Project Report

Molecular Identification of Mushroom Species in Italy: An Ongoing Project Aimed at Reinforcing the Control Measures of an Increasingly Appreciated Sustainable Food

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Abstract: Proper investment in mushroom production (farming and wild mushroom picking activities) may represent a winning strategy for many countries, including Italy, to better face the problems of food security and environmental impact, and to break away from imports, enhancing the local products. However, the risk related to the consumption of poisoning species requires governments to implement or reinforce effective control measures to protect consumers. Mushroom identification by phenotype observation is hardly applicable if morphologically-similar species, non-whole specimens, or clinical samples are involved. Genotypic analysis is a valid alternative. An ongoing research project involving the Experimental Zooprophylactic Institute of Lazio and Tuscany, the regional Mycological Inspectorate, the Tuscany Mycological Groups Association, and the Department of Veterinary Sciences of the University of Pisa aims to reinforce the collaboration among institutions for the management of mushroom poisoning. The core's project aims to develop an internal genetic database to support the identification of wild and cultivated mushroom species in the Italian territory. The database will include Internal Transcribed Spacer (ITS) sequences retrieved from official databases (the NCBI GenBank and the BOLD system) which are considered to be reliable, after a proper selection process, and sequences from specimens collected directly and identified by expert mycologists. Once it is validated, the database will be available and further implementable by the official network of national laboratories.

Keywords: mushroom; sustainable food; public health; environment safeguarding; consumers protection; official control; molecular identification; Experimental Zooprophylactic Institute

1. Introduction

1.1. Mushrooms as a Sustainable Alternative Food Source

The world population will reach an estimated 9.1 billion in 2050, which is 34 per cent higher than today [1]. Consequently, the world will have to produce 70% more food, and the demand for protein-rich food—such as meat, eggs and milk—will rise sharply. However, agricultural activities (especially croplands and pastures) represent one of the major sources of environmental pollution and waste generation [2]. In parallel, an extensive farmland abandonment has occurred over the last few years, due to many ecological and socioeconomic drivers, as well as to unadapted agricultural systems and land mismanagement leading to soil degradation, frequent flooding, overexploitation, and productivity loss [3]. This phenomenon caused a significant reduction of farmland, with negative

environmental and social consequences, and the decrease of the economic efficiency of the agricultural sector has highlighted the need to develop new strategies to rehabilitate marginal and derelict lands [4]. Looking at all of these scenarios, agri-food supply chains should focus on other available supplementary resources to protect the masses from the malnutrition, contribute to the maintenance of the rural population, enhance the marginal land, and simultaneously safeguard the environment [1,5,6].

In 2015, the United Nations developed the '2030 Agenda for Sustainable Development' and proposed some priority goals, one of which was the reduction of hunger and malnutrition for all, through the achievement of better agricultural productivity and incomes for small farmers, and the promotion of sustainable and resilient agricultural practices that help maintain ecosystems [7]. Mushroom cultivation can be viewed as an effective means to extract resources from agricultural solid recyclable waste; the most significant virtue connected to their cultivation is, in fact, that they can transform agricultural and other organic wastes into nutritious products. This waste bioconversion process is both part of an environmentally sound protection strategy and instrumental in helping to produce protein-rich food that, if properly exploited, may contribute to world sustenance and provide marketable products in impoverished areas [1,8,9]. In addition, they are currently appreciated as fat- and cholesterol-free, low-sodium foods, rich in important nutrients and antioxidants, and are considered to be excellent alternatives for vegetarian meals [5,10,11]. Among other main features, mushroom farming can be very simply carried out with low-cost materials, using low tech methods on a small plot of land that can be managed by a family or a small community, giving abundant chances to the jobless and contributing to the better exploitation of marginal areas [1,8,9]. The enhancement of the mushroom food sector may also help the internal market of countries with special mushroom vocations in breaking away from imports by relaunching the local products. In Italy, where the productions are at a comparatively lower scale in order to match the demands of the internal market, and a huge quantity of mushrooms are imported; farmers should be encouraged to take up mushroom cultivation as a business enterprise. This may also be assumed to be a valid solution to address the youth unemployment issue and curb the rate of rural–urban migration. Given the abundance and the variability of highly-appreciated local species, the activity of picking mushrooms—which mostly plays a recreational role or is essentially addressed at the local sale—should also be raised to a higher market value.

