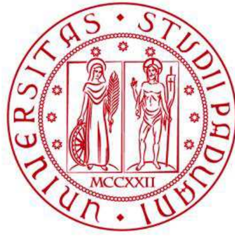

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SERIES XXXI

A next generation sequencing approach for the study of ancient fungal specimens belonging to the Pier Andrea Saccardo collection preserved at the University of Padua

Thesis written with the financial contribution of the Padua University Centre "Botanical Garden"

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ABSTRACT

The mycological collections represent a huge source of molecular information that may be exploited to obtain important DNA data. Indeed, it has been demonstrated that DNA barcoding projects of fungarium material have the potential to enlarge the coverage of species-level DNA sequence information deposited in public databases. However, these collections are an underused resource for building up voucher-based reference datasets, due to the difficulty to obtain DNA sequences from herbarium material. The over one century old Saccardo mycological collection preserved in the herbarium of the Botanical Garden of Padua contains about 70,000 specimens including more than 4,000 type specimens. The types in this collection have been borrowed by mycologists from all over the world for morphological revisions and consequent taxonomic reclassifications, but they have never been involved in sequencing projects so far. Accordingly, the aim of this research was to apply a DNA barcoding approach to obtain internal transcribed spacer (ITS) sequences, the consensus barcode for fungal species identification, from specimens preserved in this collection. This DNA region has been identified as a suitable marker for molecular studies that involve ancient mycological material, because only short DNA regions can be obtained from ancient and degraded DNA. In addition, in case of initial PCR failure of the ITS region, it is possible to increase the amplification/sequencing success by analysing separately the two non-coding regions ITS1 and ITS2 that form the entire ITS. In this thesis work, an Illumina MiSeq sequencing method was applied to recover ITS1/ITS2 sequences overcoming the problems of the high level of DNA degradation of the Saccardo fungarium samples and the presence of contaminations by exogenous fungal DNA. The method required the setup of the steps involved in the sample preparation for the sequencing and in the bioinformatic data analysis, and then its efficacy was first tested to obtain ITS2 sequences from 36 non-type *Peziza* specimens. Despite the presence of both external fungal contamination and cross-contamination between fungarium specimens, this high-throughput sequencing method has permitted to recover ITS2 sequences from 23 out of the 36 specimens studied and also a taxonomic re-evaluation of some samples at the species level and others at genus or higher taxonomic level. Then, this next-generation sequencing (NGS) approach was used to retrieve ITS1/ITS2 sequences from type specimens belonging to the genus *Nectria* and from *Nectria*-like types classified in the collection as members of other genera. Several of these types were morphologically revised in the past by expert mycologists and placed in synonymy with other species or reclassified as members of new genera. The ITS1/ITS2 sequences were obtained

for 25 different types (30 in total considering multiple specimens) out of 76 specimens involved in the study. The combined morphological and molecular data analysis suggests that there is a need to reclassify some *Nectria*/*Nectria*-like types previously reclassified only on a morphological basis and some types never considered for taxonomic revisions. In fact, for 11 types the original species name has been confirmed, for four and five types new nomenclature combinations and synonymies have been proposed respectively, while for other five types the taxonomic assignment has been possible only at genus level. Since type specimens constitute an integral part of fungal classification and nomenclature and given the outstanding and worldwide importance of the Saccardo collection, these findings provide material for a taxonomic revision of invaluable types. Moreover, the method proposed in this research not only has provided an additional scientific value to the Saccardo collection, but it can be applied to obtain important voucher-sequences from problematic herbarium material, thus expanding the databases with well-annotated ITS barcode sequences.

INTRODUCTION

Roles and biodiversity of fungi

The Kingdom Fungi includes an enormous variety of living organisms ranging from microscopic single-celled yeasts to multicellular filamentous moulds and macroscopic mushrooms. It is recognized as an eukaryotic clade sister to the animals (Baldauf and Palmer, 1993), with organisms characterized by an absorptive, heterotrophic nutritional mode that can be accomplished by either saprotrophic, or parasitic or symbiotic life style, and cell walls composed primarily of chitin, a defining feature of this kingdom that, along with the trophic mode, separates it from plants whose cell walls are mostly composed of cellulose (Seifert, 2009). Most fungi grow as filaments with apical growth (hyphae) that branch behind their tips, thus creating a network called a mycelium; however, some grow as single-celled yeasts which reproduce by either binary fission or budding (vegetative reproduction), and some can switch between a yeast phase and a hyphal phase in response to environmental conditions (dimorphic fungi) (Deacon, 2005). Fungi typically have a haploid genome and can reproduce asexually and/or sexually, producing asexual and/or sexual spores that can survive environmental stresses and disperse for long distances. In the first case spores are produced as a result of mitosis, hence they are also called mitospores or conidia, in the second one spores are produced as a result of meiosis and are called meiospores (Deacon, 2005).

Fungi are essential to the recycling of nutrients in terrestrial and marine habitats through their ability to decompose also complex components of the organic matter, such as lignin and cellulose in plant litter (Leonowicz et al., 1999), and many of them establish mutualistic symbiosis with plants to form mycorrhizas, or with algae and cyanobacteria to form lichens (Gadd, 2013). More than 80% of plant species form beneficial mutualistic mycorrhizal associations with fungi (Wang and Qiu, 2006). They can be found in every type of biome on Earth, included those with extreme conditions such as deserts or polar regions and even the bottom of oceans (e.g. Robinson, 2001; Ivarsson et al., 2018; Murgia et al., 2018). Fungi have also economic importance for medical, biotechnological and food industries. Many mushrooms are edible and different species are cultivated for a commercial purpose (e.g. *Agaricus bisporus* also known as champignon; *Pleurotus ostreatus* also known as oyster mushroom), others are used to produce several foods and beverages, including alcoholic drinks. Many fungi produce antibiotic substances like penicillins, steroids that can be used for contraceptives, and ciclosporins used as immunosuppressants in transplant surgery; others are used as commercial biological control agents against insects, nematodes or plant-pathogenic

fungi, providing alternatives to chemical pesticides (e.g. Siddiqui and Mahmood, 1996; Romero et al., 2003). Fungi also comprise pathogens causing diseases in plants, animals and humans (Klein and Tebbets, 2007). In particular, the last few decades have seen an increasing number of virulent infectious diseases in natural populations and managed landscapes. In both animals and plants, an unprecedented number of fungal diseases have recently caused some of the most severe die-offs and extinctions ever witnessed in wild species, are jeopardizing food security, and have led to an upsurge of fatal outcomes of invasive infections in humans (Fisher et al., 2012; Debourgogne et al., 2016). Therefore, understanding the fungal dimensions of biodiversity is not solely an academic exercise; it also has direct implications for the welfare of human societies (Peay et al., 2016).

Recently, Spatafora et al. (2017) recognized eight phyla within the fungal kingdom: Ascomycota, Basidiomycota, Zoopagomycota, Mucoromycota, Blastocladiomycota, Chytridiomycota, Microsporidia and Cryptomycota (**Figure 1**). In addition to the eight phyla, they proposed 12 subphyla and 46 classes. Ascomycota and Basidiomycota which together form the subkingdom Dikarya (i.e. fungi characterized by having a portion of their life cycle with paired nuclei, the dikaryon, representing two different mating compatibility groups), are globally recognized as the two largest and well-known fungal phyla (Hibbett et al., 2007). However, it seems that the infra-kingdom classification of this taxon is much more complex. In fact, Tedersoo et al. (2018) proposed an alternative higher-level classification of Fungi comprising nine subkingdoms, 18 phyla, 23 subphyla, 74 classes, 215 orders, 731 families and 5377 genera. This wide difference of opinion shows how the real subdivision of this kingdom is still not clear and debated.

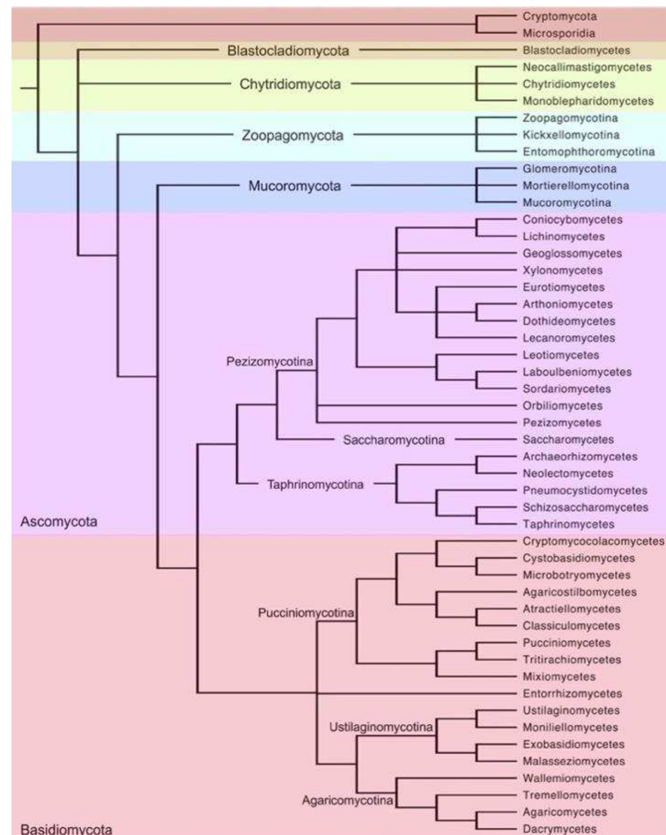

Figure 1. Fungal tree of life based on published multi-gene and genome-scale phylogenies. Adapted from Spatafora et al. (2017).

As it regards the number of existing fungal species, numerous studies have been conducted to estimate the biodiversity of this kingdom. In 1991, Hawksworth published a landmark paper providing an estimate of the number of fungal species on Earth based primarily on vascular plant: fungus ratios in different regions (Hawksworth, 1991). The estimate of 1.5 million species was accepted as a reasonable working hypothesis; however, it was later considered too conservative by Hawksworth himself (Hawksworth, 2012). Other estimates of the global number of fungal species, based on data deriving from different molecular approaches, have predicted as many as 5.1 million fungal species (O'Brien et al., 2005; Blackwell, 2011) with a minimum of 712,000 species (Schmit and Mueller, 2007). However, a recent revision of fungal diversity proposes to replace previous estimates of global fungal species richness with a range of 2.2 to 3.8 million, calculated on the basis of plant/fungus species ratios and DNA studies of environmental samples, that exceeds the estimated number of plants by more than six times (Hawksworth and Lücking, 2017). Despite Fungi being one of the most important groups of organisms on the planet, and notwithstanding they crucial roles played by them in ecological systems (Fisher et al., 2012; Martínez-García et al., 2017; Bani et al., 2018), in terms of biodiversity it is a group of poorly

described organisms and only 120,000 of the estimated fungal species have been formally described so far (Hawksworth and Lücking, 2017).

The gap between morphological description and molecular information

Morphological characters, such as the characteristics of the spore-producing structures or of the sexual/asexual spores, have long been used as the sole features taken into account in mycological taxonomy. If two fungal organisms showed differences in reproductive morphology, then they were classified as two different species. In the same way the species were grouped in genera or higher taxonomic ranks based on differences in one or more morphological characters considered to be of particular discriminatory value. Many monographs using morphology alone to separate families, genera, species and important higher-level taxonomic groups based on morphological traits were published (Hyde et al., 2011). However, the characters considered useful for the classification often varied among mycologists thus causing continuous taxonomic rearrangements in many groups of fungi.

Even today a valid description of a fungal species requires a detailed morphological characterization and morphological approaches are still used to classify fungi at order- or family-level. Nevertheless, relying only on phenotypic characters for the identification of fungi at species level can be problematic. For example, different fungal species can have indistinguishable morphological characters due to hybridization (Olson and Stenlid, 2002), cryptic speciation (Salgado-Salazar et al., 2017) or convergent evolution phenomena (Brun and Silar, 2010). A morphological identification is also difficult in fungi with a dimorphic life cycle, such as in yeast-mycelial transitions, that can prevent a correct morphological identification to species-level or in fungi which do not sporulate in culture, not providing phenotypic characters for the identification (Raja et al., 2017). As a consequence, DNA sequence-based identification (DNA barcoding) has emerged for identifying fungal species and now DNA sequences are routinely used in fungal taxonomy at all levels, phylogenetic inference and species identification (Kõljalg et al., 2005; Hibbett et al., 2007).

DNA sequence-based identification has revealed that several morphological characters previously considered to be indicative of relatedness do not necessarily predict phylogenetic relations (Lumbsch and Huhndorf, 2007) or, on the contrary, that different species defined on the basis of morphological features exhibit the same DNA sequences in many loci. Many fungal species, for instance in the Ascomycota, present a life cycle characterized by morphologically different asexual and sexual reproductive stages, called anamorph and

teleomorph respectively. The two forms of the same fungus may have two different Latin names (dual nomenclature) because, when describing new species, mycologists recover and describe mostly only one of the two stages; accordingly, the chance of describing and classifying the asexual state for an already existing and classified sexual fungus is high. Nevertheless, the application of molecular approaches is providing useful information for establishing anamorph–teleomorph connections, allowing to recognize asexual and sexual forms of the same fungus as the same species (e.g. Liu et al., 2002). In addition, using molecular information, cryptic species have been discovered within already described morphological species (e.g. Salgado-Salazar et al., 2017).

Despite the important increasing role of DNA sequences in fungal systematics and taxonomy, at present there is a big gap between fungal species description and molecular information deposited in public databases. For approximately 85,000 of the 120,000 described fungal species there is no DNA sequence in public DNA databases so far. This means that only 35,000 correctly identified fungal species are represented by DNA sequences, corresponding to a mere 1% of the estimated total number of species (Hawksworth and Lücking, 2017). This lack of fungal molecular information was also observed by Hibbett et al. (2011) who estimated that 74.4% of the 11,960 newly described fungal species catalogued by *Index Fungorum* (http://www.indexfungorum.org/) from 1999 to 2009 were not represented by molecular sequences.

On the other hand, metagenome sequencing of environmental samples is providing scientists with a large number of datasets composed of internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA, the molecular marker mostly used in fungal studies and recently recognized as primary DNA barcode for the identification of fungal species (Schoch et al., 2012), but many of them have no formal species or higher-level taxonomic annotation. Environmental metabarcoding analyses would also have the potential to discover new fungal species (Hibbett et al., 2009). However, without physical specimens such sequences cannot be named as formal species because voucherless data are not reproducible and the species hypothesis cannot be tested by other researchers (Thines et al., 2018; Zamora et al. 2018).

Thus, the linkage of curated DNA sequence data to expertly identified voucher specimens is a fundamental step to fill the present gap between the different sizes of described and sequenced fungal diversity and, in this context, the fungal collections preserved in the herbaria worldwide represent a huge resource for obtaining voucher-sequences (Brock et al., 2009; Puillandre et al., 2012; Osmundson et al., 2013). In addition, voucher-sequences from

herbarium specimens can contribute to the identification of unidentified fungal sequences deposited in DNA databases (Nagy et al., 2011).

Fungal DNA barcoding

The DNA barcoding concept was proposed for the first time by Paul Hebert (Hebert et al., 2003a) as a tool for a rapid and accurate species identification based on short standardized genomic regions named DNA barcodes. Recognizing the potential of this approach, the international initiative Consortium of Barcode of Life (CBOL, http:// barcoding.si.edu) was established in 2004, which include as members natural history museums, zoos, herbaria, botanical gardens, university departments, research organizations, governmental and intergovernmental agencies with the aim to promote DNA barcoding as a global standard for the identification of biological species (Savolainen et al., 2005; Waugh, 2007). This initiative has also developed the Barcode of Life Database (BOLD, http://www.boldsystems.org) a database for the acquisition, storage, analysis and publication of DNA barcode records to provide an accurate tool for species identification. In addition, also DNA trace files (chromatograms) must be deposited together with sequences, and voucher specimens must have complete taxonomic information, place/date of collection and images (Ratnasingham and Hebert, 2007). The initial application of DNA barcoding only to the animal kingdom was rapidly extended to plants, fungi and other groups of organisms with the idea of building reference DNA barcode libraries from individuals of recognized identity for all the eukaryotic life (International Barcode of Life Project; iBOL; http://ibol.org/).

DNA barcodes are used not only to identify species already described, retrieving in this way information about them, but also to facilitate the identification of species yet to be named (Kress and Erickson, 2012). An ideal DNA barcoding region must satisfy three main criteria: 1) it has to be of appropriate sequence length (400-800 bp) in order to facilitate the capabilities of DNA extraction and sequencing, 2) it must possess conserved flanking regions for developing universal PCR primers for a wide taxonomic application, and 3) it must be different in individuals of different species and very little variable among individuals of the same species (barcode gap) (Kress and Erickson, 2008; Valentini et al., 2009).

A fragment of about 650 base pairs (bp) of the mitochondrial gene cytochrome c oxidase subunit 1 (*COI*) was proposed by Hebert et al. (2003b) and immediately accepted as standard DNA barcode for the identification of species across the whole animal kingdom. Its

suitability has been well demonstrated for many animal groups (Hebert et al., 2004; Ward et al., 2005; Wilson et al., 2017); however, it has not proved useful for plants and fungi.

In plants the *COI* sequence evolves too slowly, thus resulting very similar also in distantly related families (Kress and Erickson, 2007). A combination of the two plastid genes ribulose-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) has been recognized as the core barcode for land plants (Hollingsworth et al., 2009b). In addition, *trnH-psbA* and the ITS region were suggested as supplementary loci to increase the level of species resolution (Shaw et al., 2007; Li et al., 2011).

In fungi the *COI* was excluded as universal barcode because of the variable and unpredictable presence of introns within the region, the difficulty in designing universal primers, the lack of sequence divergence among some fungal species and the lack of mitochondria in some fungal lineages (Bullerwell and Lang, 2005; Gilmore et al., 2009; Santamaria et al., 2009). In order to find a DNA region useful for fungal species identification, Schoch and colleagues evaluated three subunits from the nuclear ribosomal RNA gene cluster (ITS; nuclear ribosomal small subunit rRNA gene, SSU or 18S; and nuclear ribosomal large subunit rRNA gene, LSU or 28S) and three protein-coding genes (largest subunit of RNA polymerase II, *RPB1*; second largest subunit of RNA polymerase II, *RPB2*; and minichromosome maintenance protein, *MCM7*) as possible barcode candidates (Schoch et al., 2012). They found that ITS outperformed the other tested molecular markers in term of primer universality, amplification success and species discrimination power therefore they proposed this marker as universal barcode of fungi. After this work the ITS was recognized as the official barcode marker for species-level identification of fungi by the CBOL. Interestingly, previous studies had already supported its suitability as fungal barcode (Dentinger et al., 2011; Kelly et al., 2011).

The fungal ITS region varies between approximately 450 and 750 bp and consists of two highly variable non-coding ITS1 and ITS2 regions separated by the 5.8S coding gene (Blaalid et al., 2013). This region is situated between the small-subunit (SSU; 18S) and the large-subunit (LSU; 28S) coding genes of the nuclear ribosomal (rDNA) operon (**Figure 2**). The overall rDNA operon includes also the intergenic spacer region (IGS), which contains the external transcribed spacer regions ETS1 and ETS2, and variably the 5S coding gene may be present within the IGS. All the coding rDNA genes are necessary for the formation of RNA molecules, which have a structural role in the ribosome (ribosomal RNA; rRNA); however, ITS1 and ITS2 are transcribed together with the coding genes but removed in the post-transcriptional processing of the rRNA. Even if the two ITS regions do not code for ribosome

components, several studies have shown that removal of the one/two ITS prior to the initiation of transcription has a deleterious effect on the formation of mature rRNAs (Iwen et al., 2005).

Thanks to the rapid evolutionary rate of the two non-coding regions the ITS has been used in both molecular systematics and ecological studies of fungi even before being declared as the consensus fungal barcode. The adoption of this region in fungal studies started over twenty years ago when White et al. (1990) published a set of primers to amplify and sequence different regions of the rDNA operon. This set comprised several universal primers (named ITS1, ITS2, ITS3 and ITS4), complementary to the conserved SSU, LSU and 5.8S sequences, for the amplification and sequencing of either the entire ITS or the ITS1/ITS2 regions separately, that are still the most used by the mycological community (White et al., 1990). The presence of the highly conserved genes has subsequently allowed the design of other primers for the ITS1 and ITS2 regions with a wide range of fungal targets to use in high-throughput DNA barcoding studies of fungal communities in various environmental samples, such as mycorrhizae, leaves, and soil (Martin and Rygiewicz, 2005; Toju et al., 2012). The presence inside the fungal genome of multiple tandemly repeated copies of the rDNA operon (including ITS) makes the ITS amplification relatively successful also from small amounts of DNA (Xu, 2016) or from ancient herbarium specimens where the DNA results heavily degraded (Osmundson et al., 2013). Another major advantage of using ITS is that public databases (GenBank, Unite) contain a large quantity of ITS reference sequences as compared to other fungal molecular markers.

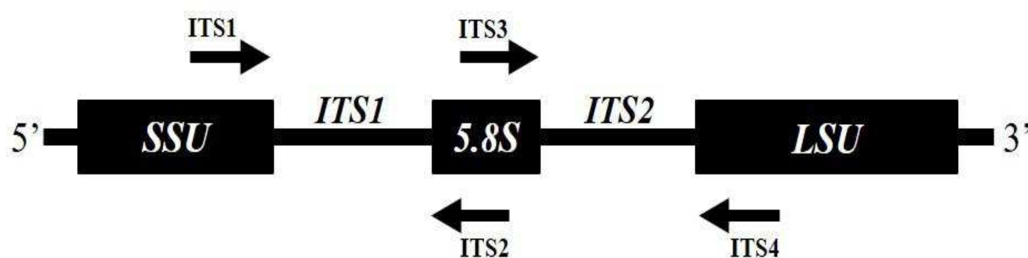

Figure 2. The internal transcribed spacer region of fungal rDNA. ITS includes two non-coding regions ITS1 and ITS2 separated by the 5.8S coding gene, flanked by the SSU and LSU coding genes. In the figure the approximate positions of the most commonly used ITS primers are reported.

Despite its recognition as the primary barcode sequence and its wide use in fungal biodiversity studies, the ITS region presents some drawbacks. It has been demonstrated that the ITS does not always work well for species discrimination. For instance, this region alone

is not variable enough to allow a correct species identification in some Ascomycota species-rich common genera such as *Cladosporium*, *Penicillium*, *Aspergillus* and *Fusarium* (Geiser et al., 2004; Schubert et al., 2007; Samson et al., 2014; Visagie et al., 2014). Other complications are linked to the inability of the most commonly used universal primers to amplify the ITS in all fungal lineages and to the presence of sequence variability among specimens of the same species and among ITS copies inside the genome of a single individual which may complicate the species identification (Smith et al., 2007; Nilsson et al., 2008; Schoch et al., 2012). As a result, the idea of combining ITS with other molecular markers (multi-locus approach) is increasingly growing among mycologists and many studies have been carried out to identify other DNA regions with suitable barcode characteristics, some focusing on specific fungal groups, others on a broad taxonomic coverage.

Protein-coding genes are commonly utilized when the ITS region is not sufficient for species identification, like for the Ascomycota genera mentioned before, and in fungal phylogenetic studies due to their better resolution at higher taxonomic levels compared to ITS (James et al., 2006; Hibbet et al., 2007). Among the protein coding genes, the first and second largest subunit of the RNA-polymerase II gene (*RPB1*, *RPB2)*, translation elongation factor 1-alpha (*TEF1α*) and beta-tubulin (*TUB2*) are used for identification and phylogenetic analysis. The combination between ITS and D1/D2 domains of the LSU is the most used as a method to discriminate fungal species and to address the phylogeny of higher ranks at the level of families and orders (Badotti et al., 2018).

Recently, a group of mycologists have tested different ribosomal and single-copy protein-coding markers in more than 1500 Ascomycota (Pezizomycotina, Saccharomycotina) and Basidiomycota (Agaricomycotina, Pucciniomycotina, Ustilaginomycotina) species of economic, phytopathological and clinical importance to assess potential candidate regions as secondary DNA fungal barcode. Among all the loci used, the authors suggested *TEF1α* as potential universal second barcoding gene and developed a novel and high-fidelity primer pair for its amplification in a broad spectrum of fungi (Stielow et al., 2015). However, a universal secondary fungal barcode has not been chosen yet and, for taxa where ITS is ineffective for identification, additional taxon-specific barcodes are being continuously tested and developed.

Fungal DNA databases

For an effective fungal identification, the mycological community needs public and well-annotated fungal DNA sequences and GenBank (https://www.ncbi.nlm.nih.gov/genbank/) represents the major repository of fungal ITS sequences produced. For the purpose of DNA-based identification, BLAST best-hit analysis (Altschul et al., 1997) is the method commonly used to verify the identity of fungal specimens. However, it has been demonstrated that BLAST search identification must be made with caution because the taxonomic reliability in GenBank seems not to be fully satisfactory due to the lack of completely annotated sequences at species-level and to the presence of misidentified sequences and named sequences coming from unpublished works that could be incorrect (Bridge et al., 2003; Nilsson et al., 2006). In addition, a substantial proportion of the publicly available sequences are chimeric or contain numerous erroneous bases or ambiguities (Abarenkov et al., 2010).

Many well curated ITS databases in terms of sequence quality and taxonomic annotation were developed and some of them are specific for certain groups of fungi (e.g. the ISHAM database for human and animal pathogenetic fungi; Irinyi and Meyer, 2015). The BOLD database (Ratnasingham and Hebert, 2007) contains some curated ITS data from fungi, but the number of sequences is very low and many of them mined from GenBank. So, this repository suffers from similar problems as GenBank such as a not fully identified sequences (Xu, 2016). Together with GenBank, another main repository of fungal sequence data is represented by UNITE (http://unite.ut.ee/; Kõljalg et al., 2005). This database, initially focused on mycorrhizal species and then expanded to other groups of fungi, is often used to identify ITS sequences in fungal community analysis (Lindahl et al., 2013). It provides identification based on reference sequences curated by experts and species hypotheses for unknown sequences based on sequence similarity (Kõljalg et al., 2005). In addition, the fungal ITS sequences are accompanied by reference identification data, collector/source and ecological data.

Given the variety of databases available and the problems linked to not well annotated sequences, the best practice is to compare the results obtained from the sequence-comparison across more than one database before defining the taxonomic affiliation of a new sequence (Raja et al., 2017).

Importance of fungaria in DNA sequencing projects

The mycological collections (or fungaria) conserved worldwide in herbaria are considered a great source of genetic material and the collection-based sequencing projects are assuming an important priority in the coming decades because these collections can host a large number of unknown or under-represented fungal lineages.

Brock et al. (2009) assessed the potential impact of systematic DNA barcoding projects from fungal herbarium collections by sequencing a set of specimens from the fungal herbarium of the Royal Botanic Gardens at Kew (UK). After the bioinformatic analysis of the 279 ITS sequences obtained, it was estimated that about 70% of the diversity in the fungal herbarium sample was not represented in molecular databases, and a further 10% of the sequences matched solely to environmental/unidentified fungi. Similarly, Osmundson et al. (2013) tried to get barcode sequences for the largest proportion of the approximately 6,000 macrofungal species preserved in the herbarium of the Museum of Natural History in Venice (IT). The obtained 1,107 ITS sequences included 416 unrepresented taxa and 398 sequences exhibited a best BLAST match to unidentified environmental sequence. These works significantly enriched the taxon representation in public databases for ITS sequences and showed also the importance of DNA barcoding projects of vouchered herbarium material to reduce the existing gap between species description and DNA sequence information in fungi.

In addition, ancient fungaria collections may contain type specimens (i.e. specimens selected to serve as a reference point when a species is first named), in particular holotypes (the single specimen designated as the type of a species by the original author at the time when the species name and description was published). The type specimen is the original material from which the description of a new species is made, and, for this reason, it is scientifically unique, invaluable and irreplaceable. Types represent the only certain link to a specific Latin binomial name, therefore, the eventual existence of molecular information from these important samples might help to solve taxonomic controversies (Liimatainen et al., 2014).

