What's for dinner?: Undescribed species of porcini in a commercial packet

Accurate diagnosis of the components of our food and a standard lexicon for clear communication is essential for regulating global food trade and identifying food frauds. Reliable identification of wild collected foods can be particularly difficult, especially when they originate in under-documented regions or belong to poorly known groups such as *Fungi*. Porcini, one of the most widely traded wild edible mushrooms in the world, are large and conspicuous and they are used as a food both on their own and in processed food products. China is a major exporter of porcini, most of it ending up in Europe. We used DNA-sequencing to identify three species of mushroom contained within a commercial packet of dried Chinese porcini purchased in London. Surprisingly, all three have never been formally described by science and required new scientific names. This demonstrates the ubiquity of unknown fungal diversity even in widely traded commercial food products from one of the most charismatic and least overlooked groups of mushrooms. Our rapid analysis and description makes it possible to reliably identify these species, allowing their harvest to be monitored and their presence tracked in the food chain.

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Introduction

Kingdom *Fungi* is one of the most diverse groups of eukaryotes with estimates ranging from 500,000 to nearly 10 million species, yet they remain vastly underdocumented (Bass & Richards, 2011). Present rates of description, which add on average about 1200 new species annually (Hibbett et al., 2011), are grossly inadequate for the task. Recent attempts to accelerate species description using short, unique DNA sequences 'DNA barcoding' (Hebert et al., 2003) and rapid, short description 'turbo-taxonomy' (Butcher et al., 2012) hold promise for meeting this enormous challenge (Riedel et al. 2013), yet they still remain marginal to traditional methods for formal diagnosis of fungal diversity.

Although taxonomists regard new fungal taxa as commonplace, they are often of little apparent consequence to human society and largely go unnoticed by the public. Like all groups of organisms, our knowledge of fungal diversity is biased towards taxa of greatest concern to ourselves, such as edible fungi. For example, wild mushrooms collected and sold as food around the world generally belong to a handful of well-known taxa (e.g. truffles and chanterelles), most of which have long histories of use in European cuisine. However, even some of these well-known groups have been shown to contain underappreciated levels of diversity. One of these, porcini, has recently been shown to be far more diverse than previously thought (Dentinger et al., 2010; Feng et al., 2012), suggesting the potential for unknown species to end up in the international food supply chain. Although no porcini are known to be poisonous, food allergens have been reported from them (Torricelli et al., 1997; Helbling et al., 2002; Castillo et al., 2013). Therefore, insufficient knowledge of the porcini species contained in food products could pose a health concern.

Porcini are estimated to have an annual worldwide consumption up to 100,000 metric tons (Hall et al., 1998). However, their harvest is restricted to wild foraging since, to date, their cultivation has failed. The high prices for this wild food foraged locally in Europe and North

America has driven the market towards less costly sources, such as China (Sitta & Floriani, 2008). According to the official website of Yunnan Province (www.yunnan.cn), the major exporter of wild mushrooms in China, locally-sourced porcini have been exported to Europe since 1973, and mushrooms of Chinese origin now account for approximately half of all dried porcini in Italy (Sitta & Floriani, 2008). The Chinese species of porcini have been shown previously to be more closely related to European *Boletus aereus* than they are to the core commercial species, *B. edulis*, with which they last shared a common ancestor millions of years ago (Dentinger et al., 2010; Feng et al., 2012).

We set out to identify the contents of dried porcini originating in Yunnan, China, commercially available in the UK using DNA barcoding and generalized mixed Yule coalescent (GMYC) analysis, a widely used approach to delimit species using single-locus data (Pons et al., 2006).

Material and Methods

A packet of dried porcini was purchased from a in southwest greater London in October 2013. Fifteen pieces of mushroom were removed arbitrarily from the packet and DNA extracted using the Sigma Extract-N-Amp kit. The full ITS region of the nrDNA was PCR-amplified using primers ITS1F and ITS4 (White et al., 1990; Gardes & Bruns, 1993). Successful amplicons were purified using ExoSAP-IT (USB, Cleveland, OH) and sequenced bidirectionally using BigDye3.1 with an ABI 3730 (Applied Biosystems, Foster City, CA). Complementary unidirectional reads were aligned and edited using Sequencher 4.2 (GeneCodes, Ann Arbor, MI).

