPE), *B. shiyong* (including TYPE), *B. nobilissimus* (including TYPE), *B. hiratsukae* (including TYPE), *B. quercophilus, B. reticuloceps, B. subcaerulescens, B. regineus, B. fibrillosus* and *B. variipes*. *Imleria badia* was chosen as the outgroup. Support values for the main clades (<75) are indicated in the pertinent nodes.

species level. One of them seems closely related to the genus *Sutorius*, and the other one to the genus *Tylopilus*. Both genera are present in China, with six *Sutorius* species and thirty-two species of *Tylopilus* known to occur in the country (Y.-C.Li & Yang, 2021). Since no identification could be obtained for these two species, their edibility is questionable and, therefore, should not be included in commercial products destined for human consumption.

Retail prices of dehydrated *Boletus* are highly variable. The products acquired for this study retailed from 30 €/Kg to 295 €/Kg. There doesn't seem to be a relationship between the quality of the dried mushrooms or the presence of *Boletus edulis* with the price per kilogram of each product; the two least expensive products (B1 and B9) contained *Boletus edulis* among other species, and the sporocarps were the least contaminated with worms and pathogens, while the most expensive one (B6) contained sporocarps in good condition, but no samples were identified as *Boletus edulis*. The presence of species other than *B. edulis* in a product labelled as such is, at best, dishonest, and should be taken seriously by the food and health authorities. Not only is it fraudulent to sell lower quality species (such as *Imleria badia*) at a *Boletus edulis*'s price, but many mushroom species considered edible could cause adverse reactions in some people (Gawlikowski et al., 2015), so it is of paramount importance to correctly identify the species in the product labels to avoid potential health risks to consumers.

5. Conclusions

Our study confirms that the use of "*Boletus edulis*" as an umbrella term for Boletaceae fungi in dried mushroom commercial products is widely used, yet it should be considered incorrect as all products analysed in this study contained a mixture of at least two different species. Furthermore, porcini products imported from China contain a mixture of *Boletus* species, but none of the samples taken in this study belonged to the European species of porcini. Therefore, more care should be taken when labelling products, as consumers need to know the species of fungi they are ingesting in order to avoid both fraud and health risks.

The importance of curating the DNA databases is highlighted by our BLAST results, which did not identify *B. bainiugan* and *B. meiweiniuganjun* in our study, even though the type sequences of these species are available online. The presence of two unknown species (*Sutorius* sp. and *Tylopilus* sp.) in a commercial food product is concerning, and proves that there is still a lot of fieldwork left to do in order to complete the barcoding databases and describing the worldwide fungal diversity (Cazabonne et al., 2022).

Dried porcini are often considered luxury products due to their high

prices; therefore, the presence of fungal pathogens and indications of decay and worm infestation is not acceptable. All three of these factors could easily be spotted in the mushrooms during the processing and packaging of the product, so the necessary establishment of control measures to avoid their presence in the final product should be straightforward.

Funding

This work was supported by Xunta de Galicia (Grupo de Referencia Competitiva, ED431C 2020/05). Mauro Rivas-Ferreiro was funded by the Spanish Ministry of Education [grant number FPU20/02280]. Funding for Open Access charge: Universidade de Vigo/CISUG.

CRediT authorship contribution statement

Mauro Rivas-Ferreiro: Conceptualization, literature review, Data curation, Formal analysis, writing. **Alberto Otero:** literature review, Methodology, Formal analysis, writing. **Paloma Morán:** Conceptualization, Funding acquisition, literature review, Formal analysis, writing, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

The authors want to thank Tixiana Albizúa-Cabaleiro for lending us a *Boletus reticulatus* sample from her collection to be sequenced and used as a reference for the species. We would also like to thank Dr. Marisa Castro Cerceda for her ever-present support and helpful advice on the conceptualization of the experiment and discussion of the results. At last, we want to thank the reviewers for the comments and suggestions that helped improve the manuscript.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109346.

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