Challenges associated with fungarium material

Fungaria are collections of dried fungal specimens with accompanying data on labels and are usually organised systematically according to a classification system. Although fungaria, such as the herbaria preserved worldwide, potentially represent an invaluable source of material for

molecular analysis, it is well known that obtaining molecular information from very old biological samples can be extremely difficult.

Ancient DNA (aDNA) is typically highly degraded into small fragments whose size is on average between 100 and 500 bp. After an organism's death, the degradation of DNA begins due to the exposure to intracellular nucleases and microorganisms, but also because of oxidative stress. In the living cells DNA is continuously damaged because of oxidative stress, but the overall integrity of DNA molecules is maintained by specific enzymes which repair these damages. When plant or fungal specimens die after the field collection, the cellular compartments where catabolic enzymes are normally sequestered break down, the enzymes are released, and the degradation of DNA begins rapidly. However, enzymatic and microbial degradation processes can be reduced with a rapid desiccation of the specimens after death. The DNA in non-living cells is subject to the same chemical processes that damage the DNA in the living cells, but the absence of a functioning repair systems causes an unavoidable DNA degradation (Pääbo et al., 2004). Besides the natural degradation of DNA that occurs in the herbarium specimens, it has been demonstrated that most of the DNA integrity depends mainly on the drying and storage methods used rather than on the age of the herbarium material. This is especially true for very old herbaria where the conservation techniques were not intended to preserve the DNA but only the specimens' morphological traits. The adopted drying methods can have different effects on the DNA quality (Staats et al., 2011) and the use of many chemicals as pesticides can lead to a high-level degradation of the specimens' DNA. For instance, fumigants were used to prevent insect damage in the herbaria and among them the mixture of methyl bromide and ethylene oxide has the worst effect in terms of DNA degradation (Kigawa et al., 2003). The conservation treatments commonly used in the past on botanical collections, using substances for their fungicidal and biocide properties, are known to have a detrimental effect on DNA integrity, therefore they can affect both extraction and amplification of herbarium DNA (Purewal, 2012).

Another great problem relative to fungarium/herbarium material is the presence of DNA contamination from external sources that makes it challenging to obtain molecular information from herbarium DNA sequencing. This contamination may occur in various circumstances: during the collection of the specimens, during the storage in the herbarium, while the samples are on loan and during the DNA manipulation processes (Verkley et al., 2015). Ancient collections are frequently contaminated by organisms like microfungi or bacteria (Brock et al., 2009; Osmundson et al., 2013). Moreover, the specimens are often not properly handled and conserved in sealed containers, but they are frequently stored in close

proximity to one another on a single herbarium sheet and then stacked in packets. This close contact between different specimens enhances the probability of cross-contamination between the samples in the packages (Yeates et al., 2016). Exogenous DNA is a complication that affects DNA barcoding projects involving ancient herbarium collections, since the presence of contaminant DNA can lead to an overrepresentation of non-target sequences during the amplification step with the consequence of either a reduced or no sequencing success. In particular, this problem is much more critical for mycological collections than for plant herbaria because normally the contamination is represented by other fungal species and the universal primers cannot discriminate between the contaminating fungal DNA and the target DNA, thus generating a mix of fungal sequences that enhance the risk of sequencing failure (Brock et al., 2009) and/or specimen misidentification.

In addition, the amount of fungarium material usable for molecular analysis is often limited because many specimens are poor in available tissue and because the sampling to yield a sufficient amount of DNA can in some cases cause irreparable damage to the herbarium specimens. When a specimen is used for molecular analysis an amount as small as possible of material should be sampled in order to preserve the specimen, and this is particularly true for type specimens. For instance, in type specimens of ascomycetous microfungi the sexual reproductive structures (ascomata) are often few, small and degraded, therefore they cannot be sampled for molecular analysis to avoid the risk of destroying them completely (personal observation). Herbarium types borrowed from other institutions for morphological revisions or molecular analyses have often been completely destroyed; so, this problem has led many herbaria, including that of the Botanical Garden of Padua, to stop the loans of the samples outside.

Since only short DNA regions can be successfully obtained from degraded DNA, the multicopy nature of the ITS region and its limited length make this region the most suitable marker for molecular studies that involve ancient mycological material. In addition, in case of initial PCR failure to amplify the entire ITS region, it is possible to increase the amplification/sequencing success by analysing the ITS1 and ITS2 separately. The ITS region was successfully amplified from a lichen herbarium sample older than 100 years (Redchenko et al., 2012) and from the type specimen of *Hygrophorus cossus* (Sowerby) Fr. (Basidiomycota, Agaricales) which had been collected in 1794 (Larsson and Jacobsson, 2004), demonstrating that ancient collections represent a source of material for molecular studies notwithstanding all the problems mentioned before.

In this context, the Pier Andrea Saccardo mycological collection conserved in the herbarium of the Botanical Garden of the University of Padua can represent a rich source of specimens suitable for a DNA barcoding project, thanks especially to the presence of a huge number of type specimens.

Pier Andrea Saccardo and his mycological collection

Pier Andrea Saccardo was born in Volpago del Montello (Treviso) on 23 March 1845 and, since an early age, he was interested in nature, in particular in botany (**Figure 3A**). He studied in Venice, Padua and Treviso and in 1864 he enrolled at the University of Padua, attending the two-year medical course, graduating in 1867 with honours in Philosophy (at that time the philosophy faculty comprised also the natural sciences). In 1866, when he was a student, Saccardo was exceptionally appointed by Roberto De Visiani, Professor of Botany at that time, as a temporary assistant to the chair of practical and theoretical botany, a task which took on a stable character the following year.

In 1872, while he collected materials in the Botanical Garden of Padua for botanical studied, Saccardo observed a large number of fungal species which encouraged him to undertake studies in the mycological field. In fact, the next year, he published his first mycological work *Mycologiae Venetae Specimen* and, between 1873 and 1877, six series of *Fungi veneti novi vel critici*, bringing the number of fungal species known in Veneto from 250 to 1,500.

In 1877 he established *Michelia*, a mycological journal in which many of his early papers were published, and two years later he became Professor of Botany at the University of Padua where he was the director of the University Botanical Garden (1879-1915). He also investigated fungi from other parts of Italy, publishing, for instance, *Fungi italici autographice delineati* (1877-1886), a work with 1,500 colour illustrations of microfungi; fungi from other parts of Europe and also exotic fungi. In a very short time, he became one of the most important reference point for the Italian and International mycology, developing a dense network of collaborations and exchange of letters and fungal material with mycologists from all over the world. He also acquired experience with the systematics of different groups of fungi, in particular Ascomycota. With the first work about Pyrenomycetes published in 1875, he devised a classification system called “carpological system”. This classification method based on the morphological characteristics of the sexual reproductive structure

(fruiting body, sporophore), in particular on the features of the spores inside the fruiting body (shape, colour and dimension), was used until 1950.

The greatest Saccardo's contribution to mycology was given with his *Sylloge Fungorum husque cognitorum omnium* (1822-1931), a monumental work in 25 volumes, where all the fungal species known at that time (78,316 different species) were nominally collected, accompanied by a brief description and arranged systematically. Saccardo personally supervised the drafting of the first twenty volumes; then, when he died in 1920, the work was completed by his collaborators with other five volumes in 1931. Thanks to this work he was considered the "Linnaeus of fungi" (Lazzari, 1973; Corte et al., 2012).

After his death (1920), Saccardo left his personal mycological herbarium in addition with a mycological library and a rich chart of mycological arguments. The collection, preserved in the herbarium of the Botanical Garden of the University of Padua (**Figure 3B**), reflected Saccardo's passion for Ascomycota which make up the majority of the collection, and represented the foundation of all his scientific publications, most of which were committed to the description of new species. The herbarium was first put together by Saccardo with samples collected around Treviso and Padua (Botanical Garden and surroundings); then it was enriched with Italian specimens sent to him by colleagues and with mycological collections edited by researchers around the world. The collection, started around 1874, is composed by almost 70,000 specimens comprising over 18,500 different species. However, the real scientific value of this collection consists in more than 4,000 type specimens, mostly assigned to Ascomycota. These specimens have a huge scientific importance because they were used to describe a new species for the first time. They have long been borrowed and used by mycologists from all over the world for morphological studies and taxonomic revisions, but now it is no longer possible to send the Saccardo herbarium material outside of the Botanical Garden of Padua because some types were irreparably damaged or lost after the loan. Furthermore, the herbarium includes approximately 65 more or less complete mycological *exsiccata* and special collections and specimens from more than 40 private herbaria (Gola, 1930). Inside the herbarium, the fungal dried material was placed in paper bags and stored on herbarium sheets subdivided into packages representing the different genera to which the fungal specimens were assigned. The fungal specimens in the collection are also accompanied by a papery label with the name of the fungal species and the place and the year in which the specimen was collected (if known). In many papery labels Saccardo reported with pencil drawings the morphological characters, especially those of spores, used for the specimen identification (**Figure 3C, D, E**). In addition, the samples inside the

collection were stored for optimal conservation of the specimens' morphological traits and not to preserve the DNA integrity. In fact, up to twenty years ago, the entire collection was treated with fumigants twice a year (herbarium curator communication), while the samples were singularly treated with dichlorodiphenyltrichloroethane (DDT), naphthalene or with mercuric chloride (information reported in some papery labels and through direct observation of DDT/ naphthalene crystals above the samples). All these treatments are known to have negative effects on DNA (Kigawa et al., 2003; Purewal, 2012).

Figure 2. A Pier Andrea Saccardo; **B** Herbarium of the Padua Botanical Garden where the Saccardo collection is preserved; **C** Package with fungal specimens belonging to a specific genus; **D** Herbarium sheet with fungal specimens; **E** Image of a fungal specimen preserved in the collection with drawings of some morphological traits**.**

Purpose of the research

The mycological collections around the world contain fungal specimens of great historical and scientific value that represent a remarkable and irreplaceable source of information. The large repositories of identified specimens preserved in the mycological collections provide the opportunity to fill the existing gap between the number of described fungal species and their molecular information. In fact, it has been demonstrated that DNA barcoding projects of vouchered herbarium material have the potential to enlarge the coverage of species-level DNA sequence information deposited in public databases with new vouchered-sequences and ameliorate the problem of unknown sequences coming from fungal community analysis (Brock et al., 2009; Osmundson et al., 2013). Despite their importance, the mycological collections are an underused and underrated resource for building up voucher-based reference datasets, and this because of to the difficulty to obtain good DNA sequences from herbarium material.

The Saccardo mycological collection contains about 70,000 specimens including more than 4,000 type specimens. These specimens have always been borrowed from expert mycologists for morphological revisions and consequent taxonomic reclassifications, but they have never been involved in sequencing projects before. Accordingly, the aim of this research was to apply a DNA barcoding approach to obtain important molecular information mainly from the type specimens preserved in the Saccardo collection. To recover ITS sequences from fungal specimens stored in this collection, I applied a high-throughput sequencing method (Illumina MiSeq 2 x 300 bp) to try to overcome the problems linked to the high degradation of the herbarium DNA and to the presence of a huge exogenous DNA contamination observed with the first results based on the conventional Sanger- and cloning-based methods. This method is commonly used in fungal community analysis because fragments in the size range of ITS1 or ITS2 (about 300 bp; Tedersoo et al., 2015) can be easily sequenced on the Illumina MiSeq platform (Schmidt et al., 2013) and, to optimize its use, different tagged amplicons from several samples can be mixed and sequenced in a single run (Lindahal et al., 2013). To verify the efficacy of the designed method, it was first tested to obtain DNA sequences from non-type (therefore less precious) specimens and then applied to more precious type specimens. In addition, this work fits into a larger project that aims to provide an additional scientific value to the Saccardo collection by building a DNA reference-database accessible to other scientists from all over the world without having to send the specimens to other laboratories, thus avoiding the risk of losing precious samples as it sometimes happened in the past.

CHAPTER 1

Next Generation Sequencing of Ancient Fungal Specimens: The Case of the Saccardo Mycological Herbarium

Mycological herbarium collections are considered an important source for fungal DNA-barcoding but, unfortunately, ancient herbarium samples have both time and conservation related DNA damages, besides exogenous DNA contamination, that make nucleic acid extraction, amplification and sequencing challenging. In this chapter, the results of DNA extraction, ITS2 amplification and Illumina MiSeq sequencing of 36 specimens from the Saccardo Mycological Herbarium assigned to the genus *Peziza* are reported. High-throughput sequencing was chosen as an alternative to the conventional Sanger- and cloning-based methods to overcome the high fragmentation of the ancient DNA and the massive occurrence of non-target DNA from fungal contaminants. This approach has permitted the assignment of ITS2 sequences to 23 out of the 36 specimens studied in this work and a taxonomic study of the samples that has resulted in a re-evaluation of some of them at species level and others at genus or higher taxonomic level. These results highlight the possibility to apply the technique presented in this work also to the type specimens stored in the Saccardo collection in order to relate a DNA sequence to these important samples.

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Next Generation Sequencing of Ancient Fungal Specimens: The Case of the Saccardo Mycological Herbarium

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Despite their essential role in the environment, the number of known fungal species is still low compared to the recent estimates of the fungal biodiversity (from 2.2 to 3.8 million species), principally because of their often cryptic or ambiguous morphological traits. Recent studies have reported that only about approx. 35,000 correctly identified fungal species are represented by DNA sequences in public databases. This corresponds to a mere 1% of the estimated total number of species. Thus, the linkage of curated DNA sequence data to properly identified voucher specimens is of fundamental importance to fill the present gap between the different sizes of described and sequenced fungal diversity. For this purpose, mycological herbarium collections are considered an important source for fungal DNA-barcoding, and collection-based sequencing is a relevant priority for the coming decades. Unfortunately, ancient herbarium samples have both time and conservation related DNA damages, besides exogenous DNA contamination, that make nucleic acid extraction and amplification challenging. Here, we present the results of DNA extraction, ITS2 amplification and Illumina MiSeq sequencing of 36 specimens from the Saccardo Mycological Herbarium that were collected in the late XIX century and assigned to the genus *Peziza*. High-throughput sequencing was chosen as an alternative to the conventional Sanger- and cloning-based methods to overcome the high fragmentation of the ancient DNA and the massive occurrence of non-target DNA from fungal contaminants. Our approach has permitted the assignment of ITS2 sequences to 23 out of the 36 specimens studied in this work, thus providing a univocal DNA sequence for those one century old samples. Furthermore, the ITS2 sequence analysis has permitted a taxonomic study of the samples that has resulted in a re-evaluation of five samples at the species level and 18 samples at genus or higher level. Our results highlight the possibility to apply the technique presented in this work also to the old and more precious type specimens in order to relate a DNA sequence to these important samples, coupling the traditional morphological description of the species with a DNA sequence.

Keywords: ancient DNA, DNA barcoding, fungi, herbarium collection, NGS sequencing, nuclear ribosomal internal transcribed spacer, Pier Andrea Saccardo

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INTRODUCTION

In terms of biodiversity fungi are one of the least described groups of multicellular eukaryotes even though they comprise 2.2–3.8 million species (Hawksworth and Lücking, 2017). In fact, despite their important role in ecological systems (Fisher et al., 2012; Martínez-García et al., 2017; Bani et al., 2018), only a small fraction of the estimated species has been formally described (approx. 120,000), and for many of them (approx. 85,000) no DNA sequences have been deposited in public DNA databases so far (Hawksworth and Lücking, 2017). On the other hand, fungal metagenome sequencing is providing scientists with a large number of datasets composed of sequences from environmental/uncultured fungi with no or poor taxonomic annotation. Thus, the linkage of curated DNA sequence data to expertly identified voucher specimens is a fundamental step to fill the present gap between the different sizes of described and sequenced fungal diversity.

In 2012, the Consortium for the Barcode of Life recognized the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster as the primary fungal barcode marker (Schoch et al., 2012). This region includes the highly variable non-coding ITS1 and ITS2 regions separated by the 5.8S coding gene and situated between 18S (small subunit; SSU) and 28S (large subunit; LSU) coding genes. Thanks to the rapid evolutionary rate of the ITS1 and ITS2 non-coding regions their sequences have been used for over 20 years in the identification of fungal species (Nilsson et al., 2008). On the other hand, the highly conserved 18S, 5.8S, and 28S genes have allowed the development of universal primers for PCR amplification and sequencing of either the entire ITS region or the ITS1 and ITS2 regions separately (White et al., 1990; Gardes and Bruns, 1993; Martin and Rygiewicz, 2005; Toju et al., 2012). An additional advantage of this DNA marker is that the fungal genome contains multiple tandemly repeated copies of the ribosomal RNA gene cluster (including the ITS), thus making possible the amplification of this region from small amounts of DNA (Xu, 2016). The sequencing of these marker regions can be easily applied to living organism material, therefore it can be particularly useful also for DNA barcoding of mycological collections (Osmundson et al., 2013).

The fungi collections conserved worldwide in herbaria are considered a great source of genetic material and the collection-based sequencing projects are assuming an important priority in the coming decades because the herbaria can host a large number of unknown or under represented fungal lineages (Brock et al., 2009; Osmundson et al., 2013). In addition, ancient herbarium collections may contain type specimens, in particular holotypes. These specimens have great scientific value because they represent the only certain link to a specific Latin binomial name; therefore, the eventual existence of molecular information from these important samples might help to solve taxonomic controversies (Liimatainen et al., 2014; Mutanen et al., 2014). However, it is well known that obtaining molecular information from very old biological material can be particularly challenging, the main problem being the fragmentation of the ancient DNA (aDNA). The nucleic acids of old biological samples are often degraded into small fragments whose size is generally less than 500 bp (Pääbo et al., 2004; Dabney et al., 2013). Although the DNA of the herbarium specimens naturally degrades in the course of time, it has been demonstrated that the fragmentation degree of the aDNA depends mainly on the methods used to collect, dry and store the specimens rather than on the age of the herbarium material (Erkens et al., 2008). This is especially true for old herbaria where the specimens were never collected and stored for optimal DNA conservation but rather for optimal conservation of the specimens' morphological traits. For example, the adopted drying methods can have different effects on the DNA quality, and the use of some chemicals as pesticides can lead to a high-level degradation of the specimens' DNA (Kigawa et al., 2003; Nagy, 2010; Staats et al., 2011).

Another obstacle that makes DNA extraction and sequencing from herbarium samples challenging is the presence of contaminant material associated to the conserved specimens. Ancient collections are frequently contaminated by other organisms, like airborne fungi or bacteria. Moreover, the specimens are often not properly handled and conserved in sealed containers but rather are stored on paper sheets stacked in herbarium packages. This close contact between different specimens enhances the probability of cross-contamination between the samples in the packages (Yeates et al., 2016). The presence of exogenous fungal DNA is an issue affecting sequencing projects involving ancient mycological collections, since contaminant DNA may outcompete the target DNA during the amplification step. Furthermore, the universal primers adopted in the barcoding procedure cannot discriminate the target DNA from the more recent and intact exogenous DNA, and this can lead to an overrepresentation of non-target sequences with the consequence of either a reduced or no sequencing success.

In addition, the amount of herbarium material usable for DNA extraction is often limited because many specimens are poor in available tissue and because the sampling to yield a sufficient amount of DNA can in some cases cause irreparable damage to the herbarium specimens. Notwithstanding such problems, DNA was successfully extracted and amplified from lichen and plant herbarium samples older than 100 years (Lehtonen and Christenhusz, 2010; Redchenko et al., 2012) and from fungal samples older than 200 years (Larsson and Jacobsson, 2004). These results demonstrate that ancient collections could still represent a source of material for molecular studies.

In this sense, the Pier Andrea Saccardo mycological collection conserved in the herbarium of the Botanical Garden of the University of Padua can represent a rich source of specimens suitable for DNA barcoding. This collection, started by P.A. Saccardo around 1874, contains almost 70,000 fungal specimens encompassing over 18,500 different species which have not been subjected to molecular studies so far. The herbarium was first put together by Saccardo with samples collected by himself around Treviso (his home town) and Padua (Botanical Garden and surroundings); then it was enriched with Italian and foreign specimens sent to him by colleagues and with whole mycological collections presented to him by mycologists from around the world. During his day, Saccardo was recognized as an expert mycologist, as shown by the many specimens that were sent

to him from scientists for sample identification. The Saccardo collection has a special scientific importance due to the presence of about 4,500 type specimens (mostly assigned to Ascomycota) that have been used for the first morphological descriptions of new fungal species, and these specimens have long been used by mycologists from all over the world for morphological studies and taxonomic revisions. Unfortunately, for this reason some types of the Saccardo collection have been damaged or lost.

Because of its importance for mycologists, and in order to preserve the scientific value of the Saccardo collection, a long-term DNA barcoding project has been initiated to obtain molecular data from the most important type specimens with the scope of making the results available to the scientific community. To overcome all the above described issues related to ancient DNA analysis, we decided to apply a high throughput sequencing method yielding a large number of sequences per sample. This technique, commonly used for community analysis, has already been used for the sequencing of old insect type specimens, partially demonstrating the feasibility of the sequencing method (Prosser et al., 2016). Preliminary results show that the high degree of DNA fragmentation of the Saccardo samples renders the amplification of the entire ITS sequence (about 600 bp) difficult. Therefore, we focused on the amplification and sequencing of the ITS2 region only. Although ITS1 and ITS2 regions (respectively, ranging from 100 to 280 bp and from 120 to 280 bp; Tedersoo et al., 2015) give similar results in fungal metabarcoding studies (Bazzicalupo et al., 2012; Blaalid et al., 2013), the ITS2 fragment is generally less variable in length compared with ITS1 and lacks the problem of co-amplification of a 5′ SSU intron that is common in many ascomycetes (Lindahl et al., 2013). ITS2 has also a relatively conserved secondary structure among eukaryotes, which potentially enables scientists to perform higher level phylogenetic comparisons (Koetschan et al., 2010), and it is somewhat better represented than ITS1 in databases (Nilsson et al., 2009).

Here we present the results of DNA extraction, ITS2 amplification, sequencing and data analysis of 36 non-type specimens classified by Saccardo as members of the genus *Peziza* (Ascomycota, Pezizomycetes, Pezizales, Pezizaceae). Given the precious nature of the type specimens present in the collection, non-type samples were selected to set up and test the methods for a future application to the more valuable type specimens. The results obtained in this work appear encouraging since for a large part of the samples it was possible to obtain the target sequence, allowing us to re-evaluate the taxonomic position of each sequenced sample.

MATERIALS AND METHODS

Sampling

A total of 36 *Peziza* specimens (28 different species) was sampled from the 323 *Peziza* specimens (166 different species including 8 types) collected in the Saccardo mycological herbarium, an example of which is shown in **Figure 1**. The samples for sequencing were selected to cover the size range (from 1 mm to 50 mm) and different conservation conditions (intact or fragmentated ascoma). For the present study no type material was used and for some species multiple specimens were analyzed (**Table 1**). Care was taken to preserve the overall integrity of each specimen taken from this historical and scientifically important fungal collection, with the permission and supervision of the herbarium curator. The specimens were sampled by removing a tiny piece of dried ascoma tissue using sterilized tweezers and scalpel.

DNA Extraction, PCR Amplification and Library Preparation

DNA was extracted using a CTAB method (Rogers and Bendich, 1985; Doyle and Doyle, 1990) slightly modified in order to obtain DNA with suitable quality from small amounts of material. The fungal tissue was ground into a fine powder in a 1.5 mL microfuge tube by using a pestle with quartz sand and liquid nitrogen. The ground tissue was mixed with 1 mL of prewarmed (65°C) CTAB extraction buffer (2% w/v CTAB; 100 mM Tris-HCl; 20 mM EDTA; 1.4 M NaCl; 2% w/v polyvinylpyrrolidone K30) with the addition of proteinase K (1 mg/mL) and RNase A (10 mg/mL), and then incubated at 65°C overnight. After a centrifugation at max speed for 10 min in a microfuge, the liquid phase was transferred to a new 2 mL microfuge tube and 800 μL of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed several times by inversion and centrifuged for 10 min at 10,000 rpm. The upper liquid phase was moved to a new microfuge tube and 600 μL of chloroform:isoamyl alcohol (24:1) was added, mixed several times by inversion and centrifuged for 10 min at 10,000 rpm (this step was repeated twice).

DNA was precipitated by adding ice-cold isopropanol (2/3 of the recovered volume) and incubated at −20°C for 2 h. After a centrifuge at 10,000 rpm for 10 min, the DNA pellet was washed with 300 μL of ice-cold 70% ethanol, resuspended in 40 μL of sterile water and, after an incubation overnight at 4°C, stored at −20°C. DNA concentration and purity (absorbance at 230, 260, and 280 nm) were assessed using both NanoDrop spectrophotometer (Thermo Fisher Scientific) and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The extracted DNA from some *Peziza* samples (*P. adae* s_01, *P. badia* s_17 and *P. cochleata* s_31) was compared with the DNA extracted from a fresh fungus (*Agaricus bisporus*), running the same amount of DNA samples in agarose gel (0.8% agarose in TAE buffer, stained with Eurosafe DNA dying, Euroclone).

In addition, to have a more precise overview on the integrity of the nucleic acid obtained from the *Peziza* specimens, the length of the extracted aDNA fragments was determined by microcapillarity electrophoresis using the Agilent Bioanalyzer (Agilent Technology) following the manufacturer's instructions.

Amplification of the nuclear ribosomal ITS2 region for the Illumina sequencing was carried out using a two-step PCR process. In the first PCR the region of interest was amplified using the universal ITS3/ITS4 primers built on the conserved regions flanking the ITS2 (White et al., 1990), while in the second PCR the products of the first amplification were amplified using ITS3mod/ITS4 tagged primers in order to add different 5 bp identifier tags to distinguish sequences from each specimen (Voyron et al., 2016). In this work 18 different tags were used,

FIGURE 1 | Pictures of some representative *Peziza* samples investigated in this work and their herbarium label. **(A)** *P. craterium;* **(B)** *P. crenata;* **(C)** *P. vesiculosa*, and **(D)** *P. brunnea.* In the last panel, beside the original label a more recent morphological revision is present, and a detail of the fungus is visualized with a 30x enlargement. Except where differently indicated, the black bars represent 10 mm.

and the second PCR was done in four replicates for each couple of tagged primers.

The first PCR reaction was carried out in a total volume of 25 μL including 5 μL of 5X Wonder Taq reaction buffer (5 mM dNTPs, 15 mM $MgCl_2$; Euroclone), 0.5 μL of bovine serum albumin (BSA, 10 mg/mL), 0.5 μL each of two primers (10 μM), 0.5 μL of Wonder Taq (5 U/μL), 2 μL of genomic DNA and ddH_2O to reach the final volume. The PCR conditions used were: 95°C for 3 min; 35 cycles of 95°C for 30 s, 52°C for 40 s and 72°C for 45 s; 72°C for 5 min. The second PCR reaction was performed similarly to the previous amplification except for the absence of the BSA, the use of 2 μL of the first PCR amplicons as template and the use of the tagged primers. The PCR products were checked in agarose gel (1.2% agarose in TAE buffer, stained with Eurosafe DNA dying, Euroclone), and the four replicates of each sample were pooled together and purified using the PureLink PCR Purification Kit (Invitrogen). After the quantification with Qubit, the purified amplicons were mixed in equimolar amount to prepare two sequencing libraries of 18 samples each. The libraries were sent to IGATech (Udine, Italy) for a paired-end sequencing using the Illumina MiSeq technology (2 × 300 bp).

NGS Data Processing, OTU Identification and Taxonomic Analysis

Raw data of the Illumina sequencing were analyzed using the Illumina pipeline developed for a community analysis of orchid

TABLE 1 | List of *Peziza* specimens involved in this study.