1.2. The Official Control System for the Management of Mushroom Poisoning in Italy

The development of the mushroom sector in Italy, as well as in other countries, may be hindered by the fact that mushroom poisoning cases currently represent a public health concern [12–14]. Several species of mushrooms are in fact known to be poisonous, and their consumption frequently leads to intoxications that can affect the gastrointestinal, neurological, renal, and hepatic systems, depending on the involved species, and in some serious cases, the liver suffers irreparable damage, such that organ transplantation is necessary [15–17]. Most poisoning accidents are due to the incorrect identification of species that is often made through empirical and traditional knowledge, as some poisonous species have a similar physical appearance in color, size, and general morphology to their edible counterparts [16]. In addition, some edible species can be toxic in certain circumstances which may not be predictable [18], especially if they are improperly collected, transported, stored, and cooked [19]. The yearly global incidence of intoxication is not completely defined. However, several studies have provided data on poisoning accidents during the few last years, in American, Asian and European countries [15,20–26], which are not-unusually assumed to be underdiagnosed and/or underreported. In Italy, the Poison Control Centre (PCC) of Milan, which represents the national reference center for mushroom intoxications, received 15,864 requests for advice about poisoning from 1998 to 2017, with 46 recorded deaths; the actual number of cases is estimated to be greater [27].

Despite the specific risk related to mushroom consumption, no dedicated EU legislation on their commerce is currently provided. In order to face this legislation gap and

better manage poisoning incidents within their national territory, European countries are therefore trying to introduce specific legislation [8]. In Italy, the Law of 23 August 1993 n. 352 [28] and the Presidential Decree of 14 July 1995 n. 376 (DPR 376/1995) [29], are the key laws on mushrooms in the national legislation. The first establishes the national guidelines and delegates to the Regions the task of regulating mushroom collection in their territory [28]. The second establishes the market rules of epigeal mushrooms and indicates the wild or cultivated species that can be placed on the market (48 species, listed in Annex I) in their fresh state [29]. The Regions may also integrate the list of species by inserting others that are locally recognized as suitable for consumption, upon notification to the Ministry of Health. Other species from other countries may also be placed on the market, if they are recognized as edible by the competent authorities of the country of origin. The commercialization of wild mushrooms intended for retail (not cultivated ones) is allowed after a health certification provided by the Local Health Authorities (LHA). Currently, around 150 mushroom species are counted in the Italian national list. Moreover, the mycological inspectorates have been set up [29] and placed under the Italian LHA; they are composed of a team of experts in the morphological identification of fungi, and deal with the official control of mushrooms whilst also providing a free recognition service for those picked up by private citizens. In presence of samples that do not show the morphological key features (e.g., non-whole specimens, matrices such as vomit or faeces), additional analytical investigations are carried out by the territorial Experimental Zooprophylactic Institutes (EZIs) [30]. The EZIs, as part of the National Health Service, act as technical and operational tool for animal health, the health control and quality of food of animal origin, farm hygiene, and related activities. The EZIs are located in all Italian Regions, and form a network of public laboratories at the national and regional level. All EZIs laboratories for the official control of feed and foodstuffs are accredited to perform laboratory diagnostics, epidemiological surveillance, research and training in the areas of animal health and welfare, zoonoses, diseases that are communicable from animals to humans, and food safety, in compliance with the quality standards established by the European Union. With the Italian Ministerial Decree of 27 February 2008 [30], the official control activities of unprocessed food of vegetal origin were assigned to EZIs. Despite this legislative framework defining the organizing of the official controls, the high incidence of poisoning cases may be related to the fact that, however, people can be left with some responsibility for their decisions to eat mushrooms they have misidentified. Therefore, the risk related to mushroom collection and consumption by citizens is still not properly managed.