New sequences were combined with 22 related sequences downloaded from GenBank corresponding to “Boletus sp. nov. 2”(EU231965-66; Dentinger et al., 2010)/”Boletus sp. nov. 6”(JN563907-08, -09, -11-13, -17; Feng et al., 2012), “Boletus sp. nov. 3”(EU231964; Dentinger et al., 2010)/”Boletus sp. nov. 7”(JQ172782-83, JN563901-06; Feng et al., 2012), and “Boletus

sp. nov. 5"(JQ563914-16, -18-19; Feng et al., 2012). A total of 38 ingroup sequences and one outgroup sequence (*Boletus aereus*, UDB000940) were aligned using MUSCLE (Edgar, 2004) in SeaView v4.4.0 (Galtier, Gouy & Gautier, 1996) and the terminal gaps converted to missing data. A maximum likelihood tree was generated under a GTR+G substitution model using the Pthreads parallelized version of RAxML v7.0.3 (Stamatakis, 2006; Ott et al., 2007) with nonparametric rapid bootstrapping set to automatically terminate with the 'autoMRE' function. A GMYC analysis using the single method (Pons et al., 2006; Fujisawa & Barraclough, 2013) was conducted with the 'splits' package (v1.0-18) in R version 2.15.0 (R Development Core Team 2009) on an ultrametric tree generated using BEAST v1.8.0 (Drummond et al., 2012). The BEAST analysis applied a rate-smoothing algorithm using an uncorrelated lognormal relaxed clock model (Drummond et al., 2006), the GTR+G substitution model, speciation under a Yule process, the 'ucld.mean' prior set to a gamma distribution with a shape of .001 and a scale of 1000 with all other priors set to default values, and 10 million generations sampling every 1000 generations. An ultrametric starting tree was provided using the best ML tree from RAxML with branches transformed using non-parametric rate smoothing in TreeEdit v1.0a10 on The perl script Burntrees [Nylander J.A.A., http://www.abc.se/~nylander/burntrees/burntrees.html] was used to sample every 98 trees from the stationary posterior distribution in the BEAST analysis after the first 250 were discarded as the burn-in. These 100 trees were imported for Bayesian GMYC (bGMYC) analysis in R (Reid & Carstens, 2012). Twenty-six GMYC models were evaluated within the 95% confidence and significant clusters were described as new taxa using the 'turbo-taxonomy' approach (Butcher et al. 2012), facilitated by the rapid e-publishing tool available through Index Fungorum (www.indexfungorum.org). Voucher material was deposited in the fungarium at the Royal Botanic Gardens, Kew (K) and all sequences were submitted to GenBank (KF815926-937, KF854281-283).

Results and Discussion

The GMYC model with the greatest significant ML score included three ML clusters (1-10 clusters with 95% confidence) plus the root (4 ML entities; 2-23 with 95% confidence). GMYC supports for the three ML clusters were weak, low bGMYC posterior probabilities indicated a substantial level of phylogenetic uncertainty, while the maximum likelihood bootstraps supported reciprocal monophyly (79%, 76% and 100% for each cluster respectively; Figure 1). This result suggests that GMYC may be particularly sensitive to phylogenetic uncertainty, even though it distinguished the same three clades supported by ML bootstrapping. The phylogenetic uncertainty in this dataset is almost certainly caused by a high ratio of autapomorphic substitutions and insertion/deletion events to phylogenetically informative changes. These autoapomorphies translate into longer terminal branch lengths relative to internal nodes, which reduces the distinction of within and between cluster branching patterns, a phenomenon that is known to affect GMYC supports (Fujisawa & Barraclough, 2013). These autapomorphies may indicate true variation in the ITS region, although our own observations suggest they may instead be the result of sequencing and editing errors in the sequences downloaded from GenBank, for which we did not have the original trace files to confirm. Such errors can have large impacts on phylogenetic inference when the number of phylogenetically informative sites is small, such as in ITS sequences of recently diverged fungi, underscoring the importance of careful scrutiny during sequence preparation.