Sample number	Herbarium specimen	Species current name	Sequences per sample	Sequences of the target fungus (%)	Sequence taxonomic assignment	GenBank accession number	Morphological assignment
s_01	*Peziza adae* Sadler	*Peziza domiciliana* Cooke	18,535	32	*Peziza* sp.	MH144155	*Peziza* sp.
s_02	*Peziza adusta* Schulzer		40,863	74	*Urnula* sp.	MH144156	*Urnula* sp.
s_03	*Peziza agassizii* Berk. & M.A. Curtis	*Lachnellula agassizii* Dennis	6,583				
s_04	*Peziza aluticolor* Berk. (1)		8,106	79	*Cookeina* sp.	MH144157	*Cookeina* sp.
s_05	*Peziza aluticolor* Berk. (2)		35,027	95	*Cookeina* sp.	MH144158	*Cookeina* sp.
s_06	*Peziza ammophila* Dr. & Mont.		7,364				
s_07	*Peziza ampliata* Pers.	*Peziza ampliata* Pers.	33,759	34	*Peziza* sp.	MH144160	*Peziza* sp.
s_08	*Peziza amplispora* Cooke & Peck		25,617	22	Helvellaceae	MH144161	*Peziza* sp.
s_09	*Peziza ancilis* Pers. (1)	*Discina ancilis* Sacc.	25,691	4	Helvellaceae	MH144162	*Helvella leucomelaena* complex
s_10	*Peziza ancilis* Pers. (2)	*Discina ancilis* Sacc.	22,552	61	Helvellaceae	MH144163	*Helvella leucomelaena* complex
s_11	*Peziza anomala* Pers.	*Merismodes anomala* Singer.	12,461				
s_12	*Peziza applanata* Fries (1)	*Peziza depressa* Pers.	26,110				
s_13	*Peziza applanata* Fries (2)	*Peziza depressa* Pers.	2,392				
s_14	*Peziza aranea* De Notaris	*Arachnopeziza aranea* Boud.	9,019				
s_15	*Peziza arctispora* Cooke & W. Phillips	*Ciliaria arctispora* Boud.	9,480	10	*Wilcoxina* sp.	MH144164	*Wilcoxina* sp.
s_16	*Peziza aurantia* Fuckel (2)	*Aleuria aurantia* Fuckel	40,010	29	*Aleuria aurantia*	MH144165	*Aleuria aurantia*
s_17	*Peziza badia* Pers. (1)	*Peziza badia* Pers.	27,557	12	*Peziza* sp.	MH144167	*Peziza* sp.
s_18	*Peziza brunnea* Alb. & Schwein.	*Sphaerosporella brunnea* Svrcek & Kubicka	24,749	19	*Wilcoxina* sp.	MH144170	*Wilcoxina* sp.
s_19	*Peziza aeruginosa* Pers.		11,579				
s_20	*Peziza amphora* Quél.	*Helvella solitaria* P. Karsten	42,550	54	Helvellaceae	MH144159	*Helvella leucomelaena* complex
s_21	*Peziza arundinariae* Berk. & M.A. Curtis	*Discocurtisia arundinariae* Nannf.	7,480				
s_22	*Peziza atriella* Cooke	*Belonopsis atriella* Lindau	59				
s_23	*Peziza aurantia* Fuckel (1)	*Aleuria aurantia* Fuckel	46,547	72	*Aleuria aurantia*	MH144166	*Aleuria aurantia*
s_24	*Peziza aurelia* Pers.	*Arachnopeziza aurelia* Fuckel	6,998				
s_25	*Peziza badia* Pers. (2)	*Peziza badia* Pers.	23,416	11	*Peziza ampelina*	MH144168	*Peziza ampelina*
s_26	*Peziza badia* Pers. (3)	*Peziza badia* Pers.	2,320				
s_27	*Peziza badia* Pers. (4)	*Peziza badia* Pers.	550				
s_28	*Peziza bicucullata* Boud.	*Aleuria bicucullata* Boud.	5,102	35	Helvellaceae	MH144169	*Helvella leucomelaena* complex
s_29	*Peziza cerea* Bull. (=*Pustularia vesiculosa var. cerea* Rehm)		44,338	45	*Peziza vesiculosa*	MH144171	*Peziza vesiculosa*

(Continued)

TABLE 1 | Continued

Sample number	Herbarium specimen	Species current name	Sequences per sample	Sequences of the target fungus (%)	Sequence taxonomic assignment	GenBank accession number	Morphological assignment
s_30	*Peziza coccinea* Jacq.	*Sarcoscypha coccinea* Boud.	47,978	58	*Sarcoscypha coccinea*	MH144172	*Sarcoscypha coccinea*
s_31	*Peziza cochleata* Huds.	*Wynnella silvicola* Nannf.	32,886	9	*Peziza varia*	MH144173	*Peziza varia*
s_32	*Peziza corium* Weber. (1)	*Helvella corium* Massee	27,803				
s_33	*Peziza corium* Weber. (2)	*Helvella corium* Massee	35,792	16	*Helvella* sp.	MH144174	*Helvella* sp.
s_34	*Peziza coronaria var. macrocalyx* Riess	*Sarcosphaera coronaria* J. Schröt.	28,863	29	*Peziza* sp.	MH144175	*Peziza* sp.
s_35	*Peziza craterium* Schwein.	*Urnula craterium* Fries	42,955	76	*Urnula* sp.	MH144176	*Urnula* sp.
s_36	*Peziza crenata* (=*Peziza cupularis* L.)	(=*Tarzetta cupularis* Svrček.)	39,083	3	*Tarzetta* sp.	MH144177	*Tarzetta* sp.

The current species name and the result of the sequence assignment, taxonomic evaluation and morphological analysis, are reported for each sample.

mycorrhizal soil fungi (Voyron et al., 2016) as guideline. Forward and reverse reads from each library were merged using PEAR v0.9.10 (Zhang et al., 2014) with the quality score threshold set at 28, the minimum length of reads after trimming set at 150 bp and the minimum overlap size set at 100 bp. The merged reads were processed using the software package QIIME v1.9.1 (Caporaso et al., 2010). To avoid incorrect assignment, demultiplexing of sequences was performed with a maximum number of errors in the tag sequence of 0. At the same time, the sequences were processed using a minimum sequence length cutoff of 150 bp, minimum quality score of 28, a sliding window test of quality score of 50, a maximum length of homopolymers of 13, a maximum number of ambiguous bases of 0 and a maximum number of mismatches in forward and reverse primers of 3, and then reoriented when necessary to 5′ to 3′.

Sequences were dereplicated with VSEARCH v2.3.4 (Rognes et al., 2016) to form clusters with an identity of 100% and the ITS2 variable region was extracted using ITSx software (http://microbiology.se/software/itsx/; Bengtsson-Palme et al., 2013). The ITSx was used to eliminate the conserved regions where the primers are located as these regions can introduce bias during the subsequent clustering and taxonomic assignment, because they increase similarity among sequences (Bálint et al., 2014). In addition, setting the option -t F (list of organism group - Fungi) all the ITS2 regions not detected as Fungi were eliminated. The picking of the Operational Taxonomic Units (OTUs) was performed with a 98% of similarity clustering using VSEARCH and the clusters with less than 10 sequences were discarded.

Chimera sequences were then removed using a *de novo* chimera detection with UCHIME algorithm (Edgar et al., 2011) implemented in the VSEARCH pipeline. The last release of the UNITE dataset version 7.2 (https://unite.ut.ee) for QIIME was used as reference for the taxonomic assignment of OTUs (Abarenkov et al., 2010; Kõljalg et al., 2013) and, consequently, for the identification of specimen target sequence. The OTU abundance table was created with VSEARCH, considering a 98% of identity, to determine the number of times an OTU was found in each sample pooled in the libraries. The sum of these numbers for a sample gives the total sequences found for that specific sample. Because a number of OTUs returned no matches compared with the UNITE database, we also compared these sequences with those deposited in NCBI GenBank, excluding uncultured/environmental sample sequences, using the BLASTN algorithm (BLAST-search, http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1997) and the first 10 hits were evaluated considering consistency between hits, *E*-value, similarity higher than 96%, query coverage, organism source and publication status of the hits.

The OTU abundance plots were obtained using the R package Phyloseq (McMurdie and Holmes, 2013) that combined the OTU abundance table and the UNITE taxonomic assignment of the OTUs.

Molecular Phylogenetic Analysis

Sequences obtained from the *Peziza* specimens were further investigated by a phylogenetic analysis in order to complement and confirm or revise the previous taxonomic assignment. The sequences used to infer the phylogenetic analysis were chosen on the bases of BLASTN results isolating a sub-dataset of taxonomically close well annotated sequences, and according to the outcomes of recent phylogenetic studies (Harrington and Potter, 1997; Hansen et al., 2002; Weinstein et al., 2002; Hansen and Pfister, 2006; Perry et al., 2007; Ekanayaka et al., 2016).

ITS2 sequences of each dataset were aligned using the default options in MUSCLE (Edgar, 2004) as implemented in MEGA7 (Kumar et al., 2016). The phylogenetic analysis was conducted in MEGA7 by Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar, 2000) to obtain a more accurate identification of the fungal specimens. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite

Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value (1000 bootstrap replicates). A discrete Gamma distribution was used to model evolutionary rate differences among sites. The phylogenetic trees were drawn to scale, with branch lengths measured in the number of substitutions per site and supporting values (MLB, Maximum likelihood bootstrap values) below 50 were generally regarded unreliable and hidden. In this way, six separated phylograms were obtained relatively to the families Pyronemataceae, Pezizaceae, Sarcosomataceae, and Helvellaceae and to the genera *Cookeina* and *Sarcoscypha* of the family Sarcoscyphaceae.

Morphological Analysis

Microscopic structures were examined in dried material using a Leitz Diaplan light microscope with 40× or 100× (immersion oil) objectives. Dried material was rehydrated in water or 3% KOH. Hymenial elements were studied by teasing apart a piece of hymenium with a scalpel. Structural features of the excipulum were studied using vertical, median sections made by hand. Other chemicals used were Melzer's reagent and Cotton blue in lactic acid. The spore size was obtained by measuring 30 spores judged to be mature.

RESULTS AND DISCUSSION

DNA Extraction

The adopted protocol has permitted the extraction of satisfactory amounts of DNA with a concentration ranging from 10 to 200 ng per mg of sample. The purity ratio 260/280 ranged between 1.4 and 2.1 while the ratio 260/230 ranged between 0.7 and 2.1. These values indicate a low level of protein contamination whereas they show the presence of phenolic contaminants and/or other molecules, such as fungal secondary metabolites absorbing at 230 nm. Since the samples used in this study are more than one century old, we expected a certain grade of DNA degradation due to the time and the conservation conditions. The comparison between some *Peziza* ancient DNA and the DNA from fresh fungal tissue (*Agaricus bisporus*) extracted using the CTAB method previously described is reported in **Figure S1A**. The herbarium DNA resulted in highly-degraded fragments shorter than 500 bp (**Figure S1A**, lanes 2, 3, 4), while the DNA extracted from fresh material appears as a sharp band, the length of which is larger than 10 Kb (**Figure S1A**, lane 1). The fragmentation level of the extracted aDNA was further investigated using the Bioanalyzer to obtain a more accurate analysis. The resulting electrophoregrams of three representative samples show the fragment length distribution demonstrating a severe DNA degradation (**Figures S1B–D**). The sample quality analysis and the comparison between aDNA and DNA from fresh tissue demonstrated that the optimized extraction protocol used in this work allows the extraction of DNA from such challenging samples.

NGS Sequencing and Operational Taxonomic Units (OTUs) Analysis

Libraries sequencing generated a total of 4,481,568 raw reads: 2,029,302 reads for library 1 and 2,452,266 reads for library 2. The reads were merged so that 977,339 and 1,138,933 assembled reads were obtained (96% in the library 1 and 93% in the library 2). After demultiplexing and quality filtering the number of reads decreased to 474,221 and 560,754 for library 1 and 2, respectively. The dereplication step produced a number of 22,734 and 25,337 clustered sequences. On these two different datasets, the analysis using the ITSx software recognized 19,427 (library 1) and 21,955 (library 2) fungal ITS2 sequences. These sequences were grouped into OTUs with a 98% identity and chimera-checked, and the result was a total of 106 OTUs for library 1 and 101 OTUs for library 2. The number of fungal sequences in each sample, reported in **Table 1**, was obtained from the OTU abundance table. The general OTU taxonomic assignment revealed the presence of fungal organisms belonging to Basidiomycota and Ascomycota phyla in all the original herbarium specimens (**Figure 2**). As expected by considering both the old age and the lack of precautions during the manipulation of the various specimens throughout the herbarium long life, the majority of the contaminants is represented by common airborne molds such as *Aspergillus* and *Penicillium* (Ascomycota, Eurotiomycetes, Eurotiales, Aspergillaceae); *Epicoccum, Alternaria* and *Cladosporium* (Ascomycota, Dothideomycetes, Pleosporales, Didymellaceae), as well as xerophilic fungi such as *Wallemia* spp. (Basidiomycota, Wallemiomycetes, Wallemiales, Wallemiaceae; Zalar et al., 2005) (**Figure 3**). The taxonomic assignment results showed that the herbarium samples present external (airborne mold species) and intra-herbarium DNA contaminations (**Figure S2**).

In order to assign the correct sequence to the analyzed Saccardo samples, we evaluated the sequencing results by taking into account the modern taxonomy of the specimens as reported in the *Index Fungorum* (http://www.indexfungorum.org) and the number of reads per OTUs in order to discriminate between the target sequence and possible contaminations. During the years, many fungal species were moved to other different genera or placed in synonymy with other existing species due to the continuous morphological revisions and the development of molecular techniques for their identification. We have found that the current name of some *Peziza* species used in this work differs from the taxonomic annotation reported in the Saccardo collection (**Table 1**). For example, some of the collection species are now placed in synonymy with other *Peziza* species (e.g., *P. applanata* = *P. depressa*); others are considered as members of another family in the Ascomycota order Pezizales (e.g., *P. aurantia* = *Aleuria aurantia,* from Pezizaceae to Pyronemataceae family), or of a different order (i.e., *P. aranea* = *Arachnopeziza aranea*, from Pezizales to Helotiales order) or even as members of the Basidiomycota phylum (e.g., *P. anomala* = *Merismodes anomala*). Therefore, an updated assignment of the ancient samples according to the modern taxonomy can be of great help in identifying the correct sequence of the specimens, in particular when a contamination between herbarium samples is present.

Despite the high contamination level of the examined samples, the NGS sequencing yielded good results because it has allowed us to assign ITS2 sequences to 23 out of the 36 Saccardo herbarium specimens studied in this work (**Table 1**). In particular, the ITS2 sequence analysis permitted the identification

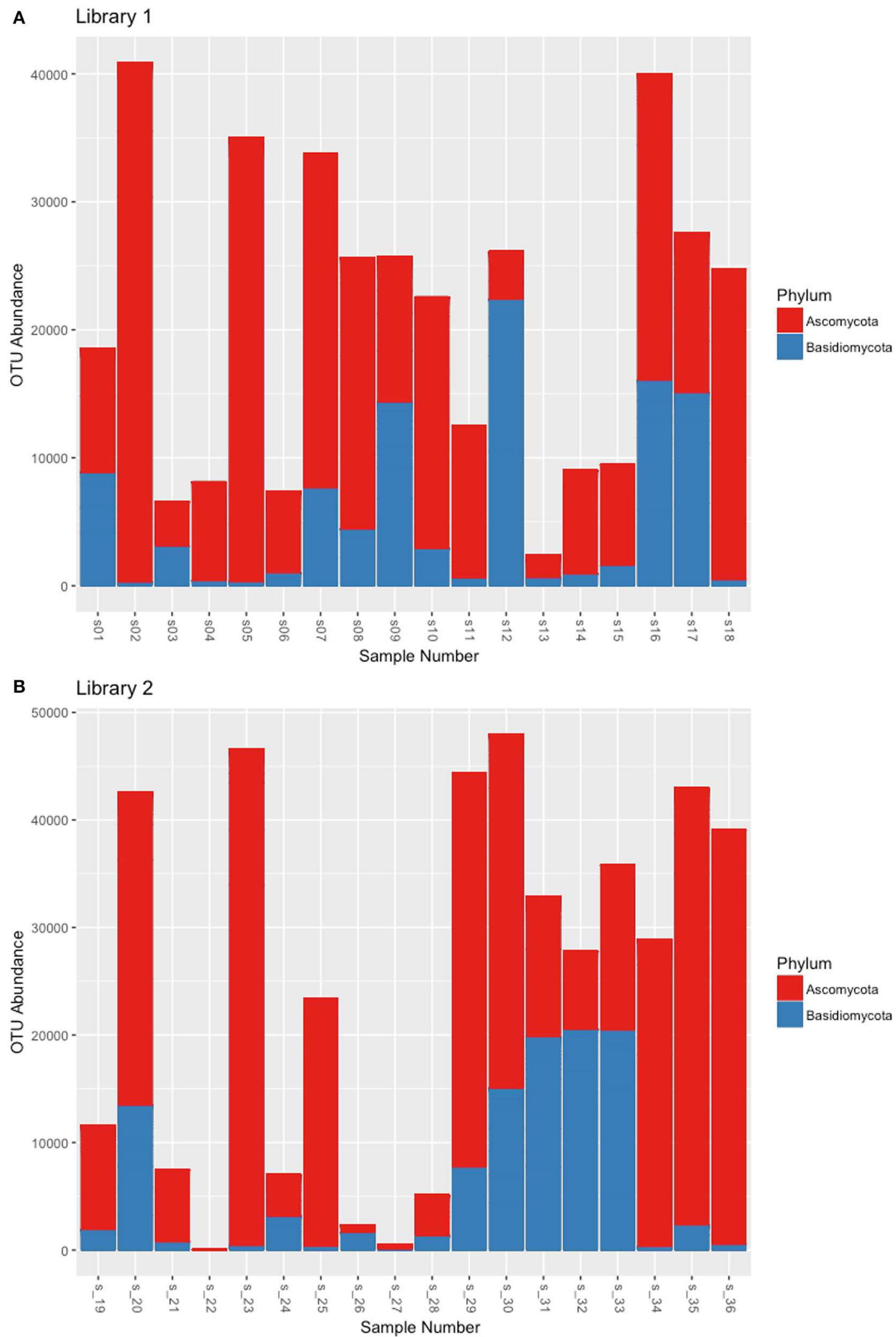

FIGURE 2 | OTU distribution among the Ascomycota and Basidiomycota phyla that resulted from the NGS experiments. **(A)** OTUs obtained from the reads of Library 1 samples, **(B)** OTUs obtained from the reads of Library 2 samples.

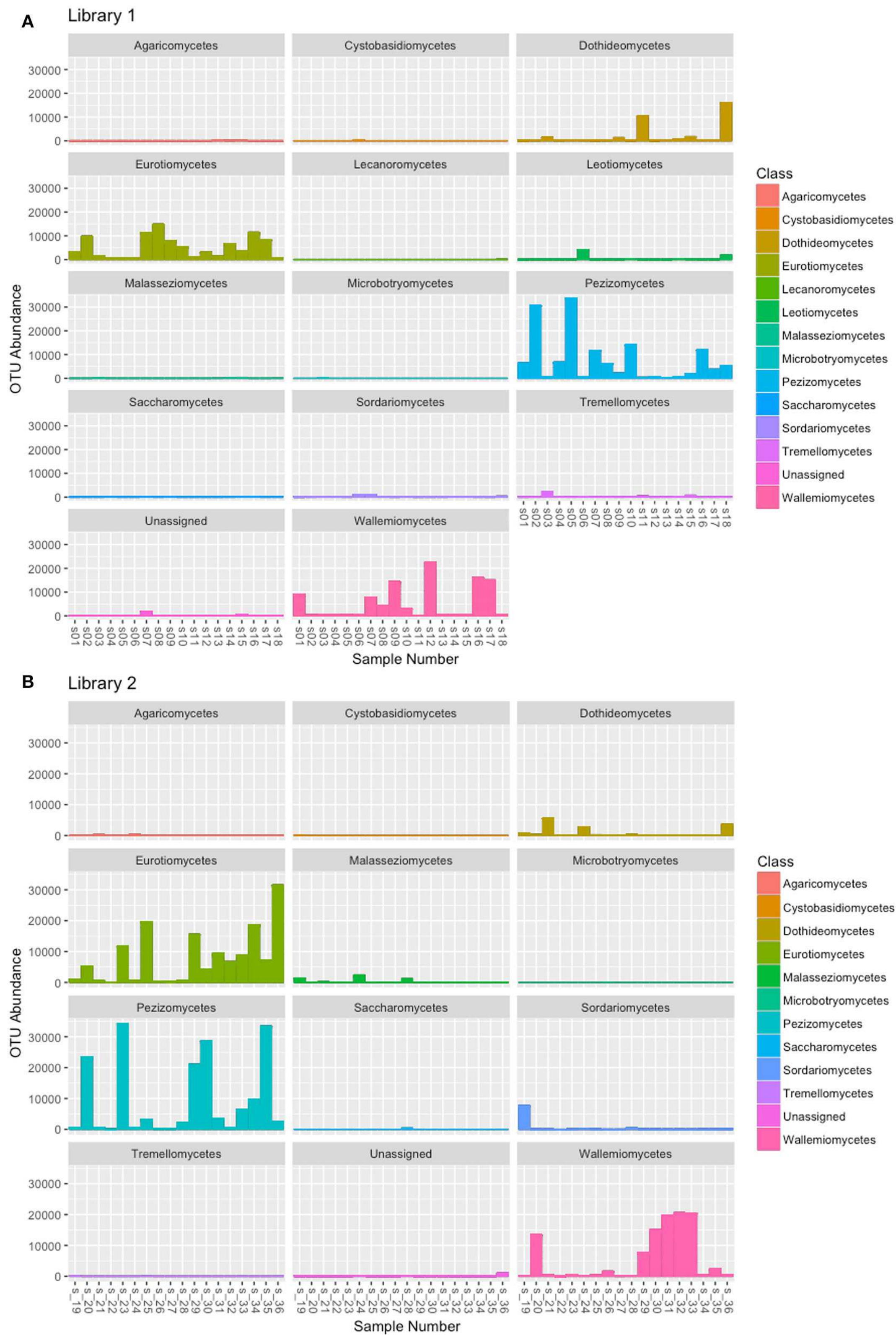

FIGURE 3 | OTU abundance in the fungal classes obtained by the taxonomic assignment of each sample. **(A)** OTUs obtained from the reads of Library 1 samples, **(B)** OTUs obtained from the reads of Library 2 samples.

of five samples at the species level and 13 samples only at genus level, while five samples resulted unassigned but were confirmed as belonging to the order Pezizales. For 13 analyzed samples we were unable to assign any sequence.

Taxonomic Assignment of the Analyzed Specimens

The taxonomic assignment has been obtained combining the sequence analysis, phylogenetic analysis and morphological analysis. The results (**Table 1**) show the different scenarios that can be encountered when sequencing old herbarium specimens. Morphological analysis has always confirmed molecular inference with the exception of *P. amplispora* (s_08), in which there was a discrepancy between the two methods of analysis used (**Table 1** and discussion below).

Samples With an Assigned Sequence and a Taxonomic Evaluation at Species Level

Peziza aurantia

The taxonomic assignment, reported in **Table 1**, confirmed the identity of *P. aurantia* (s_16, s_23) specimens giving as result the name of the species reported in the *Index fungorum* (*Aleuria aurantia;* Ascomycota, Pezizomycetes, Pezizales, Pyronemataceae). The result was also confirmed by the phylogenetic analysis reported in the Pyronemataceae tree (**Figure 4**) where the sequences clustered with the *Aleuria aurantia* sequences in a highly supported clade (MLB = 93%).

Peziza cerea

In the case of *P. cerea* (s_29), a deeper study of species synonyms, literature and phylogenetic analysis was necessary to confirm the identity of this herbarium sample. The herbarium specimen placed under the name *P. cerea* by Saccardo has a papery label where the fungus was previously classified as *Pustularia vesiculosa var. cerea* (**Figure 1C**). The name *Peziza cerea* sensu Bulliard has been suggested as synonym of *Peziza vesiculosa* by Hansen (Hansen et al., 2002). The NGS sequencing approach provided a sequence that has been recognized as derived from the target fungus and the UNITE taxonomic assignment indicates that the ITS2 sequence belongs to *Peziza vesiculosa*. The Pezizaceae phylogenetic analysis clearly confirmed this result by placing the specimen sequence in the highly supported (MLB = 97%) *Peziza vesiculosa* clade (**Figure 5**).

Peziza badia (2)

This sample produced a high number of sequences and about 11% of them were recognized as belonging to the target fungus. The modern fungal taxonomy confirms the name of the species and sequences annotated as *P. badia* are present in GenBank. However, the taxonomy assignment identified the fungus as *P. ampelina*, a result also confirmed by the phylogenetic analysis that placed the sequence in the highly supported *P. ampelina* clade (MLB = 99%) (**Figure 5**).

Peziza coccinea

The taxonomic analysis of the sequence assigned to the target fungus revealed that the specimen is a *Sarcoscypha* sp., congruent with the current name *Sarcoscypha coccinea*. Moreover, a more detailed phylogenetic analysis of the sample using selected sequences of the genus *Sarcoscypha* revealed that all the *S. coccinea* sequences clustered together and the analyzed specimen sequence appeared among them in the *S. coccinea* clade (MLB = 63%) (**Figure 6**). This result underscores that our sample belongs to the species *S. coccinea,* thus confirming and refining its taxonomical assignment.

Peziza cochleata

A large number of sequences (32,886) were obtained from *P. cochleata* but only few of them (9%) were assigned to the target fungus. Both taxonomic and phylogenetic analysis revealed that the target sequence groups with the ITS2 sequences of *P. varia* in a highly supported clade (MLB = 95%) (**Figure 5**). This result has demonstrated that the analyzed specimen is not actually a *P. cochleata*, that the modern taxonomy reports as *Wynnella silvicola* (Ascomycota, Pezizomycetes, Pezizales, Helvellaceae).

The molecular analysis (UNITE taxonomic assignment and phylogenetic analysis) and the morphological revision of *P. badia* 2 (s_25) and *P. cochleata* (s_31) did not confirm the previous classification.

All the above findings represent the best scenario that could be obtained by the molecular analysis of our ancient herbarium specimens, and this was possible because the sequences obtained for the target fungi matched with well annotated sequences in public databases. When the old sample identification is not confirmed by the molecular analysis, also in the presence of correct DNA sequences, a morphological revision is recommended to support the new molecular identification.

Samples With an Assigned Sequence and a Taxonomic Evaluation at Genus Level

P. adae (s_01), *P. adusta* (s_02), *P. aluticolor* (s_04, s_05), *P. ampliata* (s_07), *P. arctispora* (s_15), *P. badia* 1 (s_17), *P. brunnea* (s_18), *P. coccinea* (s_30), *P. coronaria var. macrocalyx* (s_34), *P. corium 2* (s_33), *P. craterium* (s_35) and *P. crenata* (s_36) samples yielded a good number of sequences (**Table 1**), but, although the identification of the sample-associated sequence was possible, the taxonomic assignment did not allow species identification due to the lack of well annotated reference sequences in online databases.