1.3. Traditional and Molecular Approaches to Mushroom Species Identification

Mushroom species identification is traditionally based on the observation of phenotypic characteristics. It is performed by observing the macroscopic structures (the shape of the cap, the margins of the cap, the structure on the surface of caps, the shape of the stipe, and the position of the stipe) or the microscopic structure (spores) under the microscope (by determining their shape, colour and sizes). Spores can also be observed through spore printing, which is used in most mushrooms with a distinct cap. Mushrooms are also classified based on what substrate they get their nutrition from. The role of experts in mycology has been of great weight since ancient times, and their growing skills over the centuries have allowed the refinement of mushroom taxonomy, leading to the current knowledges on these organisms [31]. A proper mushroom identification is fundamental to the avoidance of poisoning accidents; in this respect, the mycologist plays an even greater key role. Factually, recent research in decision-making invites us to consider the use of simple rules of thumb, or "fast and frugal" heuristics, suggesting that good judgments do not necessarily require complex cognitive processing, and that—particularly in environments with high degrees of uncertainty—people often resort to simple heuristics when making decisions [31]. However, the phenotypic characteristics traditionally applied for mushrooms are strongly influenced by the environmental conditions, and they can often

be misleading due to the phenomena of hybridization, cryptic speciation, and convergent evolution [32,33]. Therefore, such a traditional approach, although it is capable of allocating mushrooms at the ordinal or familial level, may not always perform well for lower-level (species) classifications [33,34].

Species identification is especially tricky if non-whole specimens are involved, such as market products or samples examined in the context of clinical diagnosis, which are generally not well preserved [35]. In order to counter the abovementioned difficulties, the use of DNA-based methods has become increasingly popular [33]. The Internal Transcribed Spacer (ITS), a nuclear DNA region nearly 700 bp long and containing two non-coding regions (ITS-1 and ITS-2) separated by the highly-conserved small subunit 5.8S rRNA gene [36] has been chosen as the molecular marker for the identification of fungi due to its high inter-species variation [37]. This molecular marker has been used not only in taxonomical studies but also in the food research field, for the detection of mushroom species in commercial products [38]. As a rule, molecular methods based on genetic marker sequencing rely on comparisons of DNA sequences from publicly available reference libraries, which should be opportunely validated and reliable. This approach has therefore created a surge in the number of mushroom ITS sequences in the NIH genetic sequence database GenBank (https://www.ncbi.nlm.nih.gov/genbank/). In 2012, almost 172,000 sequences belonging to 2500 genera, and 15,500 species were available [37]. In addition, the Barcode of Life Data System (BOLD) database (http://www.boldsystems.org/) is used. This database, which was initially established for biodiversity monitoring [39], is now widely used for forensic purposes [40,41]. However, pitfalls in the abovementioned online depositories have been reported [40–42]. As regards mushrooms, Nilsson et al. [43] proved that about 20% of the entries deposited in GenBank were incorrectly identified at the species level, and that most entries lacked descriptive and up-to-date annotations, especially regarding the taxonomic names. In order to remedy this problem, which was even highlighted in other studies aimed at species identification from other food sources [44–46], a preliminary analysis to ensure the data accuracy by removing the poorly-reliable sequences is recommended [45,46]. On the other hand, BOLD, although it contains more curated data, is still not adequately populated. Another limit in the molecular identification of mushrooms by the use of an online database is that most fungal species that have been described (about 70%) thus far have not been sequenced yet [33]. All of these issues decrease the authenticity of the data and the reliability of the analysis.

2. Project Objective: Reinforcing the Integrated Approach to Ensure Mushroom Safety at the National Level

The EZI of Lazio and Tuscany, the regional Mycological Inspectorate, the Tuscany Mycological Groups Association (TMGA) and the Department of Veterinary Sciences of the University of Pisa implemented a research project that aimed to develop an analytical method to optimize both the management of poisoning incidents and the official control activity of nationally-marketed mushroom products. In particular, the project provides the setup of an ITS-based genetic dataset to support the identification of the wild and cultivated mushroom species in the Italian territory. The output of the project is completely pursuant to the requirement of Article 98 of the Regulation (EU) n. 2017/625 regarding the 'Responsibilities and tasks of the European Union reference centers for the authenticity and integrity of the agri-food chain' [47]. In fact, the EU reference centres for the authenticity and integrity of the agri-food chain are responsible for different tasks, among which is: "Where necessary, establishing and maintaining collections or databases of authenticated reference materials". In particular, the EU reference centres collaborate with the national reference laboratories for the development and the validation of new methods. The project aims to provide technical support to the National Health Service's expert personnel for the identification of mushroom species. Once set up and validated, the internal database will be made available to all of the national EZI network, and its access will be extended to other official laboratories operating at the international level. In addition, it can be subsequently implemented with new reference sequences of species of interest in other