Three species could be identified based on corroboration of ML-supported reciprocal monophyly and GMYC clustering, and these corresponded to lineages previously reported in phylogenetic analyses (Dentinger et al., 2010; Feng et al., 2012, Sitta & Floriani, 2008), but none of which were formally named or described. Review of recent treatments of Chinese boletes also did not provide names for these taxa, which have been treated as a handful of species that occur in Europe and North America (Zang, 2006). New names were formally published on 12 October

2013 (see http://www.indexfungorum.org/Publications/Index%20Fungorum%20no.29.pdf for terse descriptions[1], voucher information, and GenBank accessions corresponding to these taxa).

Together with improvements in single-locus diagnosis leading to more robust inferences of evolutionary significant units (Butcher et al., 2012), rapid survey and diagnosis of vast communities of undescribed diversity is initiating a revolution in taxonomy (Riedel et al., 2013). This is particularly true for *Fungi*, which are hyperdiverse and largely cryptic, requiring indirect detection with environmental sequencing for documenting their true diversity (Taylor et al. 2014, Lücking et al. 2014). As a consequence, a vast quantity of fungal diversity is only known from DNA sequences, and these are accumulating in public databases at incredibly rapid rates (Hibbett et al., 2011). Turbo-taxonomy is an important improvement to efficiency in reconciling molecular diagnosis with a standard application of names that enable universal communication about biodiversity. Together, DNA sequence-based diagnosis and turbo-taxonomy catalyze description of new species, thereby greatly accelerating the rate at which diversity can be documented and recognized. Although descriptions based on features of organisms that are readily observed without specialized techniques are ideal, this is not always possible and descriptions based on features of DNA sequences could be automated to satisfy rules on naming. Automated pipelines that integrate analysis, taxonomy, and nomenclature will soon accelerate this revolution, enabling us to capture the most comprehensive baseline information on global organismal diversity possible. Given estimated rates of species extinction from 0.1-5% per year (Costello et al., 2013), and using recent estimates of global fungal diversity of ~6 million species (Taylor et al., 2014), extinction rates may exceed description rates in *Fungi* by up to 5 times. An 'integrative fast track' approach (Riedel et al. 2013) offers the only tractable solution presently available to filling this

1 The numbers reported in the original descriptions should be multiplied by 2.43 to achieve correct measurements of cells and spores.

knowledge gap. And as has been shown here with the three new species of porcini in a widely available commercial product, this knowledge gap can and does have direct impacts on our lives.

Conclusions

Our analysis of 15 pieces of dried porcini mushrooms from a single commercial packet showed three species corresponding to lineages that although previously reported in phylogenetic analyses have never been formally named or described until now. The recognition of these species enables them to be monitored in foods and facilitates countries' adherence to international agreements on exploitation of wildlife, i.e. the Convention on Biological Diversity.

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Figure 1. Ultrametric tree rooted with *Boletus aereus* and with branch lengths transformed using the uncorrelated relaxed clock model in BEAST. The relationship of *Boletus edulis* to the dataset is depicted using a dashed line. Clades with dark red branches represent the three maximum likelihood clusters in the GMYC model with the greatest ML score calculated using the single method in the 'splits' package in R. Terminal labels in blue represent sequences derived from individual pieces of mushroom sampled from a commercial packet of porcini. Pie charts on branches show maximum likelihood bootstraps ('MLBS'; lightest red), GMYC supports [19] ('GMYC'; medium red), and posterior probabilities of the cluster as calculated using bGMYC ('bGMYC'; darkest red).

Figure 1

What s for dinner?: Undescribed species in commercial porcini from China Ultrametric tree rooted with *Boletus aereus* and with branch lengths transformed using the uncorrelated relaxed clock model in BEAST.

Ultrametric tree rooted with *Boletus aereus* and with branch lengths transformed using the uncorrelated relaxed clock model in BEAST. The relationship of *Boletus edulis* to the dataset is depicted using a dashed line. Clades with dark red branches represent the three maximum likelihood clusters in the GMYC model with the greatest ML score calculated using the single method in the 'splits' package in R. Terminal labels in blue represent sequences derived from individual pieces of mushroom sampled from a commercial packet of porcini. Pie charts on branches show maximum likelihood bootstraps ('MLBS'; lightest red), GMYC supports [19] ('GMYC'; medium red), and posterior probabilities of the cluster as calculated using bGMYC ('bGMYC'; darkest red).