Peziza adae

Since the taxonomic assignment of the specimen by UNITE comparison was unsuccessful, the sequence of *P. adae* was compared with the NCBI (GenBank) database. The BLASTN analysis showed that the sequence has the highest similarity value (100%) with some unidentified sequences annotated as Fungal sp. (HM123315, MG915365), *Peziza* sp. (KY462519) and Pezizomycetes sp. (KX909164). The phylogenetic analysis confirmed the result by placing the sequence in the same clade (MLB = 95%) as the above cited NCBI sequences (**Figure 5**). This shows that the specimen belongs to the family Pezizaceae and probably to the genus *Peziza*. So, the sequencing permitted to assign a sequence to our sample, but the taxonomic analysis

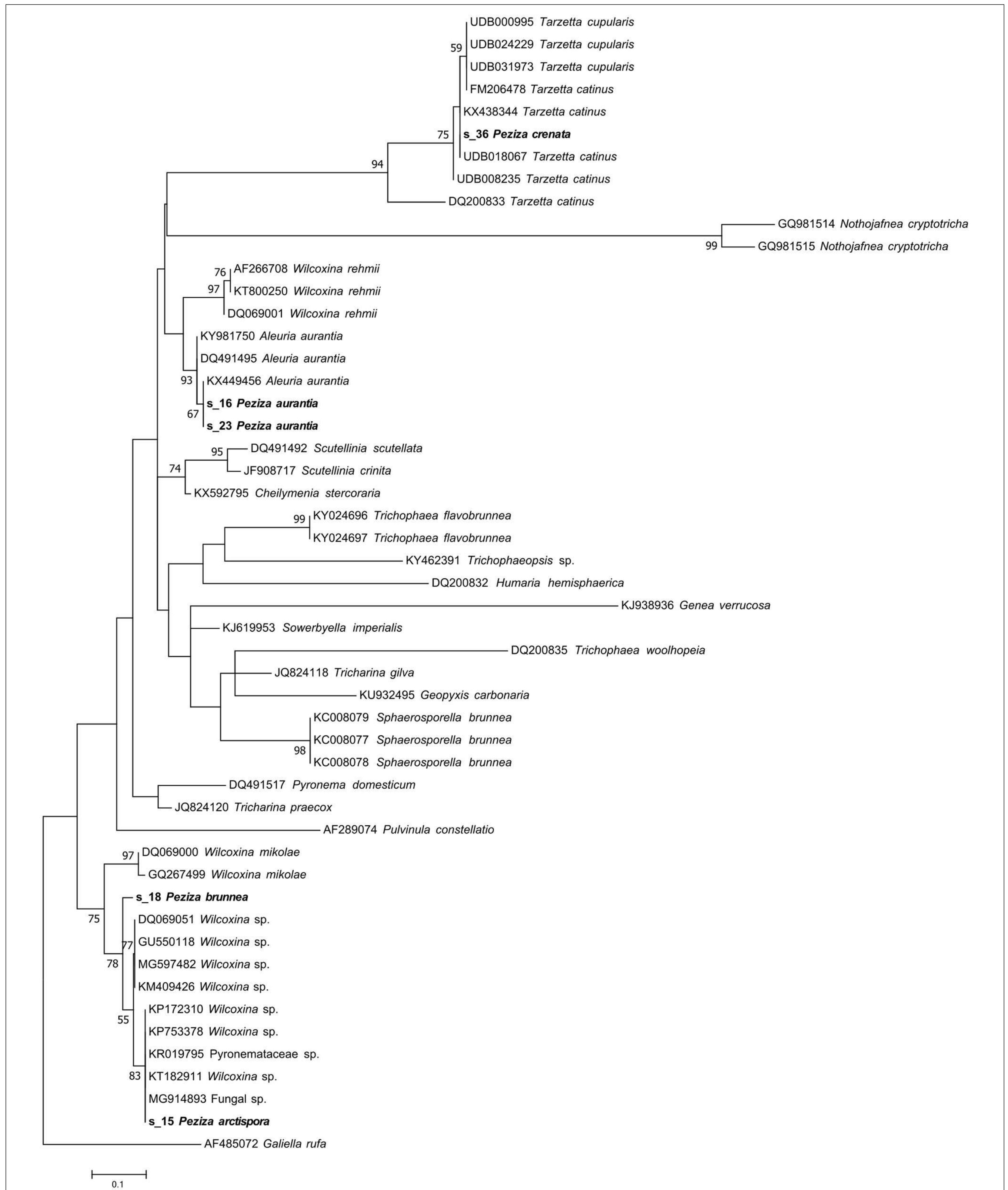

FIGURE 4 | Phylogram generated from maximum likelihood analysis of sequences of the family Pyronemataceae based on ITS2 sequence data. Maximum likelihood bootstrap values ≥50 are given above the nodes. The Accession Number indicates the sequences retrieved from the public databases. The tree was rooted with *Galiella rufa* (Ascomycota, Pezizomycetes, Pezizales, Sarcosomataceae). The Saccardo specimens are evidenced in bold.

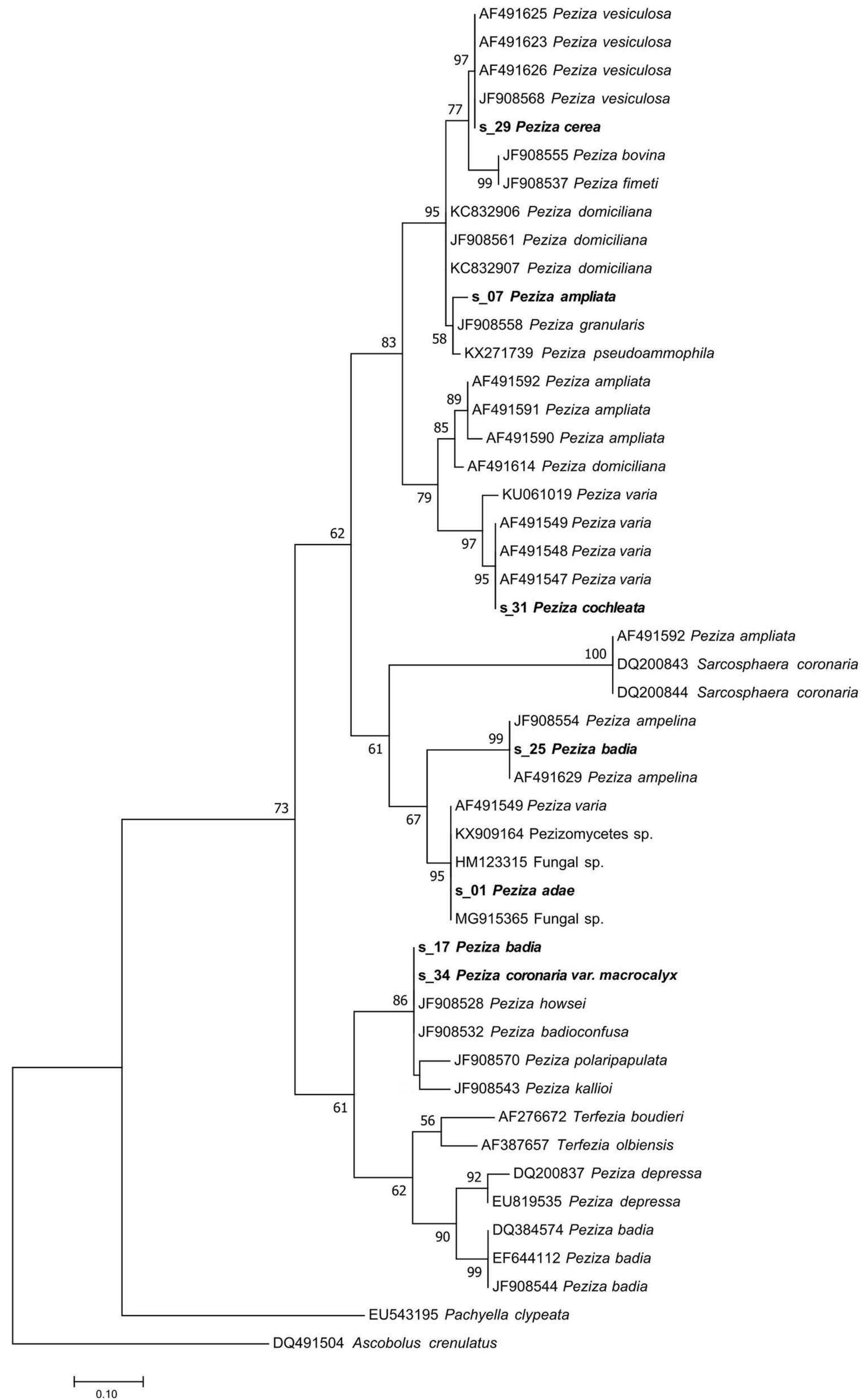

FIGURE 5 | Phylogram generated from maximum likelihood analysis of sequences of the family Pezizaceae based on ITS2 sequence data. Maximum likelihood bootstrap values $\geq$50 are given above the nodes. The Accession Number indicates the sequences retrieved from the public databases. The tree was rooted with *Ascobolus crenulatus* (Ascomycota, Pezizomycetes, Pezizales, Ascobolaceae). The Saccardo specimens are evidenced in bold.

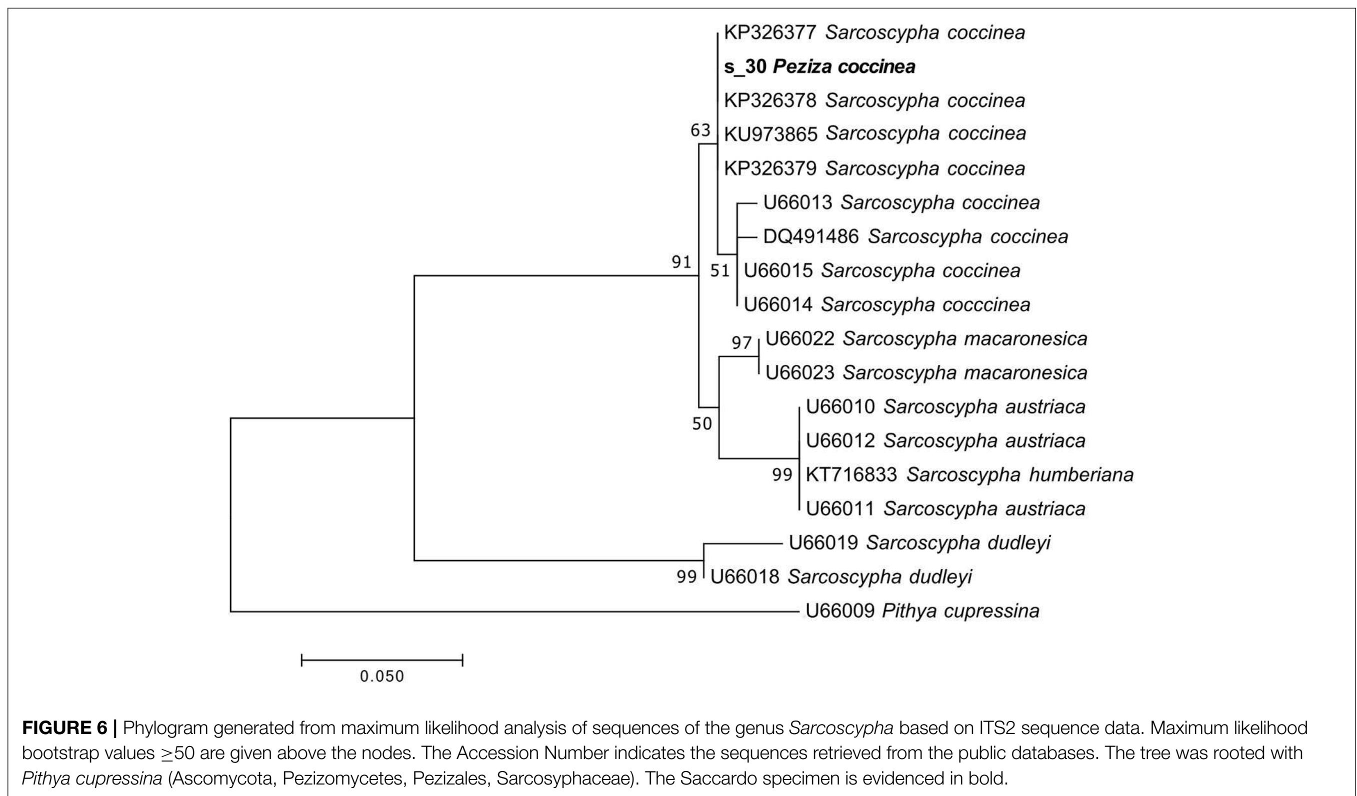

FIGURE 6 | Phylogram generated from maximum likelihood analysis of sequences of the genus *Sarcoscypha* based on ITS2 sequence data. Maximum likelihood bootstrap values ≥50 are given above the nodes. The Accession Number indicates the sequences retrieved from the public databases. The tree was rooted with *Pithya cupressina* (Ascomycota, Pezizomycetes, Pezizales, Sarcosyphaceae). The Saccardo specimen is evidenced in bold.

did not allow us to clearly identify the species of this herbarium specimen.

Peziza ampliata

The sequence of the sample classified as *P. ampliata* has been identified by the taxonomic assignment as *P. domiciliana*. However, a BLASTN analysis showed that the similarity value between the sequence found and *P. domiciliana* sequences in the NCBI database was, in our opinion, not high enough (96%) to allow the annotation of the sample as *P. domiciliana*. It is also notable that some sequences of *P. ampliata* are present in the NCBI database. In agreement with our observations, the phylogenetic analysis placed our sample in an unsupported minor clade (MLB = 58%) composed by different *Peziza* species (*P. granularis* JF908558, *P. pseudoammophila* KX271739) which is part of a well-supported major clade (MLB = 95%) consisting of collections of *P. domiciliana*, *P. fimeti*, *P. bovina* and *P. vesiculosa* (s_29 *P. cerea* included) (**Figure 5**). This major clade is sister (MLB = 83%) to a clade formed by the collections of *P. ampliata* present in the NCBI database and the collections of *P. varia* (s_31 *P. cochleata* included). This suggests that the sample of the collection is not *P. ampliata* but probably a species more related to *P. domiciliana* without any reference ITS2 sequences in the public databases. For this reason, we can only confirm that this specimen belongs to the genus *Peziza,* as originally annotated by Saccardo without being able to assign it species name.

Peziza badia (1) and Peziza coronaria var. macrocalyx

The analysis of the reads produced by these samples has permitted to assign a sequence to the analyzed fungi. However, the sequences found for the two specimens are identical and they have the same nucleotide identity with the ITS2 sequences of two different *Peziza* species: *P. badioconfusa* (JF908532) and *P. howsei* (JF908528). Probably the two samples classified as two different species in the Saccardo collection are the same species, but we are not able to add details about the species identification, apart from placing them in the *Peziza* genus. Anyway, the presence of deposited sequences of *P. badia* and *Sarcosphaera coronaria* (Ascomycota, Pezizomycetes, Pezizales, Pezizaceae), the current name of *P. coronaria var. macrocalyx*, excludes the previous identification of the specimens reported in the herbarium samples. This idea is also confirmed by the phylogenetic analysis of the family Pezizaceae where the sequences of the two specimens were grouped together and far from the ITS2 of *P. badia* and *S. coronaria* (**Figure 5**).

Peziza aluticolor (1 and 2)

For both of the samples present in the herbarium we were able to obtain a large number of sequences (79 and 95% for specimen 1 and 2, respectively) of the target fungi. From the *Index Fungorum* it seems that this species has not changed name although Seaver (1913) considered *P. aluticolor* as a synonym of *Cookeina colensoi* (Ascomycota, Pezizomycetes, Pezizales, Sarcoscyphaceae). The taxonomic assignment identified our samples as *Cookeina garethjonesii*. However, although the phylogenetic analysis using the ITS2 sequences confirmed that both samples actually belong to the genus *Cookeina*, a species-level identification is impossible because in the phylogram the sequences of *C. garethjonesii* appear in the same highly supported clade (MLB = 97%) with some sequences of *C. speciosa* (**Figure 7**).

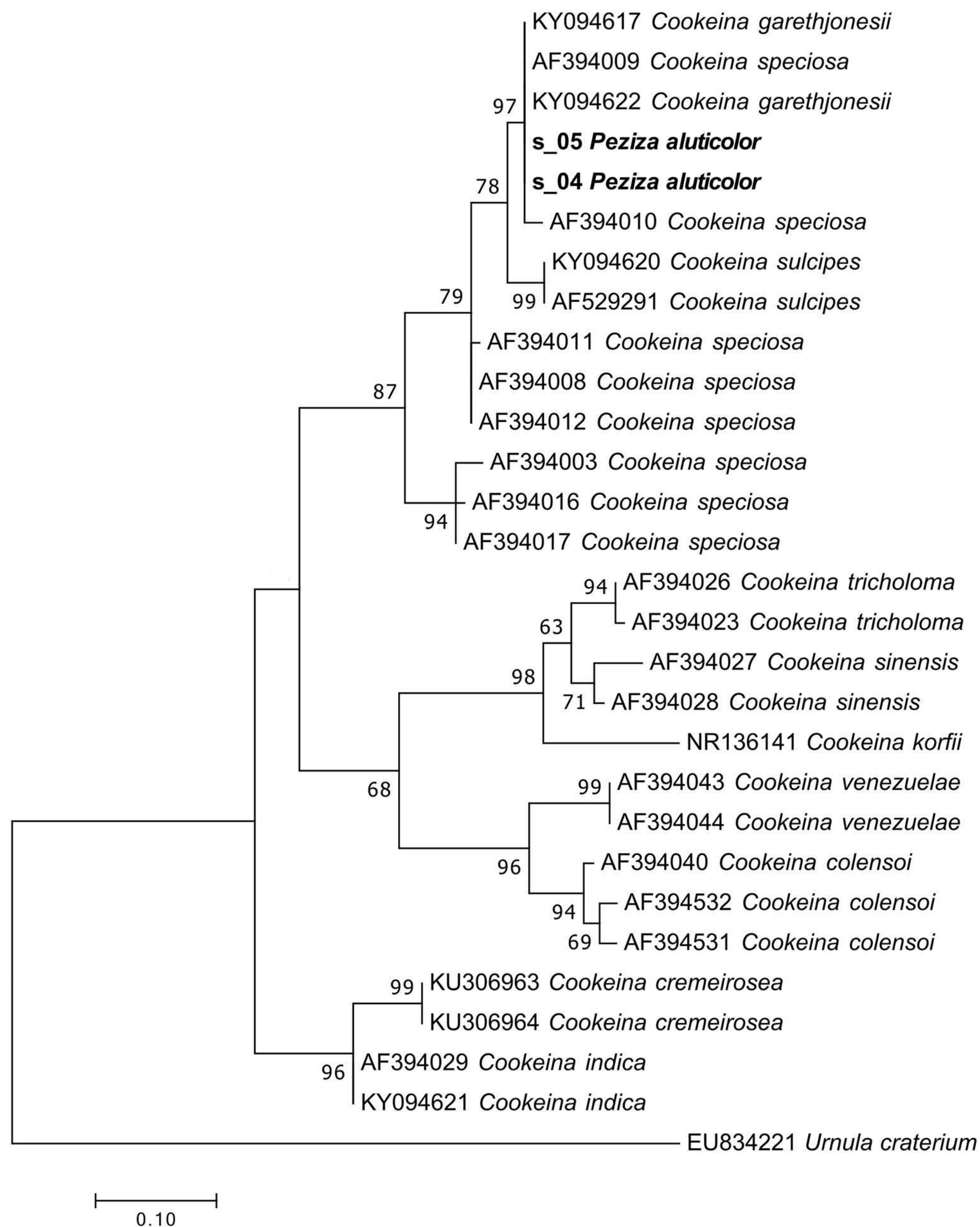

FIGURE 7 | Phylogram generated from maximum likelihood analysis of sequences of the genus *Cookeina* based on ITS2 sequence data. Maximum likelihood bootstrap values ≥50 are given above the nodes. The Accession Number indicates the sequences retrieved from the public databases. The tree was rooted with *Urnula craterium* (Ascomycota, Pezizomycetes, Pezizales, Sarcosomataceae). The Saccardo specimens are evidenced in bold.

Peziza adusta and Peziza craterium

These specimens were originally annotated as two distinct species, but the herbarium label of *P. craterium* has an annotation that places it in synonymy with *P. adusta* (**Figure 1A**). However, the UNITE taxonomic assignment annotated the *P. adusta* sequence as *Strumella coryneoidea* (Ascomycota, Pezizomycetes, Pezizales, Sarcosomataceae) whereas the sequence of the specimen *P. craterium* (current name *Urnula craterium*) only as a Sarcosomataceae. Interestingly, *U. craterium* and *S. coryneoidea* are two different names for the same species (Davidson, 1950).

Phylogenetic analysis of the specimen sequences and of some representative sequences of the family Sarcosomataceae divided the two *S. coryneoidea* (the only ones present in GenBank) in two distinct clades, separating our sequences in these two clades (**Figure 8**). The positions in the tree of the two *Strumella* sequences support a previous observation about a possible misidentification of one of them (Köpcke et al., 2002); unfortunately, all these considerations make us confident to assign the two herbarium specimens only to the genus *Urnula*. As reported in **Table 1**, the

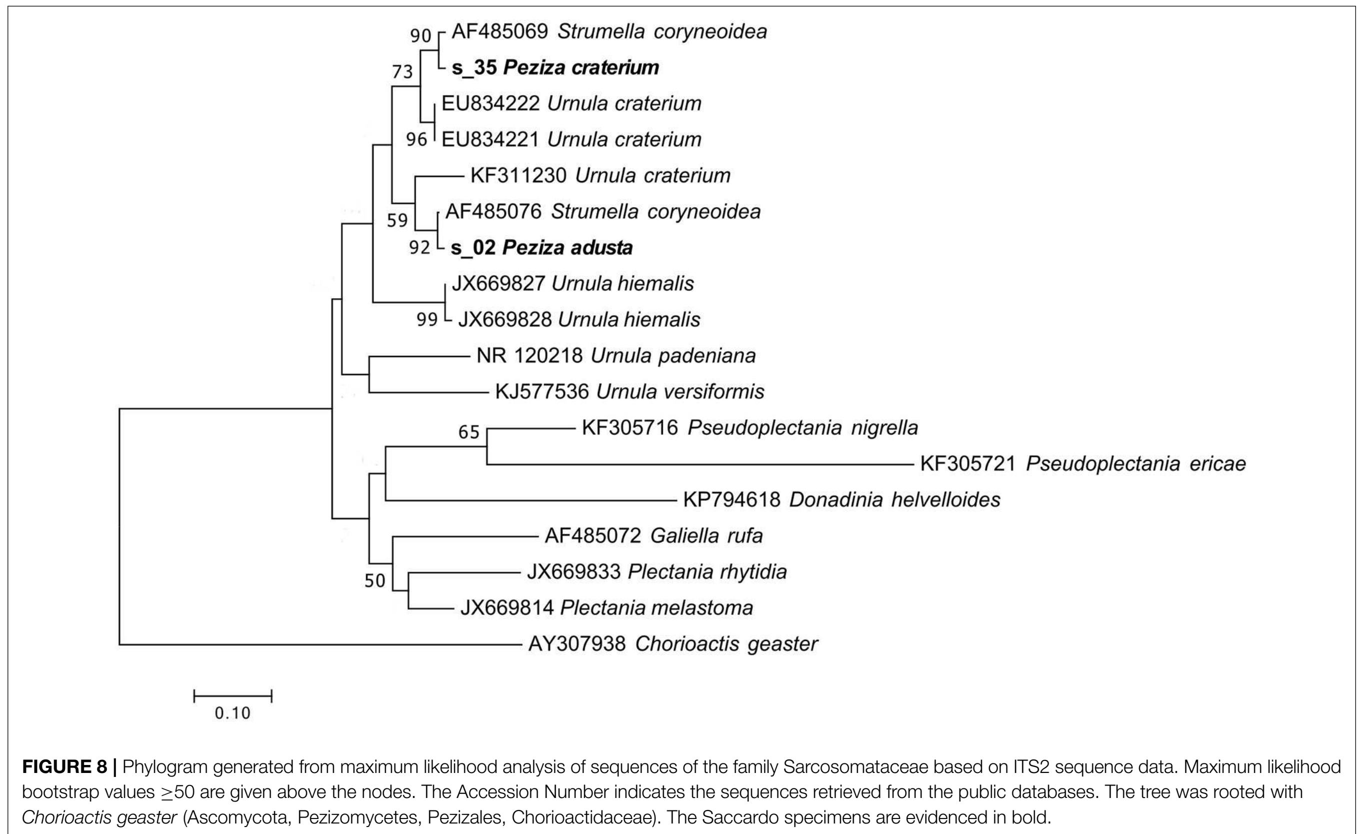

FIGURE 8 | Phylogram generated from maximum likelihood analysis of sequences of the family Sarcosomataceae based on ITS2 sequence data. Maximum likelihood bootstrap values ≥50 are given above the nodes. The Accession Number indicates the sequences retrieved from the public databases. The tree was rooted with *Chorioactis geaster* (Ascomycota, Pezizomycetes, Pezizales, Chorioactidaceae). The Saccardo specimens are evidenced in bold.

morphological analysis has been useful to confirm the genus assignment.

Peziza arctispora and Peziza brunnea

Sequences related to the target fungus *P. arctispora* (approx. 10% of the total of read number) provided as best taxonomic assignment a generic Pyronemataceae (Ascomycota, Pezizomycetes, Pezizales), while the specimen annotated by Saccardo as *P. brunnea* (**Figure 1D**; current name *Sphaerosporella brunnea;* Ascomycota, Pezizomycetes, Pezizales, Pyronemataceae) produced 24,749 sequences and 19% of these were assigned to *Trichophaea* sp. (Ascomycota, Pezizomycetes, Pezizales, Pyronemataceae).

Phylogenetic analysis of these two samples revealed that both ITS2 sequences grouped within a *Wilcoxina* cluster, but the tree topology is not clear enough to permit further taxonomical considerations (**Figure 4**). On the other hand, our ITS2 sequence analysis has not validated a revision from a morphological point of view made in 1974 (H.J. Larsen) that recognized *P. brunnea* as *Nothojafnea cryptotricha* (Ascomycota, Pezizomycetes, Pezizales, Pyronemataceae). This is another example that shows how important the role of molecular analysis is in the identification or revision of fungal species.

Peziza corium (2)

NGS sequencing of the *P. corium* (2) specimen produced 35,792 sequences, 16% assigned to the target fungus. The taxonomic assignment of the sample ITS2 sequence gave as result a generic Pezizales annotation while the phylogenetic analysis placed the sequence in a clade of Helvellaceae between two groups of *Helvella* species (**Figure 9**). The position of the sample sequence makes impossible a more detailed identification based on the ITS2, with the result of a *Helvella* sp. sequence assignment.

Peziza crenata

The herbarium label of this specimen shows a handwritten annotation where Saccardo himself posited a morphological similarity with *P. cupularis* (**Figure 1B**) and this species is reported in the *Index Fungorum* as *Tarzetta cupularis* (Ascomycota, Pezizomycetes, Pezizales, Pyronemataceae). The NGS analysis assigned the samples to the genus *Tarzetta,* in agreement with the suggestion made by Saccardo. Even though the taxonomic analysis has not provided any information about the species, this result could be still useful for a further analysis of the sample. In fact, the assigned sequence was compared to others of the Pyronemataceae family by phylogenetic analysis in order to obtain a more detailed molecular identification of the specimen. The resulting phylogenetic cladogram (**Figure 4**) confirmed the genus assignment but not a species-level identification.

Samples With a Recognized Target Sequence but Where a Taxonomic Assignment Was Not Possible

For *P. amplispora* (s_08), *P. ancilis* (s_09, s_10), *P. amphora* (s_20) and *P. bicucullata* (s_28) specimens we are able to assign a sequence, but the taxonomic identification cannot be done.