regions. As an operational tool that is able to quickly respond in case of intoxication, it will work as a support for the analysis of clinical samples. In fact, a proper molecular identification can guide the most appropriate medical treatment. Moreover, it can support the official control activities that aim to verify the actual market condition, detect fraud incidents, and safeguard consumers. The project also involves targeted activities aimed at citizens' information: academic lectures within the course of the food inspection of the Department of Veterinary Science of Pisa will be provided, and training courses for pickers, farmers interested in the sector, food business operators (FBOs), and laboratories' technical staff will be organized.

3. Methodologies

The project is articulated into different work packages (WPs). WP1: a bibliographic search aimed at identifying the mushroom species to be included in the ITS-based dataset was performed; in detail, the following species were selected: (1) mushrooms species involved in poisoning incidents that occurred in the Tuscany region during the ten-year period 2007–2017 and reported in a list provided by the Tuscany PCC (when only the genus was listed, all of the congeneric species were considered); (2) species recognized by expert mycologists as morphologically similar to those reported in the PCC list and present in the territory; (3) edible mushroom species that are commonly picked in Tuscany, and other mushroom species (edible, inedible and toxic) that are widely distributed in the regional territory. The Mycological Group, which is especially consulted for points 2 and 3, also reviewed the correctness of the list reported in point 1 by removing some species that, to date, are extremely rare in the regional territory. The correctness of the species nomenclature was also verified by consulting the on-line databases Index Fungorum (http://www.indexfungorum.org/) and MycoBank (http://www.mycobank.org/), owned by the International Mycological Association and constantly updated with all of the mycological nomenclatural novelties. Target species listed with obsolete or non-valid nomenclature were replaced with valid ones. The Mycological Group also contributed to the categorization of the selected species into the following categories: edible, suspected inedible, inedible, toxic, and fatal. WP2: A preliminary analysis of the official genetic databases was performed. In detail, the sequences of the ITS region belonging to all the target species were retrieved from the genetic databases GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.boldsystems.org/). The sequences deposited in BOLD that were mined from GenBank were not retrieved as duplicates belonging to the same specimens. The filtering process was conducted by discarding sequences that did not include both the ITS-1 and ITS-2 regions, and sequences presenting too many degenerated bases. In addition, a phylogenetic analysis was conducted in order to eventually discard wrongly-deposited sequences that belong to misidentified specimens. WP3: The Mycological Group has been especially involved for the collection and phenotypic identification of mushroom specimens to be used for the production of reference sequences. The collection was especially focused on species the sequences of which were less represented after the filtering process described in WP2. In future months, the DNA extraction process, as well as the amplification (primer selection, PCR programs, etc.) and sequencing conditions will be rigorously set up. The newly-produced sequences will be included in the dataset together with those retrieved in WP2.

4. Conclusions

Despite the environmental and socioeconomic benefit that may arise from proper investments in the mushroom sector, the risk related to mushroom consumption requires the government implementation or reinforcement of effective management and control measures for the protection of consumers. In this light, an integrated approach that brings together different competences, such as that proposed in the presented project, represents an important chance for the improvement of the entire mushroom supply chain. In fact, once the dataset is set up, it can represent a valid tool for the National Health Service

to quickly react to poisoning accidents. Overall, 242 mushroom species belonging to 59 genera, 23 families, seven orders, two classes and two phyla were selected to be included in the dataset. Due to the sequence filtering process, 15% of the 7247 ITS-available sequences were discarded. Specimens from over 130 species were collected by the Mycological Group for the production of the reference sequences. Comparing the list of mushrooms species involved in poisoning incidents in the Tuscany region with the intoxication cases reported nationwide, a substantial species overlapping was observed. Therefore, the dataset appeared extremely versatile, and each EZI can include reference territorial species for its improvement. The dataset can be used at the national level if it is further integrated with species that are typical of other regions. An operational tool that is able to quickly respond in case of intoxication is advocated, considering the high incidence (2–3 deaths per year, on average) reported at the national level. Benefits will also arise for FBOs—especially in terms of checks on imported products—such that farmers and citizens can gain more confidence in consuming these kind of sustainable food products.

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