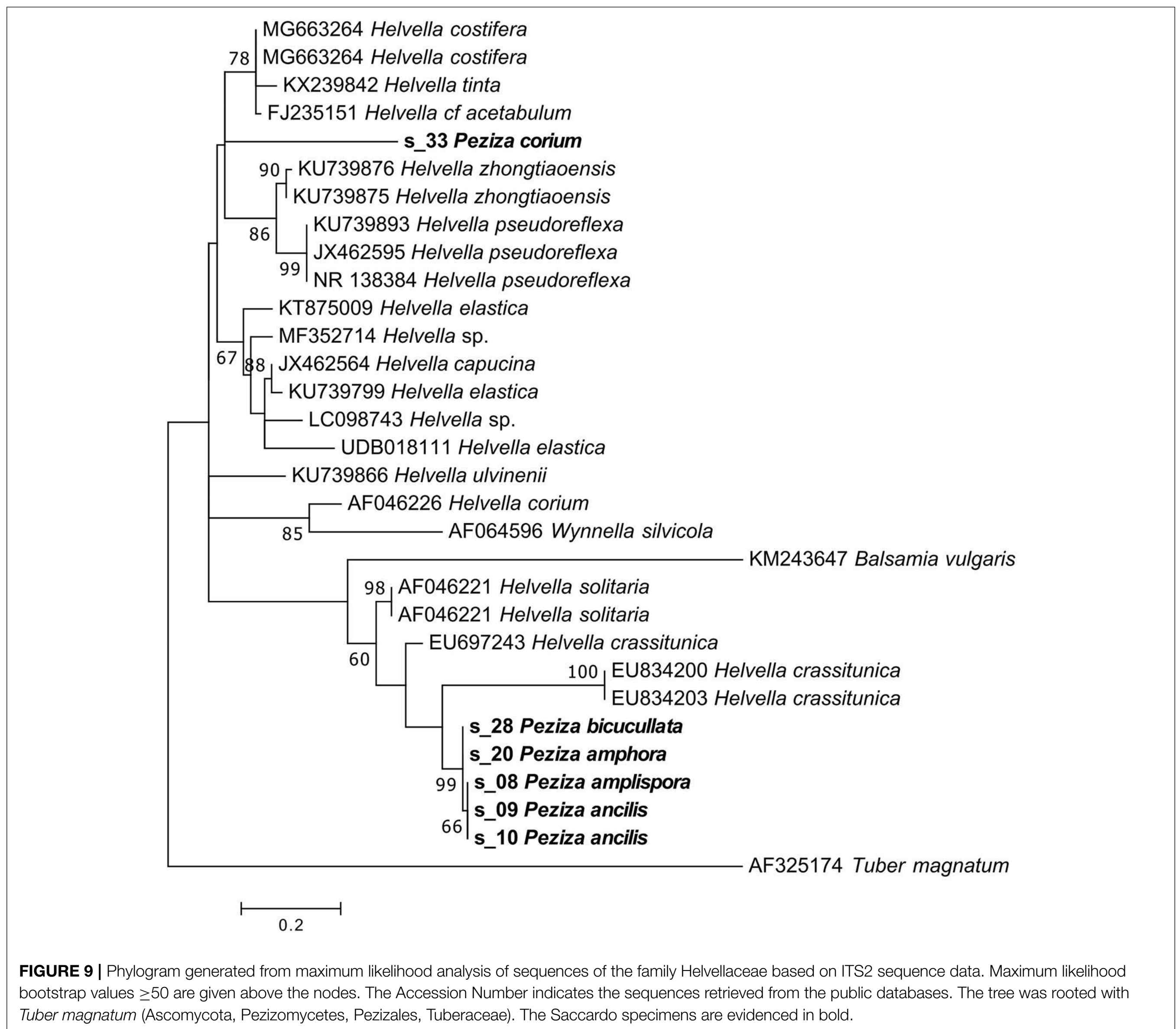

FIGURE 9 | Phylogram generated from maximum likelihood analysis of sequences of the family Helvellaceae based on ITS2 sequence data. Maximum likelihood bootstrap values ≥50 are given above the nodes. The Accession Number indicates the sequences retrieved from the public databases. The tree was rooted with *Tuber magnatum* (Ascomycota, Pezizomycetes, Pezizales, Tuberaceae). The Saccardo specimens are evidenced in bold.

Although they were determined with four different species names, the samples are probably conspecific, as indicated by the phylogenetic analysis (**Figure 9**). The phylogenetic analysis of these samples placed their sequences in a highly supported clade (MLB = 99%) separated from the *Helvella* sequences (**Figure 9**), suggesting that they could be generically annotated as Helvellaceae. This result probably depends on the fact that only a small fraction of the known fungal species is represented by molecular data in the public databases, and to such a purpose it is worth remembering that no molecular information is presently available not only for many species, but also for entire genera. The morphological analysis of the five specimens highlighted that, based on the presence of a regularly cupulate to planar apothecium with a ± prominent distinct stipe bearing blunt ribs not or scarcely continuing onto the receptacle, a non-amyloid ascus apex (in Melzer's reagent), spores 16–20 × (11) 12–14 µm with a large inner guttula, *P. ancilis* (s_09, s_10), *P. amphora* (s_20) and *P. bicucullata* (s_28) are conspecific and assignable to the *Helvella leucomelaena* complex (Skrede et al., 2017). By contrast, the specimen of *P. amplispora* (s_08), characterized by a non-stipitate apothecium, an amyloid ascus apex, large, non-guttulate spores up to 30 × 16 µm, is assignable to the genus *Peziza*. This is probably a case of cross-contamination. Therefore, the fact that the corresponding sequence is identical to those recovered from the samples belonging to the *Helvella leucomelaena* complex suggests that it arose by cross-contamination and not through amplification of the genuine *P. amplispora* (s_08) ITS2.

These examples illustrate the current gap between the number of described morphological fungal species and the sequences available in the public databases. Therefore, an

increase of the number of public sequences obtained from herbarium collections appears of paramount importance, although the ancient samples might require time and efforts to optimize the molecular analysis workflow due to high level of DNA degradation and contamination of their specimens.

Samples Where a Target Sequence Was Not Produced

The samples *P. agassizii* (s_03), *P. ammophila* (s_06), *P. anomala* (s_11), *P. applanata* 1 and 2 (s_12, s_13), *P. aranea* (s_14), *P. aeruginosa* (s_19), *P. arundinariae* (s_21), *P. atriella* (s_22), *P. aurelia* (s_24), *P. badia* 3 and 4 (s_26, s_27) and *P. corium* 1 (s_32) produced only sequences deriving from contaminating DNA (**Table 1**). The negative results of the last group of samples might be due to an inefficient amplification of the target DNA in favor of the fungal contaminants. This effect might be explained by considering that the high degradation level of the aDNA (Dabney et al., 2013) leads to a decreased amplification efficiency in the PCR with a consequent lower target ITS2 number in comparison with the contaminant ITS2 sequences.

General Considerations on the NGS Approach for the Study of Herbarium Samples

The ancient fungal collections were created by mycologists principally to have a catalog of fungi as wide as possible, and in those times, it appeared especially important to preserve the specimen's morphological characteristics. Unfortunately, this was accomplished by keeping the samples under conditions that were not the best for the conservation of the nucleic acid integrity. For example, for more than a century in the case of the Saccardo collection, the samples have been treated with chemicals against pests, but these compounds can have a detrimental effect on DNA integrity (Whitten et al., 1999). Moreover, a problem that is much more critical for mycological collection than for plant herbaria is represented by contamination of the samples with airborne environmental fungi, as the generally used universal barcode primers cannot discriminate between the contaminating DNA and the target DNA, thus generating a mix of fungal sequences that enhance the risk of sequencing failure. In fact, the classic DNA extraction followed by PCR amplification and Sanger sequencing has revealed its limitation in the case of old and highly contaminated specimens, as the Saccardo collection turned out to be, and caused poor results, frustration and time and money loss. The method we present here has been designed to overcome the technical issues inherent to ancient herbarium samples. NGS allows one to discard the large amount of non-target sequences and to obtain the ITS2 sequence of the target fungus from a minimal amount specimen biomass, thus protecting the herbarium collection.

In this work, we analyzed 36 samples belonging to *Peziza sensu* Saccardo, demonstrating that these experimental procedures and data analysis pipeline allowed in many cases a sequence assignment and a taxonomic re-evaluation of samples that are more than 100-years old. Our results underscore that accurate taxonomic assignments require comparing sequences against different databases and phylogenetic analyses. These complementary approaches have allowed us to place the sample sequences in the correct taxon. The NGS platform, which is scalable for a large number of samples, has been applied here as an important test on ancient specimens as a proof of principle for the investigation of no type specimens. The encouraging results obtained in this work suggest that this technique may be used to obtain marker sequences from the approximately 4,500 type specimens conserved in the Saccardo herbarium.

The Saccardo collection could represent a source of genetic material to increase the number of fungal sequences in public databases. Moreover, since we succeeded in the ITS2 amplification and NGS sequencing, the technique might be extended to other molecular markers, focusing on relatively short sequences to overcome the fragmentation of the ancient DNA, thus improving the taxonomic identification and the phylogenetic analysis of the samples. In this sense, next-generation sequencing of important samples can be crucial to support the traditional macro- and microscopic morphological analysis, in particular when the analysis considers poorly sequenced taxa and/or taxa with evanescent morphological traits. In particular, herbarium-derived sequences matching the target taxa only at the genus level provide interesting material and scope for taxonomic revisions based on total (morphological and molecular) evidence.

DATA AVAILABILITY STATEMENT

The ITS2 sequences annotated as obtained by Saccardo's specimens have been deposited in the NCBI database and the Accession Numbers are reported in **Table 1**. The entire datasets considered for this manuscript are not publicly available because they contain data not involved in this study but currently investigated for other purposes. Data is available upon request, and requests to access the datasets should be directed to Prof. Barbara Baldan, barbara.baldan@unipd.it.

AUTHOR CONTRIBUTIONS

NF and SN equally made the experimental work, analyzed the results, and co-wrote the manuscript. SV contributed to the setting up of the experimental procedures, helped with the data and phylogenetic analysis, discussed the results and critically read the manuscript. MG discussed the results and critically read the manuscript and AV contributed to the phylogenetic and morphological analysis, discussed the results and critically read the manuscript. GC and BB conceived the research, discussed the results and critically read the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00129/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Next generation sequencing of ancient fungal specimens: the case of the Saccardo Mycological Herbarium

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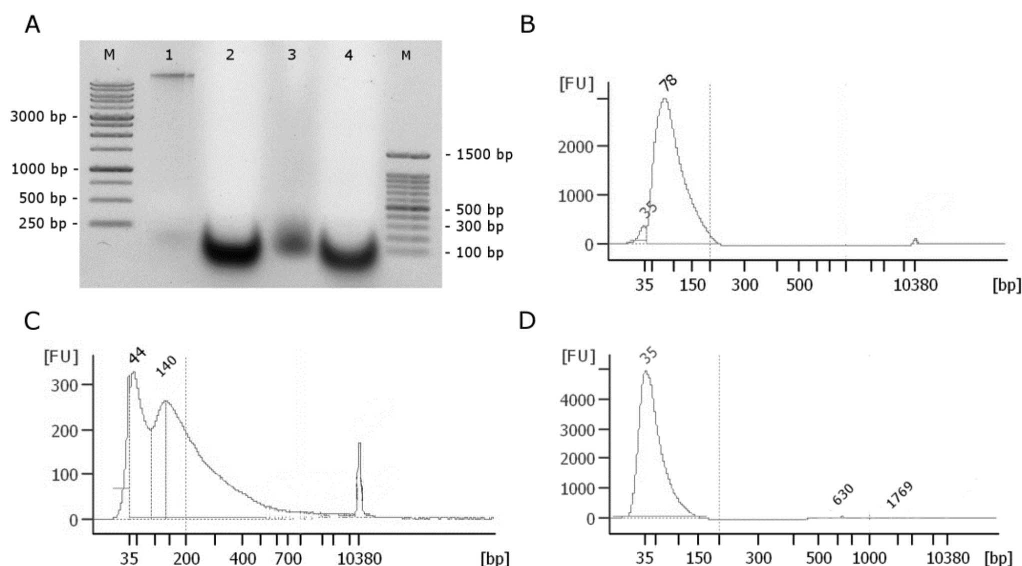
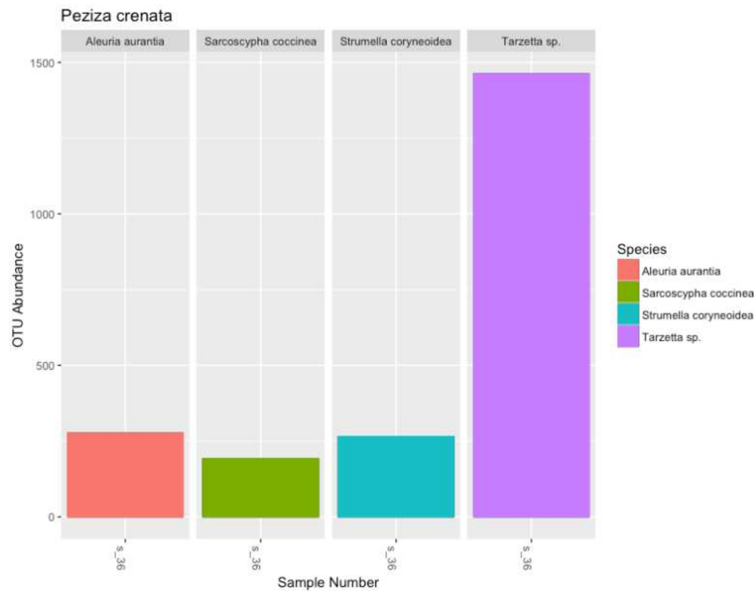
Supplementary Figures



Supplementary Figure S3. Integrity analysis of the DNA extracted from 3 *Peziza* samples studied in this work. (A) DNA electrophoresis on agarose gel of DNA extracted from a freshly collected *Agaricus bisporus* (lane 1), *Peziza adae* s_01 (lane 2), *P. badia* s_17 (lane 3) and *P. cochleata* s_31 (lane 4). A refined analysis was performed by means of Agilent Bionalyzer for *P. adae* (B), *P. badia* (C) and *P. cochleata* (D).



Supplementary Figure S2. OTU abundance showing the intra-herbarium contamination in the sample *Peziza crenata* (s_36). In addition to the sequence of the target sample (*Tarzetta* sp. in purple), our analysis has identified the presence of sequences of other three species belonging to different genera of Pezizales (*Aleuria aurantia* in orange, *Sarcoscypha coccinea* in green, *Strumella coryneoidea* in light blue). As these three species are present in the herbarium, this result is a strong evidence of the cross-contamination between specimens in the Saccardo collection.

CHAPTER 2

DNA Barcode sequencing and taxonomic revision of type specimens belonging to the genus *Nectria* and to *Nectria*-like genera preserved in the Saccardo collection

This chapter deals with the results of the Illumina MiSeq sequencing performed to recover ITS1 and ITS2 sequences from ancient type specimens classified in the Saccardo mycological collection as members of the genus *Nectria* (Fr.) Fr., but also from additional *Nectria*-like type specimens classified in the collection as members of the genera *Calonectria* De Not., *Chilonectria* Sacc., *Hypocrea* Fr., *Nectriella* Nitschke ex Fuckel, *Ophionectria* Sacc. and *Pleonectria* Sacc. In the past, many of these types were morphologically revised and placed in synonymy with other species or reclassified as members of new genera, while others were never reanalysed for reclassifications. None of these specimens has ever been involved in molecular studies so far. The Next-generation sequencing approach, applied in this work, has allowed to assign ITS1 and ITS2 sequences of 25 different *Nectria*/*Nectria*-like types (30 in total when considering multiple specimens) out of 76 specimens examined in this study. The sequences were then used for phylogenetic analysis to confirm or invalidate the reclassifications made for the types already revised and to reclassify the non-revised one. In this way, some types have been placed in synonymy with existing species, while others have been completely reclassified. This study provides new and important DNA sequences from precious fungal type material.

INTRODUCTION

Currently the genus *Nectria* is an Ascomycota group of fungi belonging to the class Sordariomycetes O.E. Erikss. & Winka, order Hypocreales Lindau family Nectriaceae Tul. and C. Tul. Fungi of this genus are pleomorphic, characterized by a *Tubercularia*-like asexual morph and a teleomorph stage producing small sexual reproductive structures named perithecia (ascocarps or ascomata containing asci, the structures that produce the ascospores internally). Normally *Nectria* perithecia are fleshy, uniloculate, generally red to bay, subglobose to globose with a smooth or warted surface and superficial on a well-developed stroma (mass of fungal hyphae, having spore-bearing structures either embedded in it or on its surface); asci are cylindrical to clavate in shape; ascospores usually ellipsoidal to fusiform, hyaline, smooth to spinulose (Hirooka et al., 2012; Lombard et al., 2015). Members of this genus, as well as all nectriaceous fungi, are typically parasites of woody plants occurring on hardwood trees and shrubs in tropical, subtropical, or temperate regions worldwide (Rossman et al., 1999; Hirooka et al., 2012). For instance, the type species of this genus, *Nectria cinnabarina* (Tode) Fr., is a plant pathogen causing a disease on apple and other hardwood trees known as "coral spot" that leads to the death of the tree branches (Hirooka et al., 2014). Furthermore, several members of the genus (such as the anamorphic species complex *Fusarium solani*) are implicated in invasive mycoses, which feature increasing incidence over the last decades, especially in patients with hematologic malignancies (Debourgogne et al., 2016).

The name *Nectria* was proposed for the first time by Fries in 1825, as an infrageneric section of the fungal genus *Hypocrea*, for species having perithecia superficially on a tomentose matrix or directly seated on the substratum. Then, in 1849, Fries himself erected *Nectria* to the generic rank giving additional descriptive characters such as the bright colour of perithecia, 8-spored asci and hyaline ascospores (Booth, 1959; Schroers, 2001). For many years the concept of *Nectria* was extremely broad and more than 1,000 species have been described and classified in *Nectria sensu lato* (Hirooka et al., 2012). In recent times, the concept of *Nectria* was restricted to species considered congeneric with the type species of the genus, *N. cinnabarina*, having a specific ascomatal wall structure and a *Tubercularia* Tode anamorph (Rossman et al., 1989,1999), and nowadays 31 different species are accepted in the genus *Nectria* (Zeng et al., 2018). As a consequence, the species excluded from *Nectria sensu lato* were placed in different or old-resurrected genera. For instance, genera such as *Neonectria* Wollenw. (family Nectriaceae) was reintroduced to accommodate *Nectria* species

having *Cylindrocarpon* Wollenw. anamorphs (Brayford et al., 2004) or *Hydropisphaera* Dumort. (family Bionectriaceae Samuels & Rossman) was resurrected for *Nectria*-like fungi previously placed in the *Nectria peziza* Berk. group (Samuels, 1976; Lechat et al., 2010); instead, other *Nectria*-like species were placed in genera of the Hypocreales families Nectriaceae and Bionectriaceae. The Bionectriaceae includes *Nectria*-like species having white, yellow, orange to tan or brown perithecia that do not change colour in KOH or lactic acid, while members of the Nectriaceae have orange-red to purple perithecia that turn dark red in KOH and yellow in lactic acid. The colour reaction of ascocarps in KOH was considered an important descriptive character to divide Bionectriaceae species from those of the family Nectriaceae (Rossman et al., 1999).

Taxonomic studies based on DNA sequence data confirmed the separation of the Bionectriaceae from the Nectriaceae and the relationships between the genera where *Nectria*-like species were segregated and the others, within the families Bionectriaceae and Nectriaceae (Rossman et al., 2001; Schroers, 2001; Hirooka et al., 2012; Lombard et al., 2015). In addition, molecular studies resurrected genera previously considered synonyms of *Nectria*, such as *Pleonectria* (now synonym of the Nectriaceae genus *Thyronectria* Sacc.; Lombard et al., 2015) and *Allantonectria* Earle (family Nectriaceae) (Rossman et al., 1999; Hirooka et al., 2012).

Pier Andrea Saccardo had an important role in the definition of the genus *Nectria* and in the descriptions of new *Nectria sensu* Saccardo species. In 1878, he restricted the description of *Nectria* to species with 1-septate ascospores and later, in his *Sylloge fungorum*, he rearranged the genus in 10 different subgenera according to the presence or absence and nature of a stroma, perithecial surface characteristics, and ascospores morphology (Booth, 1959; Schroers, 2001). Specifically, he subdivided the genus in: *Eunectria* (perithecia seated on a stroma); *Dialonectria* (perithecia not seated on a stroma/byssoid stroma); *Cryphonectria* (perithecia immersed in a stroma); *Hyphonectria* (perithecia seated on byssoid stroma); *Lepidonectria* (warted perithecia); *Lasionectria* (hairy perithecia); *Zimmermania* (perithecia with a dentate crown of hair); *Cosmospora* (ascospores verrucose); *Phaeonectria* (ascospores brown) and *Lichenonectria* (parasitic on lichens) (Samuels, 1976). Subsequently, Cooke (1884) raised all these subgenera to the generic rank, but today only *Lasionectria* (Sacc.) Cooke (Bionectriaceae; Rossman et al., 1999) is accepted as a genus in Hypocreales, while the others were placed in synonymy with older genera (Schroers, 2001). In addition, Saccardo recognised generic segregates from *Nectria*, based on the ascospore septation, such as *Calonectria* (medium length ascospores with more than one septum) and *Ophionectria* (long

ascospores and multiple septa), currently genera of Nectriaceae (Hirooka et al., 2012; Lombard et al., 2015), describing also for these genera new species that are preserved in his mycological collection.

In the Saccardo collection, the genus *Nectria* is represented by over 100 different species comprising 40 type specimens, some of them represented by multiple samples. All these specimens were deposited prior to the year 1920 and are stored on the substrates on which they were found (bark, dead wood and plant stems). Many of these types (34) were used directly by Saccardo for the morphological description of new species, while six by other contemporary mycologists to Saccardo to describe new species (named Cotypes) (**Table 1**). Later, most of these specimens were morphologically revised by expert mycologists and placed in synonymy with other existing species or reclassified as members of new genera within the families Nectriaceae and Bionectriaceae; others were not considered for subsequent morphological revisions (**Table 1**). However, none of these specimens has never been the subject of molecular studies so far.

The task of recovering molecular data from *Nectria* and *Nectria*-like types preserved in the Saccardo fungarium is extremely important because many of these fungi are plant pathogens, thus the availability of DNA barcodes from these specimens has the potential to facilitate future species identification for the purpose of plant disease diagnoses (Zhao et al., 2010). In addition, obtaining molecular information from type material can be essential, for example, when the study of modern specimens suggests synonymy or it indicates that a known species is a complex of morphologically similar taxa, or when the type represents the only known record for a specific taxon (Prosser et al., 2015), such as in the case of some species analysed in this study that are known only from the type material preserved in the Saccardo collection.

For this purpose, a high-throughput sequencing method was applied to get ITS1 and ITS2 sequences from 56 *Nectria* and 20 additional *Nectria*-like types (**Tables 1-2**), to overcome the problems linked to the ancient herbarium material already observed in a previously study on *Peziza* specimens preserved in the Saccardo collection (Forin et al., 2018).

Table 1. *Nectria* type specimens preserved in the collection. The number of the samples per type, the authors and the current name for the species (recovered from *Index Fungorum*, MycoBank (http://www.mycobank.org/) and literature) are also reported.

Herbarium name, number of samples and authors	Current name
Nectria abscondita, 2, Sacc.	*Cryphonectria abscondita* (Sacc.) Sacc. & D. Sacc. (Cryphonectriaceae Gryzenh. & M. J. Wingf.)
Nectria albofimbriata, 3, Penz. & Sacc.	*Protocreopsis albofimbriata* (Penz. & Sacc.) Yoshim. (Bionectriaceae)
Nectria ambigua, 1, Penz. & Sacc.	*Bionectria apocyni* (Peck) Schroers & Samuels (Bionectriaceae)
Nectria ambigua var. *pallens*, 1, Penz. & Sacc.	*Pezicula livida* (Berk. & Broome) Rehm (Dermateaceae Fr.)
Nectria arundinella, 1, Penz. & Sacc.	
Nectria binotiana, 1, Sacc.	
Nectria bonanseana, 2, Sacc.	
Nectria brassicae, 4, Ellis & Sacc.	*Cosmospora peponum* (Berk. & Curtis) Rossman & Samuels (Nectriaceae)
Nectria carneoflavida, 1, Penz. & Sacc.	*Bionectria apocyni* (Peck) Schroers & Samuels (Bionectriaceae)
Nectria congesta, 1, Sacc.	
Nectria coronata, 1, Penzig. & Sacc.	*Thelonectria coronata* (Penz. & Sacc.) Chaverri & Salgado (Nectriaceae)
Nectria cyanostoma, 2, Sacc. & Flageolet	*Cyanonectria cyanostoma* (Sacc. & Flageolet) Samuels & Chaverri (Nectriaceae)
Nectria dolichospora, 2, Penz. & Sacc.	*Hydropisphaera dolichospora* (Penz. & Sacc.) Rossman & Samuels (Bionectriaceae)
Nectria episphaerioides, 1, Penz. & Sacc.	*Haematonectria haematococca* (Berk. & Broome) Samuels & Nirenberg (Nectriaceae)
Nectria eustoma, 1, Penz. & Sacc.	*Thelonectria jungneri* (Henn.) Chaverri & Salgado (Nectriaceae)
Nectria flageoletiana, 1, Sacc.	
Nectria granuligera, 2, Starbäck	*Creonectria granuligera* (Starbäck) Seaver (Nectriaceae)
Nectria hirtella, 2, Sacc. & Speg.	
Nectria hypoxantha, 1, Penz. & Sacc.	*Hydropisphaera hypoxantha* (Penz. & Sacc.) Rossman & Samuels (Bionectriaceae)
Nectria illudens, 1, Berk.	*Neocosmospora illudens* (Berkeley) Lombard & Crous (Nectriaceae)
Nectria leucotricha, 2, Penz. & Sacc.	*Hydropisphaera leucotricha* (Penz. & Sacc.) Rossman & Samuels (Bionectriaceae)
Nectria mantuana, 1, Sacc.	*Lasionectria mantuana* (Sacc.) Cooke (Nectriaceae)
Nectria megalospora, 1, Sacc. & Berl.	*Perisporiopsis megalospora* (Sacc. & Berl.) Arx (Perisporiopsidaceae E. Müll. & Arx ex R. Kirschner & T.A. Hofm)
Nectria muscicola, 1, Penz. & Sacc.	
Nectria nigella, 1, Penz. & Sacc.	

Nectria perpusilla, 1, Sacc.	
Nectria pezicula, 2, Speg.	*Hydropisphaera peziza* (Tode: Fr.) Dumort. (Bionectriaceae)
Nectria peziza subsp. *reyesiana*, 1, Sacc.	*Nectria peziza* (Tode) Fr. = *Hydrophisphaera peziza* (Tode: Fr.) Dumort (Bionectriaceae)
Nectria phyllogena, 1, Sacc.	
Nectria phyllostachydis, 1, Hara	
Nectria radians, 1, Penz. & Sacc.	*Sarcopodium flavolanatum* (Berk. & Broome) Lombard & Crous (Nectriaceae)
Nectria rousseauana, 1, Sacc.	
Nectria raripila, 1, Penz. & Sacc.	*Sarcopodium raripilum* (Penz. & Sacc.) Lombard & Crous (Nectriaceae)
Nectria sordescens, 1, Sacc.	
Nectria squamuligera, 3, Sacc.	
Nectria tasmanica, 1, Berk.	*Thelonectria discophora* (Mont.) Chaverri & Salgado (Nectriaceae)
Nectria theprothala, 1, Berk.	*Endothia tephrothele* (Berk.) Höhn. (Cryphonectriaceae)
Nectria tjibodensis, 1, Penz. & Sacc.	*Sarcopodium flavolanatum* (Berk. & Broome) Lombard & Crous (Nectriaceae)
Nectria tjibodensis var. *crebrior*, 1, Penz. & Sacc.	*Actinostilbe flocculenta* (Henn. & E. Nyman) Rossman, Samuels & Seifert (Nectriaceae)
Nectria xanthostroma, 2, Penz. & Sacc.	*Polystigma xanthostroma* (Penz. & Sacc.) Gola (Phyllachoraceae Theiss. & P. Syd.)

Table 2. Additional *Nectria*-like type specimens. The number of the samples per type, the authors and the current name for the species (recovered from *Index Fungorum*, MycoBank (http://www.mycobank.org/) and literature) are also reported.

Herbarium name, number of samples and authors	Current name
Calonectria macrospora, 4, Sacc. & Speg.	
Chilonectria macrospora,1, Penz. & Sacc.	
Chilonectria romana,1, Sacc.	
Chilonectria rosellinii, 1, (Carestia) Sacc.	*Thyronectria rosellinii* (Carestia) Jaklitsch & Voglmayr (Nectriaceae)
Hypocrea discolor, 1, Sacc.	
Hypocrea fulva, 3, Penz. & Sacc.	
Nectriella bacillispora, 1, Traverso & Spessa	*Allantonectria miltina* (Mont.) Weese in Höhnel & Weese (Nectriaceae)
Nectriella maquilingica, 1, Sacc.	*Bionectria byssicola* (Berk. & Broome) Schroers & Samuels (Bionectriaceae)
Nectriella rufofusca, 1, Penz. & Sacc.	*Hydropisphaera rufofusca* (Penz. & Sacc.) Rossman& Samuels (Bionectriaceae)
Ophionectria trichia, 1, Penz. & Sacc.	
Pleonectria affinis, 1, Sacc.	
Pleonectria berolinensis, 4, Sacc.	*Thyronectria berolinensis* (Sacc.) Seaver (Nectriaceae)

MATERIALS AND METHODS

Sampling

Nectria and *Nectria*-like types, encompassing multiple specimens, were collected from the Saccardo mycological herbarium and observed under a stereomicroscope (Leica EZ4W) in order to identify and to sample the fungi on their substrates. Considering the inestimable value of these specimens, the sampling was done with the permission of the herbarium curator and a particular attention was paid in order to preserve the overall integrity of each specimen. The specimens were sampled by removing a small number of dried perithecia from the substratum (plant material or bark), without damaging them, using sterilized tweezers. The material was used both for morphological observations and for molecular analysis.

Morphological analysis

Due to the age and to non-optimal preservation of the samples in the collection, not all the morphological characters used to describe the species belonging to Nectriaceae and related families can be investigated (Rossman et al., 1999). The morphological observations were focused on characters linked to the visible teleomorph stages such as shape, dimension, and colour of the reproductive structures (perithecia), asci and ascospores.

The sampled material was placed on a slide and rehydrated by placing a drop of water directly on the perithecia for a few minutes. The macroscopic features of the perithecia were observed, photographed and described as follows: distribution on the host and shape, colour, and colour reaction to 3% potassium hydroxide (KOH) and 100% lactic acid (LA) using a stereomicroscope Leica EZ4W. If a significant colour change occurred, the sample was indicated as KOH+. If, after the first KOH test for colour reaction, a new colour change occurred using lactic acid, this was indicated as LA+. The internal microscopic characters such as asci and ascospores were observed after the colour test with KOH and LA by placing the perithecia under a cover slide and smashing them. To observe the surface of spores, cotton blue was used as the mounting staining medium. Microscopic structures were examined and photographed using a Leica ICC50W optical microscope with 40× or 100× (immersion oil) objectives. After taking the pictures, the diameter of perithecia, the length and width of asci and ascospores were measured using the software Fiji (Schindelin et al., 2012). The average of the measurements is marked in *italic* in the species description.

DNA extraction

DNA was extracted using the CTAB method described in Forin et al. (2018). The success of the DNA extraction was verified by running a small amount of the extracted DNA (3 μL stained with Eurosafe DNA dying, Euroclone) for each sample in 0.8% agarose gel in TRIS acetate-EDTA buffer. The extracted DNAs were then purified using OneStep^{TM} PCR Inhibitor Removal Kit (Zymo research) in order to remove potential contaminants that might inhibit downstream PCR reactions.

ITS1/ITS2 amplification and Illumina MiSeq libraries preparation

For the preparation of the Illumina sequencing libraries, the nuclear ribosomal ITS1 and ITS2 regions were amplified using a two-step PCR process. The first PCR was carried out using the universal primers ITS1F/ITS2 (White et al., 1990; Gardes and Bruns, 1993) for the ITS1 amplification and the universal primers ITS3/ITS4 (White et al., 1990) for the ITS2 amplification. In the second PCR the products of the first amplification of the ITS1 and ITS2 regions were amplified using the same couple of primers tagged with different 5 bp identifier tags to distinguish sequences from each specimen. The second PCR was done in four replicates for each couple of tagged primers.

The first PCR reaction was carried out in a total volume of 25 μL including 5 μL of 5X Wonder Taq reaction buffer (5 mM dNTPs, 15 mM $MgCl_2$; Euroclone), 0.5 μL of bovine serum albumin (BSA, 10 mg/mL), 0.5 μL each of two primers (10 μM), 0.5 μL of Wonder Taq (5 U/μL), 2 μL of genomic DNA and ddH_2O to reach the final volume. The PCR conditions used for the ITS1 were: 95°C for 3 min; 35 cycles of 95°C for 30 s, 53°C for 40 s and 72°C for 45 s; 72°C for 5 min. The PCR conditions used for the ITS2 were: 95°C for 3 min; 35 cycles of 95°C for 30 s, 52°C for 40 s and 72°C for 45 s; 72°C for 5 min. The success of the amplifications was checked in 1.2% agarose gel in TRIS acetate-EDTA buffer using 5 μL of the PCR products stained with Eurosafe DNA dying (Euroclone).

The second PCR reactions were performed similarly to the previous amplifications except for the absence of the BSA, the use of 2 μL of the first PCR amplicons as template and the use of the tagged primers. The success of the amplifications was checked in 1.2% agarose gel in TRIS acetate-EDTA buffer using 5 μL of the PCR products stained with Eurosafe DNA dying (Euroclone). Then, the four replicates of each sample were pooled together and purified using the PureLink PCR Purification Kit (Invitrogen). After the quantification with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), the purified amplicons were mixed in

equimolar amount to prepare different Illumina sequencing ITS1 and ITS2 libraries according to the specifications provided by the DNA sequencing service. In some cases, the use of Speedvac centrifuge was necessary to concentrate some samples to reach the correct library concentration. In addition, several specimens were placed in the libraries more than once depending on the quantity and quality of the PCR products in order to increase the chance of obtaining the target sequences. The libraries were sent to Fasteris (Geneva, Switzerland) for a paired-end sequencing using the Illumina MiSeq technology 2 x 300 bp (the libraries are reported in **Appendix 1**).

Bioinformatic analysis and sequences identification

The attribution of the reads to each sample was done directly by the DNA sequencing service, according to a list of barcodes and primers provided to them, considering no mismatch in the 5 bp tag sequences and a maximum number of two mismatches in the primer sequences. In this way, each sample was analysed separately. Forward and reverse reads were merged using PEAR v0.9.10 (Zhang et al., 2014) with the quality score threshold set at 28, the minimum length of reads after trimming set at 150 bp and the minimum overlap size set at 100 bp. Sequences were dereplicated with VSEARCH v2.3.4 (Rognes et al., 2016) to form clusters with an identity of 100%. After the dereplication, the ITSx software (http://microbiology.se/software/itsx/; Bengtsson-Palme et al., 2013) was used to extract the ITS1 and ITS2 regions. The picking of the Operational Taxonomic Units (OTUs) was performed with 98% similarity clustering using VSEARCH and the clusters with less than 10 sequences were discarded. Chimera sequences were then removed using a *de novo* chimera detection with UCHIME algorithm (Edgar et al., 2011) implemented in the VSEARCH pipeline. The OTUs obtained were compared with the sequences deposited in GenBank, excluding uncultured/environmental sample sequences, using the BLASTN algorithm (BLAST-search, http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1997) in order to identify the target sequences. The bioinformatic pipeline used is reported in **Appendix 2**.

In order to assign the correct sequence to the analysed Saccardo samples (discriminating between the target sequence and possible contaminations/coexisting species), the sequencing results were evaluated by taking into account: the modern taxonomy of the specimens; the notes reported in the papery labels associated with the samples; the new morphological observations; the number of sequences per OTUs and, in the case of specimens for which

both ITS regions were amplified, comparing the BLASTN taxonomic assignment obtained for ITS1 and ITS2.

Molecular phylogenetic analysis

Sequences obtained from the *Nectria sensu* Saccardo and *Nectria*-like specimens were further investigated by a phylogenetic analysis to obtain a more accurate identification of the fungal specimens. The sequences used for the phylogenetic analysis were chosen on the basis of BLASTN results, selecting taxonomically close well annotated and published sequences in accordance with known phylogenetic studies about the families Nectriaceae and Bionectriaceae (Schroers, 2001; Chaverri et al., 2011; Gräfenhan et al., 2011; Hirooka et al., 2012; Lombard et al., 2015; Salgado-Salazar et al., 2017). The ITS1 and ITS2 regions were extracted from the sequences downloaded from GenBank and used in the phylogenetic analysis by using the ITSx software and were then reassembled in order to exclude the conserved 5.8S region (not present in the sequence obtained with the Illumina sequencing) and any partial 18S and 28S sequences. In the same way, ITS1 and ITS2 sequences, when both identified, of the Saccardo type specimens were recombined and used in the phylogenetic analysis (from this point forward, "ITS sequence" refers to the combination of ITS1 and ITS2 sequences).

The ITS sequences of each dataset were aligned using the default parameters in Muscle (Edgar, 2004) as implemented in SeaView ver. 4.7 (Gouy et al., 2010). Maximum Likelihood trees were generated in SeaView ver. 4.7 using PhyML (Guindon et al., 2010) with generalized time-reversible (GTR) evolution model (Nei and Kumar, 2000), using Gblocks with the options for a less stringent selection (Castresana, 2000) and 1000 bootstrap replicates. The phylogenetic trees were drawn to scale, with branch lengths measured in the number of substitutions per site, and supporting values (MLB, Maximum likelihood bootstrap values) below 50 were generally regarded as unreliable and not reported on the trees.

Additional specimens involved in the study

DNA samples of the isotype *Bionectria subquaternata* (Berk. and Broome) Schroers and Samuels, and of *Hydropisphaera peziza* (Tode) Dumort. were obtained from The Westerdijk Fungal Biodiversity Institute (KNAW, Utrecht) and the entire ITS sequences were amplified using the universal primers ITS1/ITS4 (White et al., 1990). The PCR reaction was carried out in a total volume of 25 µL including 5 µL of 5X Wonder Taq reaction buffer (5 mM dNTPs,

15 mM $MgCl_2$; Euroclone), 0.5 µL of bovine serum albumin (BSA, 10 mg/mL), 0.5 µL each of two primers (10 µM), 0.5 µL of Wonder Taq (5 U/µL), 2 µL of genomic DNA and ddH_2O to reach the final volume. The PCR conditions used were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 70 s; 72°C for 7 min. The success of the amplifications was checked in 1.2% agarose gel in TRIS acetate-EDTA buffer using 5 µL of the PCR products stained with Eurosafe DNA dying (Euroclone). The PCR products were quantified with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and sent to Eurofins Genomics (Vimodrone, Italy) service for the sequencing. The sequences were used in the phylogenetic analysis to obtain a better taxonomic identification of some of the type specimens analysed.

RESULTS AND DISCUSSION

Morphological observations

This section reports the morphological descriptions of *Nectria sensu* Saccardo and *Nectria*-like types for which it was possible to identify the ITS1 or/and ITS2 sequence(s). No anamorph was observed on the specimens analysed. Only for the type specimens *Nectria cyanostoma* and *Nectriella rufofusca*, asexual spores (conidia) were observed.

Nectria albofimbriata Penzig & Saccardo, Malpighia 11: 513. 1897. **Figure 1**

≡*Protocreopsis albofimbriata* (Sacc. & Penz.) Yoshim. Doi, Bulletin of the National Science Museum, Tokyo: 117 (1978).

Specimens examined:

- Lectotype specimen of *Nectria albofimbriata* (number 436a) from Indonesia (Java, Tjibodas), on dead stems of *Elettaria* sp., 6 February 1897 (Samuels et al., 1990).
- Syntype specimen of *Nectria albofimbriata* (number 172) from Indonesia (Java, Tjibodas) (Samuels et al., 1990).

Perithecia gregarious, completely immersed in a superficial white stroma, globose, not papillate, yellowish orange, 200-280 µm diameter (n= 5); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate to fusiform, 48-*52*-59 × 10-*12*-13.7 µm (n= 10), 8-spored, ascospores biseriate. **Ascospores** fusiform, 16-*18*-19.2 × 3-*3.9*-5 µm (n= 30), 1-septate, equally subdivided in two cells, not constricted or slightly constricted at the septum, hyaline, striate with parallel striae.

NOTES - The two specimens were morphologically revised in 1977, as reported in the papery label associated with the samples. This species is now considered a member of the Bionectriaceae genus *Protocreopsis* Yoshim. Doi with the name *P. albofimbriata*. *Nectria albofimbriata* is the basionym of *P. albofimbriata* (Samuels et al., 1990; Rossman et al., 1999). A detailed description of these specimens is reported in Samuels et al. (1990).

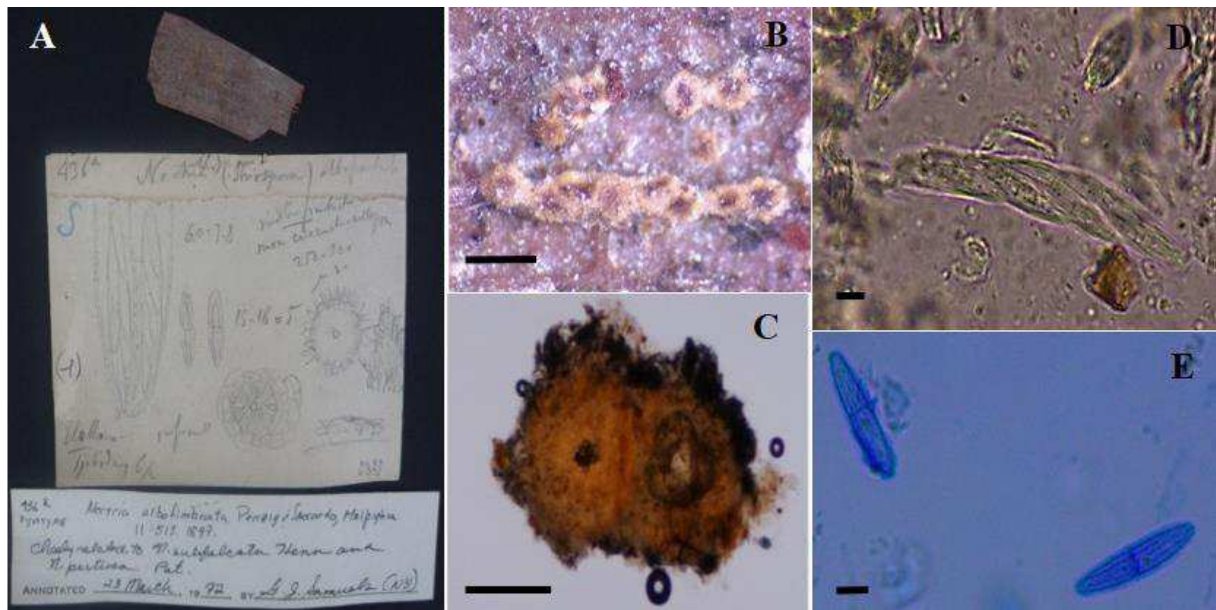


Figure 1. **A** *Nectria albofimbriata*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecia; **D** Ascus with ascospores; **E** Ascospores. Scale bars: **B**= 500 µm; **C**= 200 µm; **D-E**= 5 µm.

Nectria ambigua Penzig & Saccardo, Malpighia 11: 511. 1897. **Figure 2**

Specimen examined:

- Holotype specimen of *Nectria ambigua* from Indonesia (Java, Tjibodas), on bark (Samuels et al., 1990).

Perithecia solitary or in groups of a few, superficial on bark, yellowish orange, globose, not papillate, warted, about 450 µm diameter; not changing colour in 3% KOH and in 100% lactic acid. **Asci** narrowly clavate, 80 × 12 µm (n= 1), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoidal to fusiform, 13.6-*17.9*-21 × 4.8-*5.6*-8 µm (n= 28), 1-septate, equally subdivided in two cells, not constricted at the septum, hyaline, warted.

NOTES - The specimen was morphologically revised in 1983 and in 1997, as reported in the papery labels associated with the sample. In the first revision it was hypothesized a relationship of this specimen with *N. aureofulva* Cooke & Ellis (≡*Bionectria aureofulva*

(Cooke & Ellis) Schroers and Samuels, family Bionectriaceae; Schroers, 2001) and *N. apocyni* Peck (≡*Clonostachys apocyni* (Peck) Rossman, L. Lombard & Crous, *Bionectria apocyni* (Peck) Schroers & Samuels, family Bionectriaceae; Schroers, 2001), and its affinity with the *Nectria ochroleuca* group (Samuels et al., 1990). In the second revision it was suggested to transfer *N. ambigua* to the Bionectriaceae genus *Bionectria*. Nowadays this species is considered a synonym of *B. apocyni* (family Bionectriaceae), although with some doubts (Schroers, 2001). A detailed description of this species is reported in Samuels et al. (1990).

Figure 2. A *Nectria ambigua*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Ascus with ascospores; **D-E** Ascospores. Scale bars: **B**= 500 µm; **C**= 20 µm; **D-E**= 10 µm.

Nectria ambigua* var. *pallens Penzig & Saccardo, Malpighia 11: 511. 1897. **Figure 3**

Specimen examined:

- Holotype specimen of *Nectria ambigua* var. *pallens* from Indonesia (Java, Tjibodas), on dead rotten bark, 4 February 1897. The species results associated on the same substrate with *Nectria coronata* Penzig and Saccardo (Samuels et al., 1990).

Perithecia solitary or aggregated in groups, not immersed in a stroma, globose to subglobose-depressed, not papillate, superficial on bark, pale yellow, 240-375 µm diameter (n= 5); not changing colour in 3% KOH and 100% lactic acid. **Asci** strictly clavate, 50-*57.4*-67 × 6.5-*7.4*-9 µm (n= 10), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores**

ellipsoid to fusiform, 15.2-*16.7*-18.4 × 4-*4.9*-6.1 µm (n= 30), 1-septate, equally subdivided in two cells, not constricted at the septum, hyaline, striate with parallel striae.

NOTES - The specimen was morphologically revised in 1983 and in 1997, as reported in the papery label associated with the sample. The revisions suggest the synonymy with *Nectria subquaternata* Berk. & Broome (≡*Bionectria subquaternata*, family Bionectriaceae; Schroers, 2001). However, the synonymy between *N. ambigua* var. *pallens* and *B. subquaternata* is not reported. Samuels et al. (1990) considered *N. ambigua* var. *pallens* a synonym of *N. grammicospora* Ferd. & Winge (≡*B. grammicospora* (Ferd. & Winge) Schroers & Samuels, family Bionectriaceae; Schroers, 2001).

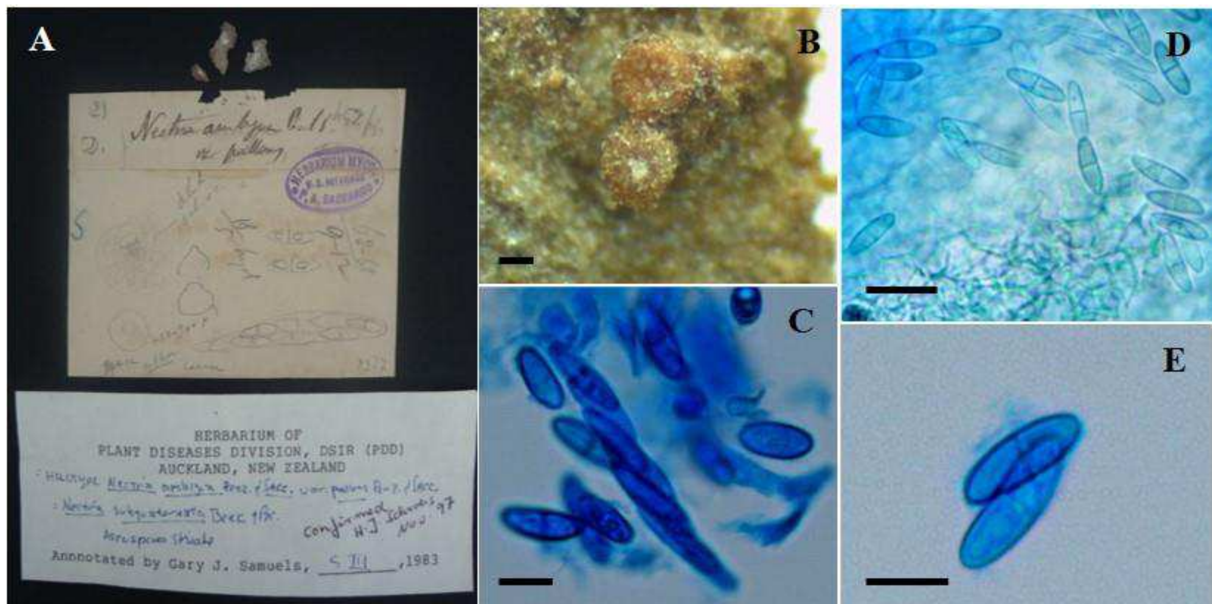


Figure 3. A *Nectria ambigua* var. *pallens*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Ascus with ascospores. **D**-**E** Ascospores. Scale bars: **B**= 100 µm; **C**= 10 µm; **D**= 20 µm; **E**= 10 µm.

Nectria congesta Saccardo, Michelia 2: 256. 1881. **Figure 4**

Specimen examined:

- Type specimen of *Nectria congesta* from Italy (Padua, Botanical Garden), on dead rhizome of *Hedychium coronarium*.

Perithecia aggregated in dense groups, partially immersed in a stroma superficial on the substrate, globose, not papillate, yellow, 190-240 µm diameter (n= 10); not changing colour in 3% KOH and 100% lactic acid. **Asci** not found. **Ascospores** ellipsoid, 15.2-*16.7*-18.4 × 4-

4.9-6.1 µm (n= 20), equally subdivided in two cells, 1-septate, not constricted or slightly constricted at the septum, warted, hyaline.

NOTES - This specimen has never been morphologically revised.

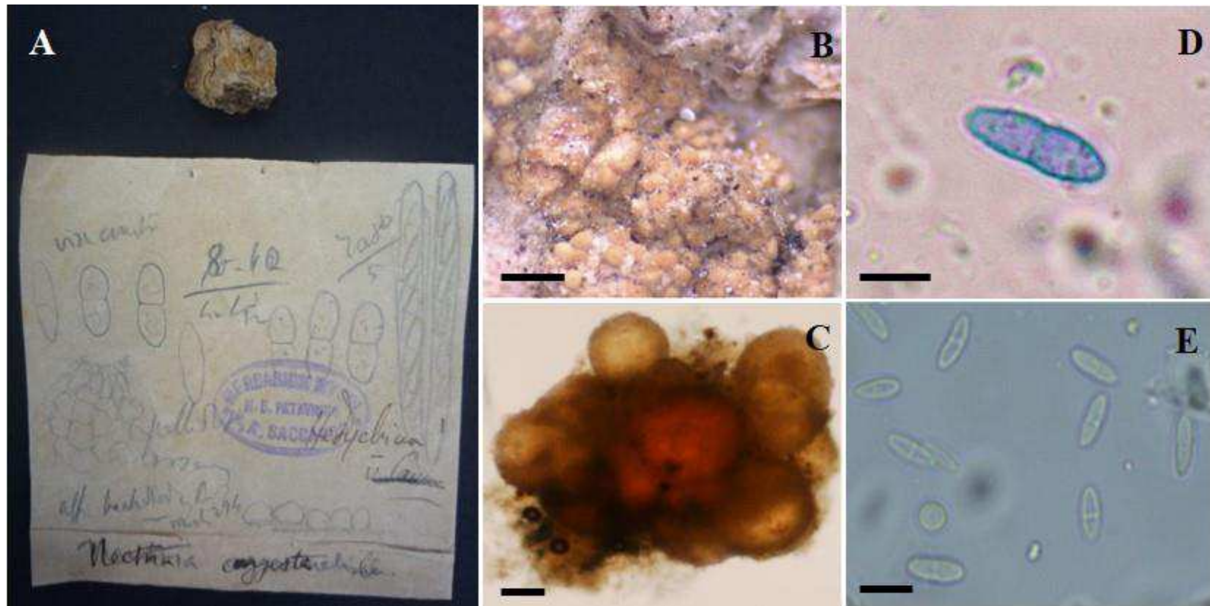


Figure 4. A *Nectria congesta*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecia; **D**-**E** Ascospores. Scale bars: **B**= 50 µm; **C**= 100 µm; **D**= 5 µm; **E**= 10 µm.

Nectria coronata Penzig & Saccardo, Malpighia 11: 510. 1897. **Figure 5**

≡*Thelonectria coronata* (Penz. & Sacc.) P. Chaverri & C. Salgado, Studies in Mycology 68: 76 (2011).

Specimen examined:

- Holotype specimen of *Nectria coronata* from Indonesia (Java, Tjibodas), on dead rotten bark. The species results associated on the same substrate with *Nectria ambigua* var. *pallens* Penzig and Saccardo (Samuels et al., 1990).

Perithecia gregarious in group, superficial, globose to pyriform, brownish-red with a darker ostiolar disc, 225-350 µm diameter; darker in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+); ostiolar disc 170 µm diameter with saccate cells which forms a fringe around the ostiolar disc giving the perithecium a coronate aspect. **Asci** not found. **Ascospores** ellipsoid to fusiform, 17.1-*19.2*-22.3 × 5.3-*6.3*-7.1 µm (n= 20), two-celled, symmetrical or eccentric, sometimes with one side curved and one side flattened, 1-septate, constricted or not constricted at the septum, hyaline, striate.

NOTES - This species now belongs to the Nectriaceae genus *Thelonectria* P. Chaverri & Salgado as *T. coronata* (Penz. & Sacc.) P. Chaverri & Salgado. *Nectria coronata* is the basionym of *T. coronata*. The anamorph of *T. coronata* is *Cylindrocarpon coronatum* Brayford & Samuels (family Nectriaceae) (Chaverri et al., 2011).

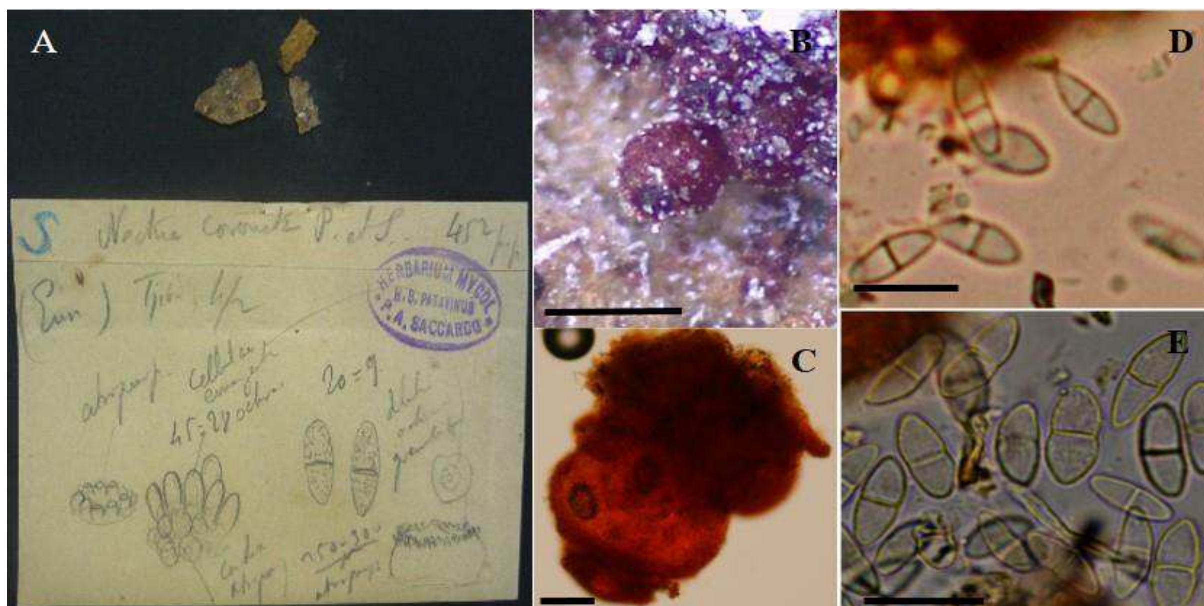


Figure 5. A *Nectria coronata*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecium; **D-E** Ascospores. Scale bars: **B**= 250 µm; **C**=200 µm; **D-E**= 20 µm.

Nectria cyanostoma Saccardo & Flageolet, Atti del Congresso Botanico di Palermo: 53. 1902. **Figure 6**

≡*Cyanonectria cyanostoma* (Sacc. & Flageolet) Samuels & Chaverri, Mycological Progress 8 (1): 56 (2009).

≡*Fusarium cyanostomum* (Saccardo & Flageolet) O'Donnell & Geiser, Phytopathology 103 (5): 404 (2013).

Specimen examined:

- Type specimen of *Nectria cyanostoma* (number 32) from France (St. Romain near Rigny), on bark of *Buxus sempervirens*.

Perithecia gregarious, superficial, reddish-brown, pyriform with darker apical region, 158-250 µm diameter (n= 10); dark-red in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+). **Asci** not found. **Ascospores** ellipsoidal to ovoidal, 9.6-*12.6*-15.2 × 4-*4.9*-5.8 µm (n= 30), 1-septate, equally subdivided in two cells, constricted at the septum, pale yellow, warted.

Macroconidia 3-5 septate: 3-septate 43 × 4 µm (n= 1); 5-septate 52-63 × 5 µm (n= 2), curved.

NOTES - This species now belongs to the Nectriaceae genus *Cyanonectria* Samuels & P. Chaverri as *C. cyanostoma*. *Nectria cyanostoma* is the basionym of *C. cyanostoma* and the type species of the genus *Cyanonectria.* The anamorph of *C. cyanostoma* is *Fusarium cyanostomum* (family Nectriaceae) (Samuels et al., 2008; Lombard et al., 2015).

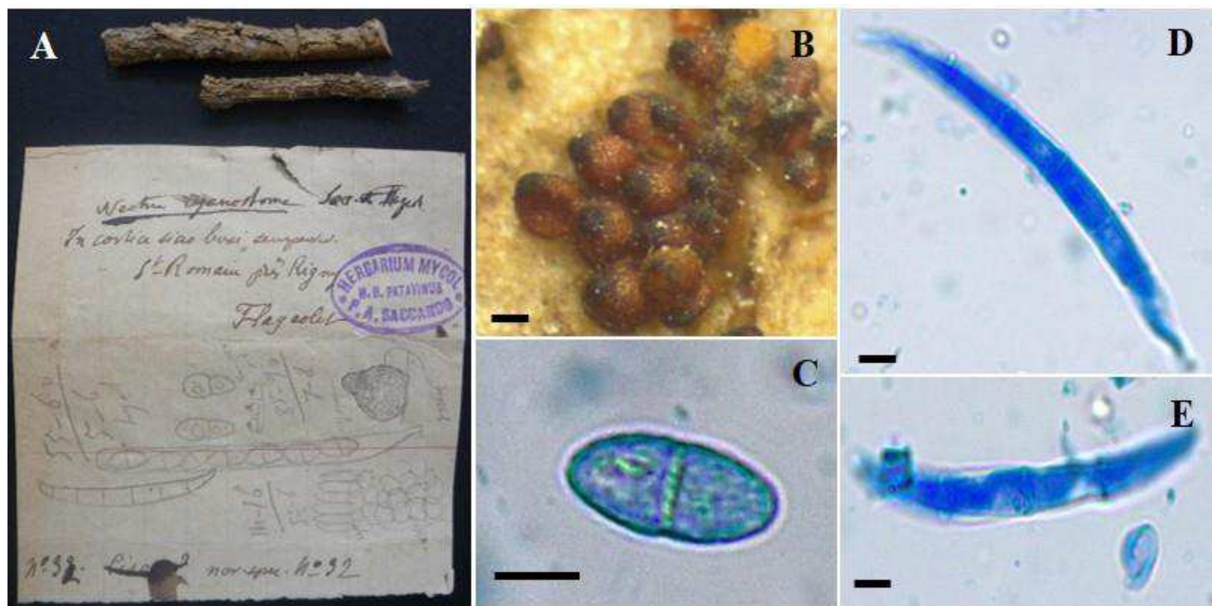


Figure 6. A *Nectria cyanostoma*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Ascospore**; D-E** Macroconidia. Scale bars: **B**= 100 µm; **C**-**D**-**E**= 5 µm.

Nectria dolichospora Penzig & Saccardo, Malpighia 11: 513. 1897. **Figure 7**

≡*Hydropisphaera dolichospora* (Penz. & Sacc.) Rossman & Samuels, Studies in Mycology 42: 30 (1999).

Specimens examined:

- Lectotype specimen of *Nectria dolichospora* (number 434) from Indonesia (Java, Tjibodas), on dead stem of *Elettaria* sp., February 1897 (Samuels et al., 1990).
- Type specimen of *Nectria dolichospora* (number 442) from Indonesia (Java, Tjibodas), on dead stem of *Elettaria* sp., February 1897.

Perithecia solitary or gregarious, superficial, brown, globose with hyphae around the perithecium base, not papillate, 187-257 µm diameter (n= 10); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate, 66-*74.6*-80 × 10-*12.6*-15 µm (n= 5), 8-spored,

ascospores biseriate. **Ascospores** ellipsoidal to fusiform, 26-*28.6*-32 × 6-*6.7*-8 µm (n= 30), straight or with one side flat and one side curved, 1-septate, equally subdivided in two cells, not constricted at the septum, hyaline, smooth to slightly striate.

NOTES - The specimens were morphologically revised in 1970, as reported in the papery label associated with the samples. Nowadays this species is considered a member of the Bionectriaceae genus *Hydropisphaera* as *H. dolichospora*. *Nectria dolichospora* is the basionym of *H. dolichospora* (Rossman et al., 1999). A detailed description of this specimen is reported in Samuels et al. (1990).

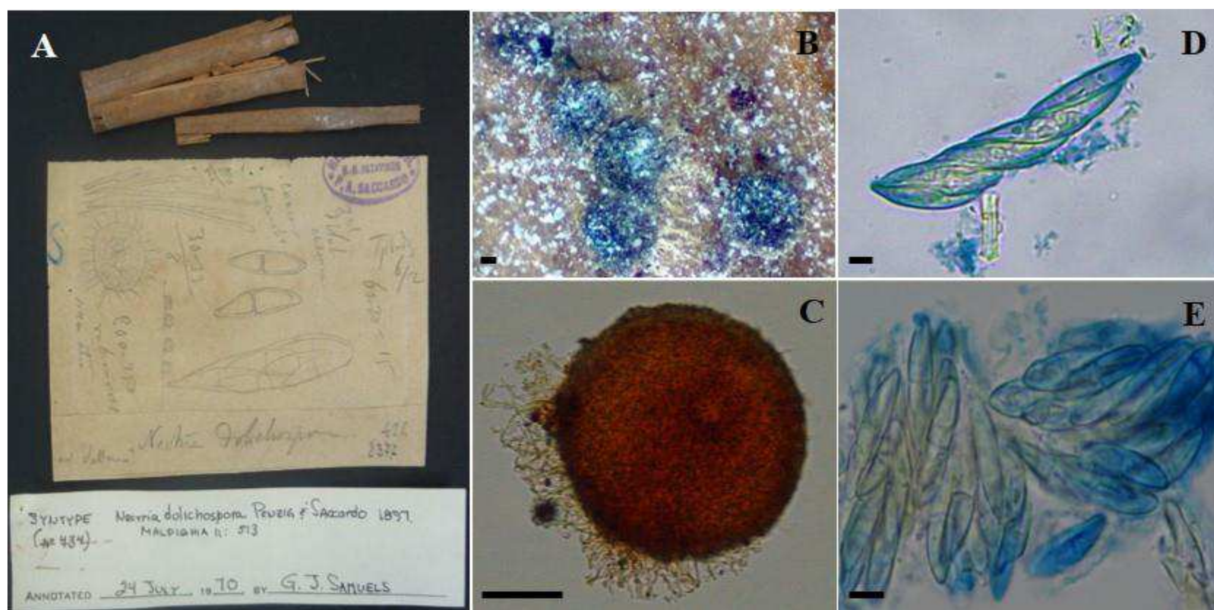


Figure 7. **A** *Nectria dolichospora*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecium; **D** Ascus with ascospores; **E** Ascospores. Scale bars: **B**= 50 µm; **C**= 100 µm; **D**= 5 µm; **E**= 10 µm.

Nectria flageoletiana Saccardo, Atti e Memorie dell'Accademia Galileiana di Scienze Lettere ed Arti in Padova 33: 161. 1917. **Figure 8**

Specimen examined:

- Type specimen of *Nectria flageoletiana* from France (Rigny), on bark of *Prunus laurocerasus*, 1916. The species results associated on the same substrate with *Tubercularia granulata* Pers. (family Nectriaceae).

Perithecia solitary or in small groups, erumpent through bark, globose and slightly sunken when dry, pale yellow, 190-255 µm diameter (n= 10); not changing colour in 3% KOH and 100% lactic acid; ostiolar openings slightly papillate. **Asci** narrowly clavate, 40-*52.7*-60 × 5-

6.2-7 µm (n= 10), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid to oblong-ellipsoidal, 7.2-*9.2*-11 × 3-*3.2*-4 µm (n= 30), equally subdivided in two cells, aseptate or 1-septate, slightly constricted at the septum, hyaline, finely roughened.

NOTES - This specimen has never been morphologically revised.

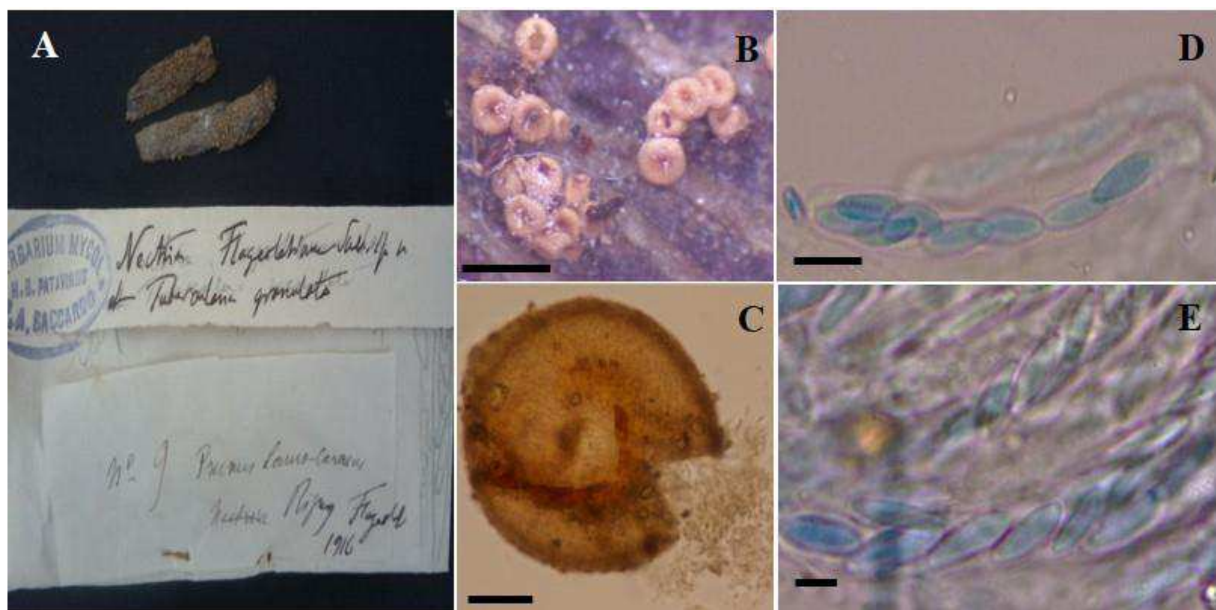


Figure 8. A *Nectria flageoletiana*, specimen and original drawings; **B** Perithecium on natural substrate; **C** Ascus with asci; **D** Asco with ascospores; **E** Ascospores. Scale bars: **B**= 500 µm; **C**= 100 µm; **D-E**= 10 µm.

Nectria granuligera Starbäck, Hedwigia 31: 308. 1892. **Figure 9**

Specimens examined:

- Cotype specimen of *Nectria granuligera* from Sweden (Botanical Garden Uppsala), on orchid bark, 1891.
- Cotype specimen of *Nectria granuligera*.

Perithecia crowd in groups, erumpent from bark, globose to subglobose, not papillate, yellowish orange, warted surface, 230-300 µm diameter (n= 8); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate 43-*49.*4-57 × 6-*6.*4-7 µm (n= 7), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid, 11.2-*2.4*-14.4 × 3.2-*3.9*-4.5 µm (n= 30), equally subdivided in two cells, 1-septate, slightly constricted at the septum, warted, hyaline.

NOTES - The papery labels associated with the samples is reported the annotation *Nectria subquaternata*. *N. subquaternata* is now a member of the Bionectriaceae genus *Bionectria* with the name *B. subquaternata.*

Figure 9. A *Nectria granuligera***; B** Perithecia on natural substrate**; C** Ascospores**; D-E** Ascus with ascospores. Scale bars: **B=** 500 µm**; C=** 10 µm**; D-E=** 5 µm.

Nectria illudens Berkeley, in Hooker, Botany of the Antarctic Voyage II. Flora of New Zealand 7: 203. 1855. **Figure 10**

≡*Neocosmospora illudens* (Berkeley) L. Lombard & Crous, Studies in Mycology 80: 227 (2015).

Specimen examined:

- Cotype specimen of *Nectria illudens* from New Zealand, on bark.

Perithecia gregarious, superficial, red-orange, warted, globose, papillate with a darker papilla, 224-349 µm diameter; dark-red in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+). **Asci** clavate 125-138 × 16.8-18.7 µm (n= 2), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoidal to fusiform, 19-*22.5*-30 × 6.3-*7.8*-12.5 µm (n= 30), straight to curved, 1-septate, equally subdivided in two cells, not constricted at the septum, finely striate.

NOTES - This species now belongs to the Nectriaceae genus *Neocosmospora* E.F. Sm. as *N. illudens*. *Nectria illudens* is the basionym of *Neocosmospora illudens.* The anamorph of *N. illudens* is *Fusarium illudens* C. Booth (Nectriaceae) (Lombard et al., 2015).

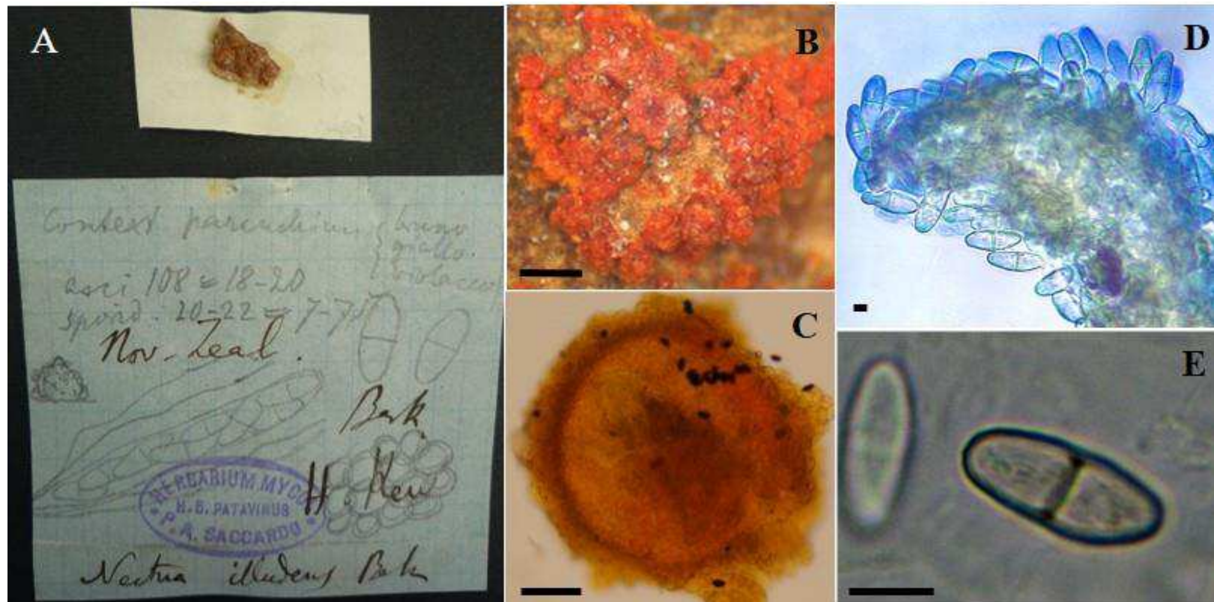


Figure 10. **A** *Nectria illudens*, *specimen and original drawings*; **B** Perithecia on natural substrate; **C** Perithecium; **D-E** Ascospores. Scale bars: **B**= 500 µm; **C**= 100 µm; **D**= 5 µm; **E**= 10 µm.

Nectria leucotricha Penzig & Saccardo; Malpighia 11: 512. 1897. **Figure 11**

≡*Hydropisphaera leucotricha* (Penz. & Sacc.) Rossman & Samuels, Studies in Mycology 42: 31 (1999).

Specimen examined:

- Lectotype specimen of *Nectria leucotricha* (number 150) from Indonesia (Java, Tjibodas), on decaying leaf of *Elettaria* sp., 6 February 1897 (Samuels et al., 1990).

Perithecia characterized by the presence of trichomes on the surface, solitary or gregarious, brown, superficial, globose with hyphae around the perithecium base, not papillate, 280-385 µm diameter (n= 10); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate**,** 48-*51.2*-53 × 7-*8.1*-8.8 µm (n= 5), 8-spored, ascospores biseriate. **Ascospores** ellipsoid, 14-*15.9*-17.3 × 4-*4.6*-5.7 µm (n= 30),1-septate, equally subdivided in two cells, not constricted at the septum, hyaline, striate.

NOTES - The specimen was morphologically revised in 1983, as reported in the papery label associated with the sample. This species is now considered a member of the Bionectriaceae

genus *Hydropisphaera* with the name *H. leucotricha*. *Nectria leucotricha* is the basionym of *H. leucotricha* (Rossman et al., 1999). A detailed description of this species is reported in Samuels et al. (1990).

Figure 11. **A** *Nectria leucotricha*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecium; **D** Ascus with ascospores; **E** Ascospores. Scale bars: **B**= 500 µm; **C**= 100 µm; **D**-**E**= 10 µm.

Nectria mantuana Saccardo, Michelia 1: 52. 1877. **Figure 12**

≡*Lasionectria mantuana* **(**Sacc.) Cooke, Grevillea 12 (64): 112 (1884).

Specimen examined:

- Holotype specimen of *Nectria mantuana* from Italy (Mantua, Migliaretto), on decorticated poplar wood, February 1873 (Rossman et al., 1999).

Perithecia solitary, superficial, brown, globose-depressed, not papillate, 164-280 µm diameter (n= 10); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate, 40-*46.6*-51 × 6-*6.7*-7 µm (n= 8), 8-spored, ascospores uniseriate. **Ascospores** ellipsoidal, 7-*9.3*-11 × 3-*3.4*-4 µm (n= 30), 1-septate, equally subdivided in two cells, not constricted at the septum, hyaline, smooth to slightly striate.

NOTES - The specimen was morphologically revised in 1993, as reported in the papery label associated with the sample. This species is now considered to be a member of the Bionectriaceae genus *Lasionectria* as *L. mantuana*. *Nectria mantuana* is the basionym of *L. mantuana* and the type species of the genus *Lasionectria* (Rossman et al., 1999).

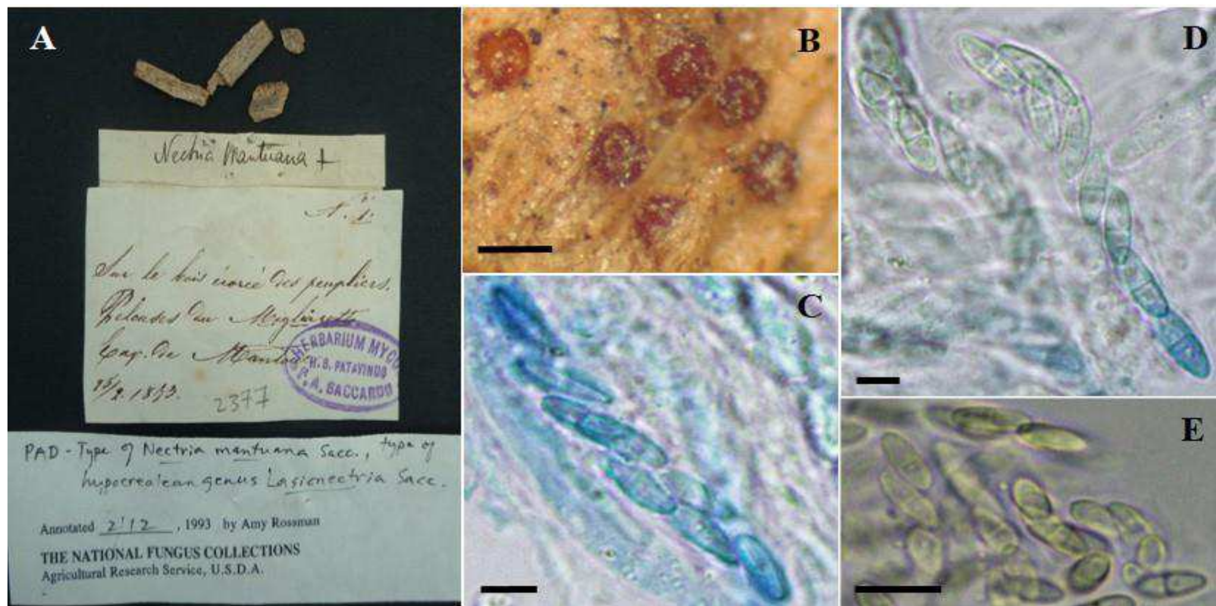


Figure 12. A *Nectria mantuana*; **B** Perithecia on natural substrate; **C-D** Asci with ascospores; **E** Ascospores. Scale bars: **B**= 250 µm; **C-D**= 10 µm; **E**= 10 µm.

Nectria pezicula Spegazzini, Michelia 1: 232. 1878. **Figure 13**

Specimen examined:

- Type specimen of *Nectria pezicula* from Italy (Conegliano, Veneto), on paper, September 1877.

Perithecia aggregated in small groups, superficial on the substratum with hyphae surrounding the ascomatal base, globose, not papillate, orange, 212-230 µm diameter (n= 3); not changing colour in 3% KOH and 100% lactic acid. **Asci** narrowly clavate, 55-*60.8*-69 × 7-*9*-12 µm (n= 10), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid to fusiform, 16-*17.9*-20 × 3-*3.8*-5 µm (n= 20), 1-septate, equally subdivided in two biguttulate cells, not constricted at the septum, hyaline, slightly striate.

NOTES - This specimen has never been morphologically revised. However, *Nectria pezicula* is now considered a synonym of *Hydropisphaera peziza* (Tode) Dumort. (family Bionectriaceae) (Rossman et al., 1999).

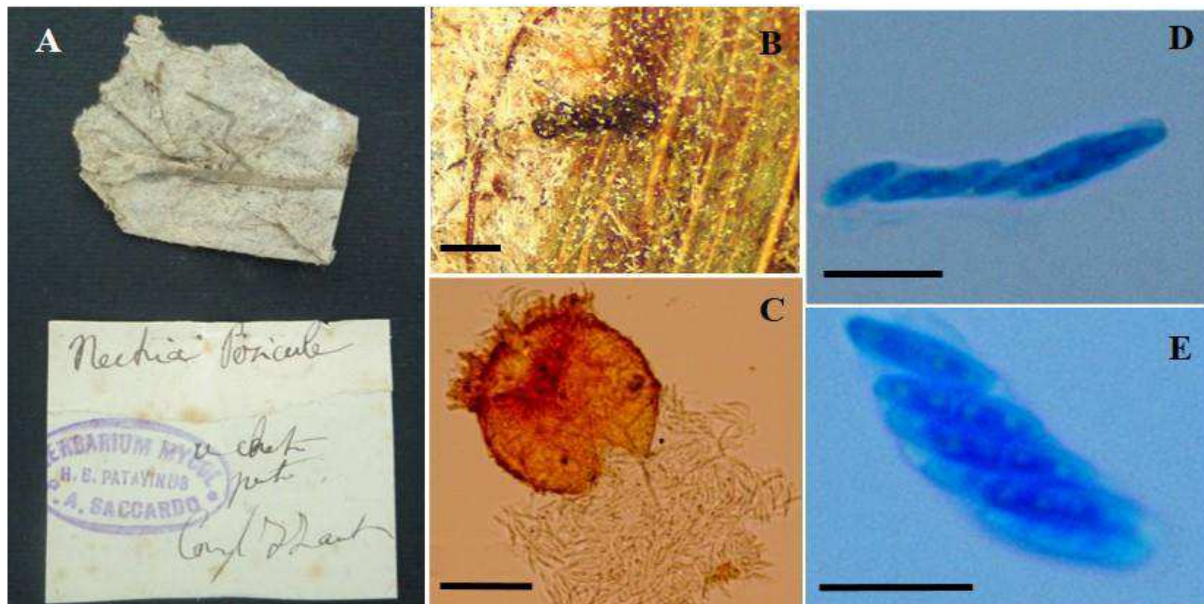


Figure 13. A *Nectria pezicula*; **B** Perithecium on natural substrate; **C** Perithecium with asci**; D** Ascus with ascospores; **E** Ascospores. Scale bars: **B**= 500 µm; **C**= 100 µm; **D-E**= 10 µm.

Nectria peziza* subsp. *reyesiana Saccardo, Annales Mycologici 12: 305. 1914. **Figure 14**

Specimen examined:

- Type specimen of *Nectria peziza* subsp. *reyesiana* from Philippine (Luzon, Los Banos), on dead rotten bark, August 1913.

Perithecia gregarious, partially immersed in a white stroma or superficial, globose, not papillate, red-orange, 234-342 µm diameter (n= 8); not changing colour in 3% KOH but yellow in 100% lactic acid (LA+). **Asci** narrowly clavate, 54-*59.6*-64 × 7-*8.4*-10 µm (n= 5), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid, 12-*13.1*-16 × 4-*4.9*-6 µm (n= 30), 1-septate, equally subdivided in two cells, not constricted at the septum, hyaline, verrucose.

NOTES - This specimen has never been morphologically revised.

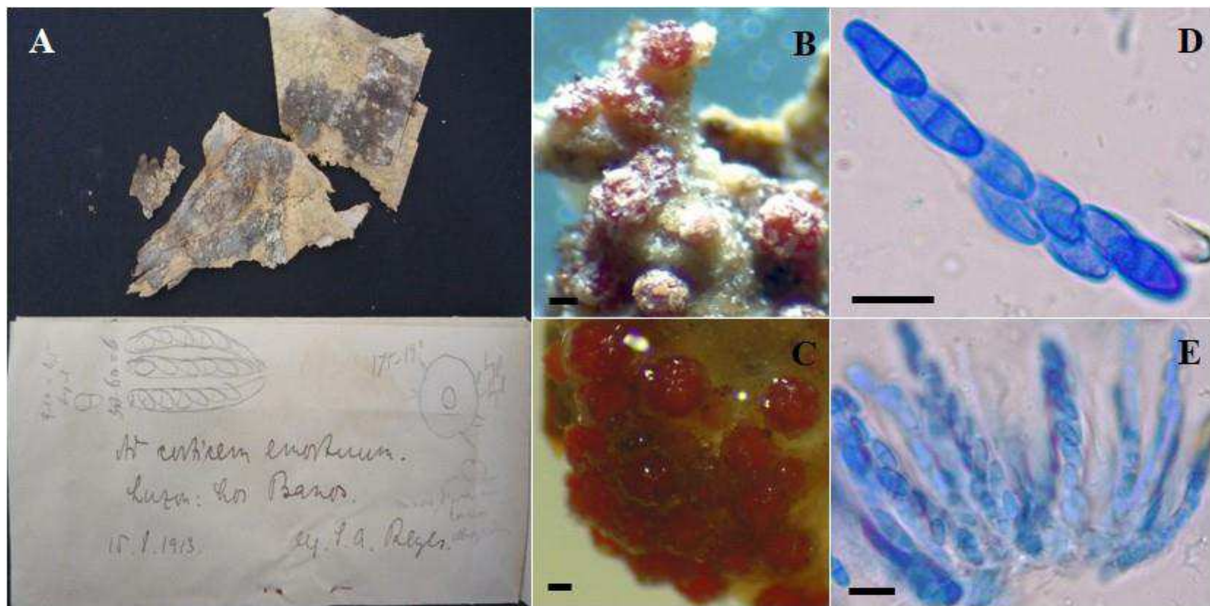


Figure 14. A *Nectria peziza* subsp. *Reyesiana*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecia; **D-E** Asci with ascospores. Scale bars: **B-C**= 100 µm; **D-E**= 10 µm.

Nectria phyllostachydis Hara, Botanical Magazine Tokyo 27: 247. 1913. **Figure 15**

Specimen examined:

- Type specimen of *Nectria phyllostachydis* from Japan (Mino, Kawanye), on *Phyllostachydis bambusoides*, 1912.

Perithecia gregarious, not immersed in a stroma, globose to subglobose-depressed, not papillate, superficial on bark, pale yellow, 175-300 µm diameter (n= 5); not changing colour in 3% KOH and 100% lactic acid. **Asci** not found. **Ascospores** ellipsoid to fusiform, 6.9-*10*-11.2 × 2.5-*3.4*-4 µm (n= 30), 1-septate, not equally subdivided in two cells, constricted at the septum, hyaline, warted.

NOTES - This specimen has never been morphologically revised.

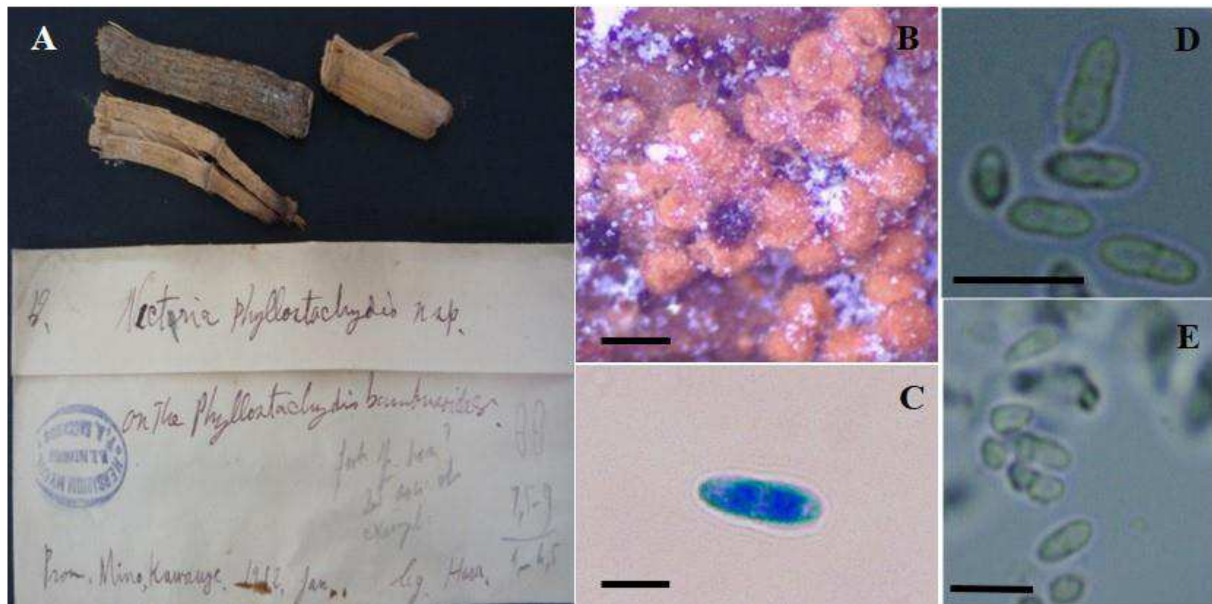


Figure 15. **A** *Nectria phyllostachidis*, specimen and original drawings; **B** Perithecia on natural substrate; **C**-**D**-**E** Ascospores. Scale bars: **B**= 250 µm; **C**= 5 µm**;** **D**-**E**= 10 µm.

Nectria raripila Penzig & Saccardo, Malpighia 15: 228. 1901. **Figure 16**

≡*Sarcopodium raripilum* (Penz. & Sacc.) L. Lombard & Crous, Studies in Mycology 80: 221 (2015).

Specimen examined:

- Type specimen of *Nectria raripila* from Indonesia (Java, Tjibodas), on *Elettaria* sp., 1898.

Perithecia scattered, solitary or in small groups, superficial on the substratum, globose to pyriform, cupulate, yellow with hyphal hairs around the perithecium, 132-180 µm diameter (n= 5); orange in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+). **Asci** clavate, 52-*62.8*-70 × 9-*12.6*-17 µm (n= 10), 8-spored, ascospores biseriate. **Ascospores** fusiform, 21-*24.7*-27 × 4-*5*-6 µm (n= 30) slightly curved, 1-septate, equally subdivided in two cells, constricted at the septum, hyaline, striate with parallel striae.

NOTES - The specimen was morphologically revised in 1983, as reported in the papery label associated with the sample. *Nectria raripila* is now considered a member of the Nectriaceae genus *Sarcopodium* Ehrenb. with the name *S. raripilum*. *Nectria raripila* is the basionym of *S. raripilum* (Lombard et al., 2015). A detailed description of this specimen is reported in Samuels et al. (1990).

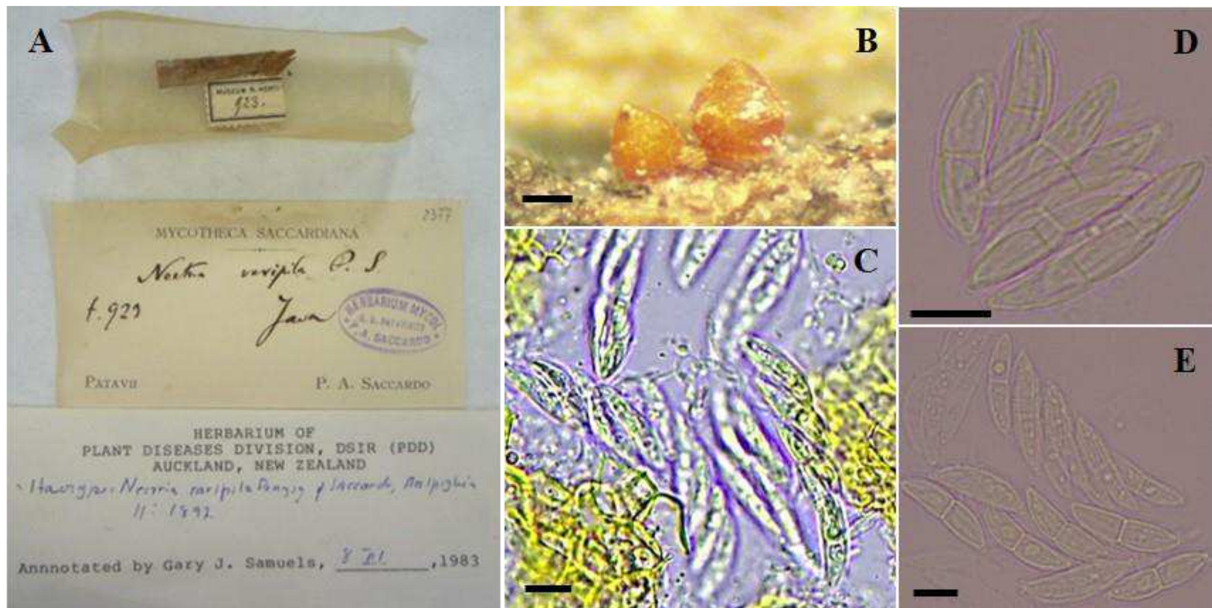


Figure 16. A *Nectria raripila*; **B** Perithecia on natural substrate; **C** Asci with ascospores; **D-E** Ascospores. Scale bars: **B**= 100 µm; **C-D-E**= 10 µm.

Nectria squamuligera Saccardo, Hedwigia 17: 156. 1878. **Figure 17**

Specimens examined:

- Type specimen of *Nectria squamuligera*, on branch bark of *Salix babylonica*.
- Type specimen of *Nectria squamuligera* from Padua (Botanical Garden, Italy), on branch bark of *Glycinis sinensis*, December 1898.
- Type specimen of *Nectria squamuligera* on branch bark of *Glycinis* sp., November 1891.

Perithecia solitary to gregarious, superficial, globose-depressed, not papillate, yellow, 230-320 µm diameter (n= 7); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate, 48-*51*-59 × 6-*7.2*-9 µm (n= 5)**,** 8-spored, ascospores biseriate above and uniseriate below**. Ascospores** ellipsoid to fusiform, 11.2-*13.5*-15.3 × 3.2-*4.2*-5.6 µm (n= 30), 1-septate, equally subdivided in two cells, not constricted or slightly constricted at the septum, warted, hyaline.

NOTES - *Nectria squamuligera* was placed in synonymy with *Nectria ochroleuca* (Schwein.) Berk. by Samuels (1976). *N. ochroleuca* now is a member of the Bionectriaceae genus *Bionectria* as *B. ochroleuca* (Schwein.) Schroers & Samuels (Rossman et al., 1999).

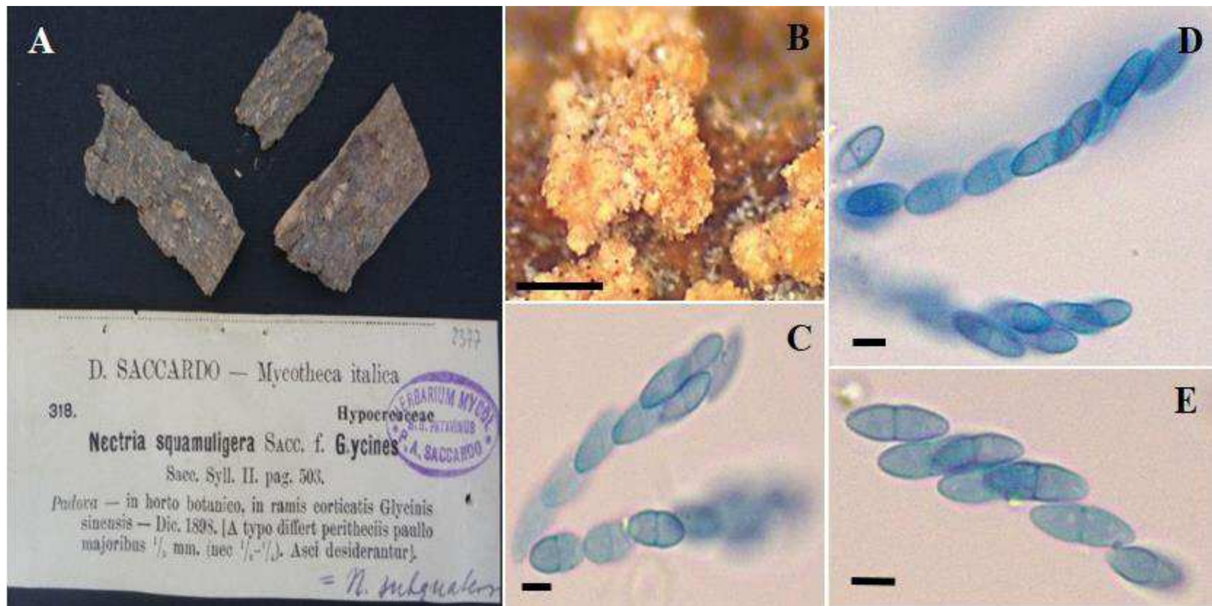


Figure 17. **A** *Nectria squamuligera*; **B** Perithecia on natural substrate; **C**-**D** Asci with ascospores; **E** Ascospores. Scale bars: **B**= 250 µm; **C**-**D**-**E**= 5 µm.

Nectria tjibodensis Penzig & Saccardo, Malpighia 11: 512. 1897. **Figure 18**

Specimen examined:

- Lectotype specimen of *Nectria tjibodensis* from Indonesia (Java, Tjibodas), on bark (Samuels et al., 1990).

Perithecia solitary or gregarious in groups, superficial on bark, globose papillate, cupulate, not collapsing when dry, red, 225-298 µm diameter (n= 10); dark red in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+). **Asci** clavate, 39-*41.4*-43 × 8-9 µm (n= 5), 8-spored, ascospores biseriate. **Ascospores** ellipsoid to fusiform, 9.6-*13.1*-16 × 3.6-*4.2*-5 µm (n= 30), 1-septate, equally subdivided in two cells, constricted at the septum, hyaline, striate with parallel striae.

NOTES - This specimen was morphologically revised in 1970, as reported in the papery label associated with the sample. Presently, *Nectria tjibodensis* is placed in synonymy with *Sarcopodium flavolanatum* (Berk. & Broome) L. Lombard & Crous (family Nectriaceae) (Lombard et al., 2015).

Figure 18. **A** *Nectria tjibodensis*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Perithecia; **D** Asci with ascospores; **E** Ascospores. Scale bars: **B**= 250 µm; **C**= 10 µm; **D**-**E**= 10 µm.

Nectria tjibodensis* var. *crebrior Saccardo, Sylloge Fungorum 14: 636. 1899. **Figure 19**

Specimen examined:

- Holotype specimen of *Nectria tjibodensis* var. *crebrior* from Indonesia (Java, Tjibodas), on dead bark, 6 March 1897 (Samuels et al., 1990).

Perithecia solitary or gregarious in groups, partially immersed in an erumpent stroma, globose to subglobose with a papilla in the middle of the perithecial apex, not collapsing when dry, red, 260-310 µm diameter (n= 10); dark red in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+). **Asci** clavate, 35-*41.8*-51 × 5-*6*-7 µm (n= 6), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid to fusiform, 10-*11.4*-13 × 3-*3.6*-4 µm (n= 30), 1-septate, equally subdivided in two cells, not or slightly constricted at the septum, hyaline, striate with parallel striae.

NOTES - This species was placed in synonymy with *Lanatonectria flocculenta* (Henn. & E. Nyman) Samuels & Rossman (family Nectriaceae) and its anamorph *Actinostilbe macalpinei* (Agnihothr. & G. C. S. Barua) Seifert & Samuels (Rossman et al., 1999). Later, Rossman et al. (2013) proposed the suppression of the genus name *Lanatonectria* Samuels & Rossman in favour of *Actinostilbe* Petch based on priority. Sutton (1981) had already synonymized *Actinostilbe* under the asexual morph genus *Sarcopodium*. Rossman et al. (2013) synonymized *L. flocculenta* (=*A. macalpinei*) under *A. flocculenta*. *A. flocculenta* should be

regarded as a synonym of *Sarcopodium macalpinei* (Agnihothr. & G. C. S. Barua) B. Sutton (Lombard et al., 2015). Consequently, *Nectria tjibodensis* var. *crebrior* should be a member of the Nectriaceae genus *Sarcopodium*, in particular a synonym of *S. macalpinei*. However, this synonymy is not reported.

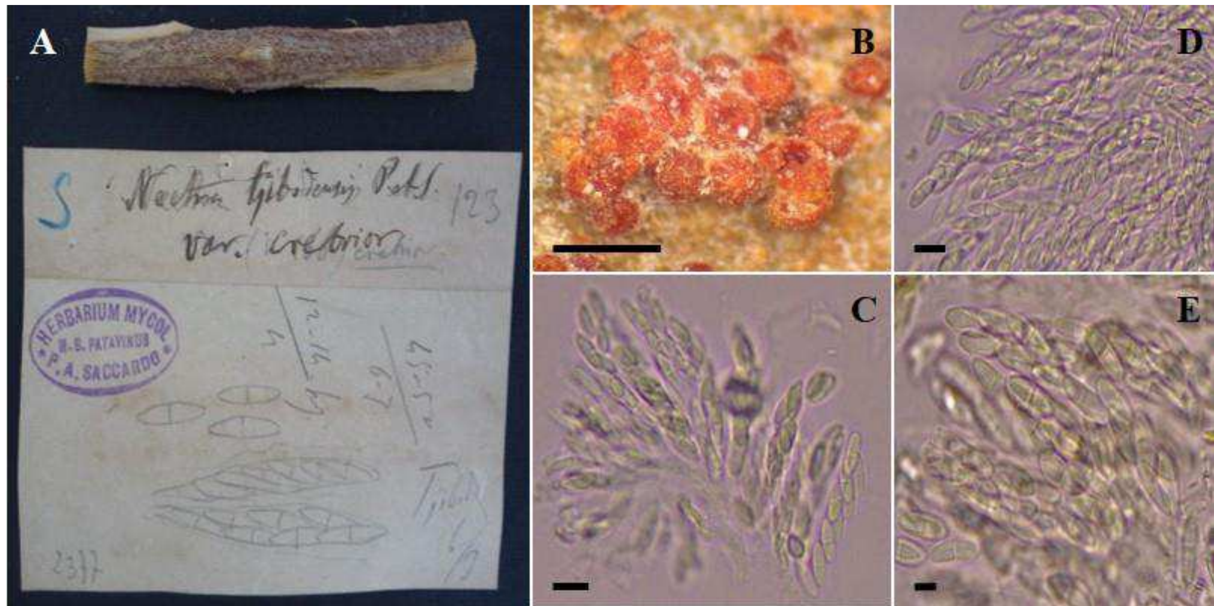


Figure 19. A *Nectria tjibodensis* var. *crebrior*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Asci with ascospores; **D-E** Ascospores. Scale bars: **B**= 500 µm; **C-D**= 10 µm; **E**= 5 µm.

Nectriella bacillispora Traverso & Spessa, Boletim da Sociedade Broteriana. 25: 172. 1910.

Figure 20

=*Allantonectria miltina* (Mont.) Weese in Höhnel & Weese, ann. Mycol. 8: 464. 1910.

Specimen examined:

- Holotype specimen of *Nectriella bacillispora* from Portugal (Botanical Garden of Coimbra), on *Fourcoya gigantea*, November 1906 (Rossman et al., 1993).

Perithecia aggregated in groups, superficial on well-developed stromata, globose, scarlet with apical region slightly darker, 178-234 µm diameter (n= 3); darker in 3% KOH (KOH+) and yellow in 100% lactic acid (LA+). **Asci** cylindrical 8-spored, 24-26 × 3 µm (n= 2), ascospores uniseriate. **Ascospores** allantoid to cylindrical, rounded at both ends, straight to slightly curved, 4-*4.5*-6 × 1– *1.4*-2 µm (n = 20), aseptate, hyaline, smooth.

NOTES - This specimen was morphologically revised in 1992 and in 2010, as reported in the papery labels associated with the sample. In both the revisions it is suggested a synonymy

with *Allantonectria miltina* (Durieu & Mont.) Weese (Hypocreales, Nectriaceae). *Nectriella bacillispora* is now considered a synonym of *A. miltina* (Hirooka et al., 2012).

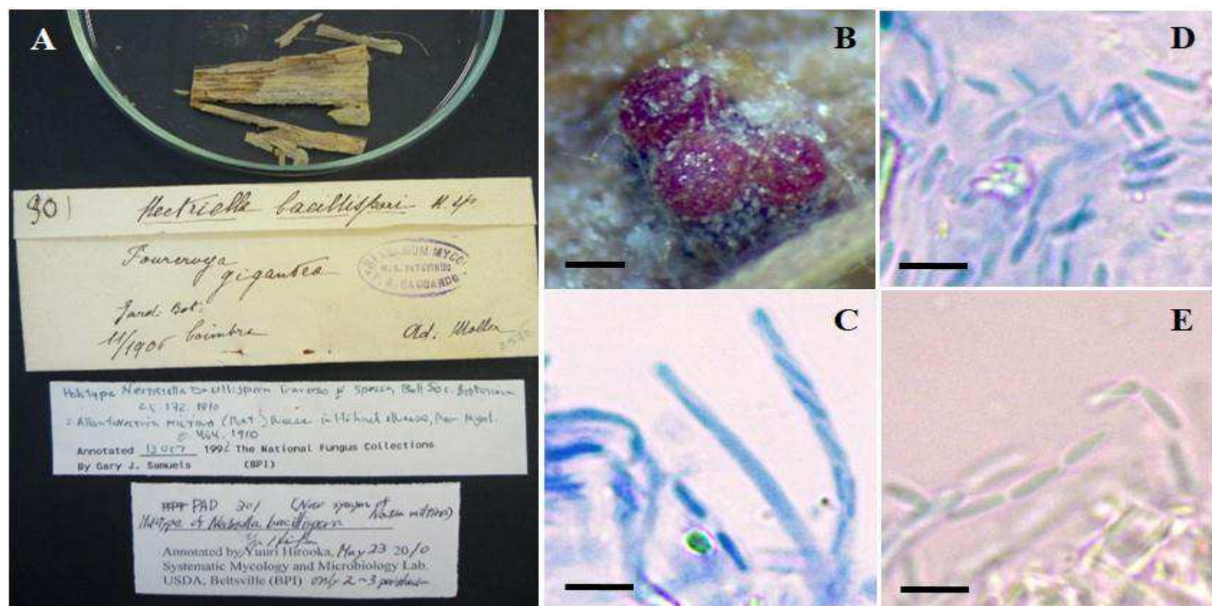


Figure 20. **A** *Nectriella bacillispora*; **B** Perithecia on natural substrate; **C** Asci with ascospores; **D**-**E** Ascospores. Scale bars: **B**= 500 µm; **C**-**D**-**E**= 5 µm.

Nectriella maquilingica Saccardo, Atti della Accademia Scientifica Veneto-Trentino-Istriana 10: 69. 1917. **Figure 21**

Specimen examined:

- Holotype specimen of *Nectriella maquilingica* from Philippines (Maquiling, Luzon), on branch bark of *Leucaena glauca*, December 1915 (Rossman et al., 1993).

Perithecia aggregated in groups, on an erumpent stroma from bark, globose, warted, pale yellow, ostiolar openings slightly papillate and darker, 254-344 µm diameter (n= 6); not changing colour in 3% KOH and 100% lactic acid. **Asci** narrowly clavate to clavate, 38-*45.3*-51 × 6-*7*-9 µm (n= 4), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid to oblong-ellipsoidal, 9-*11.1*-14 × 3-*3*-4 µm (n= 30), equally subdivided in two cells, 1-septate, not or slightly constricted at the septum, hyaline, warted.

NOTES - This specimen was morphologically revised in 1992, as reported in the papery label associated with the sample. In the revision it was suggested a synonymy with *Nectria byssicola* Berk. & Broome and this synonymy is reported by Rossman et al. (1993). *N.*

byssicola is now a member of the Bionectriaceae genus *Bionectria* with the name *B. byssicola* (Berk. & Broome) Schroers & Samuels (Rossman et al., 1999).

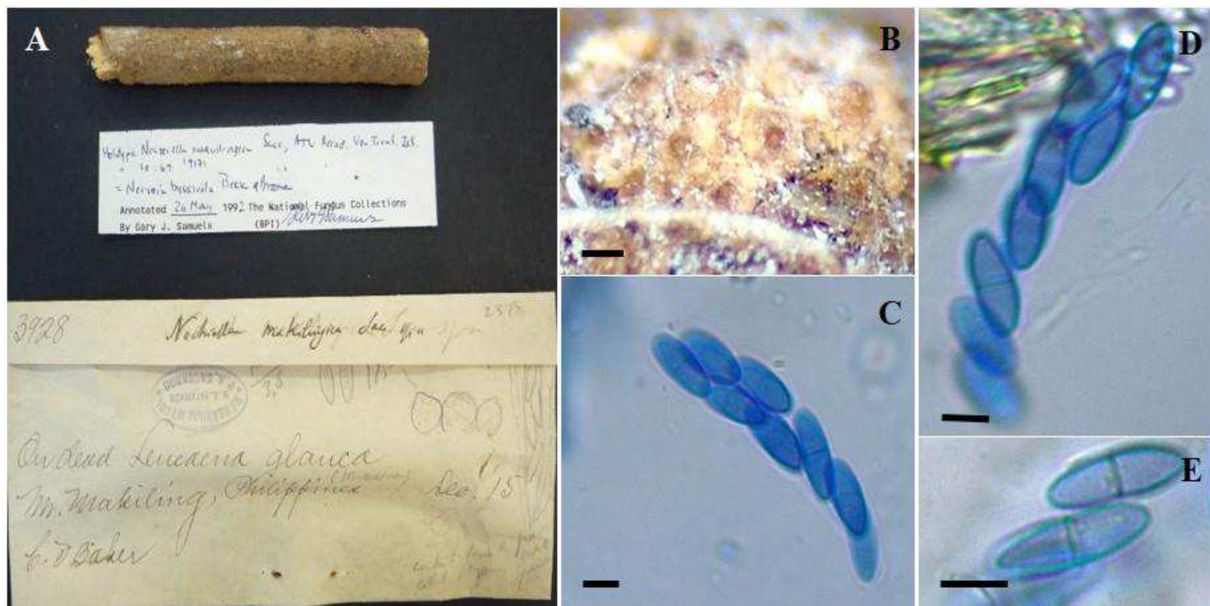


Figure 21. A *Nectriella maquilingica*, specimen and original drawings; **B** Perithecia on natural substrate; **C**-**D** Asci with ascospores; **E** Ascospores. Scale bars: **B**= 500 µm; **C**-**D**-**E**= 5 µm.

Nectriella rufofusca Penzig & Saccardo, Malpighia 11: 507. 1897. **Figure 22**

≡*Hydropisphaera rufofusca* (Penz. & Sacc.) Rossman & Samuels, Mycologia 85: 702 (1993).

Specimen examined:

- Type specimen of *Nectriella rufofusca* (number 436) from Indonesia (Java, Tjibodas), on decaying leaf of *Elettaria* sp., February 1897.

Perithecia solitary to gregarious, superficial, nonstromatic, globose, red brownish, 184-237 µm diameter (n= 5); not changing colour in 3% KOH and 100% lactic acid. **Asci** clavate, 31-*33*-36 × 4-*4.75*-6 µm (n= 4), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** ellipsoid to fusiform, 10-*11.2*-13 × 2-*2.7*-3 µm (n= 30), equally subdivided in two biguttulate cells, 1-septate, not constricted at the septum, hyaline, smooth. **Conidia** oblong-ellipsoidal, 6-*7.65*-8 × 2µm (n= 20), hyaline, smooth, unicellular.

NOTES - The specimen was morphologically revised in 1992, as reported in the papery label associated with the sample. In the revision it was suggested a synonymy with *Nectria brasiliensis* (Henn.) Höhn. *Nectriella rufofusca* is now considered a member of the

Bionectriaceae genus *Hydropisphaera* with the name *H. rufofusca* (Penz. & Sacc.) Rossman & Samuels. *Nectriella rufofusca* is the basionym of *H. rufofusca* (Rossman et al., 1999).

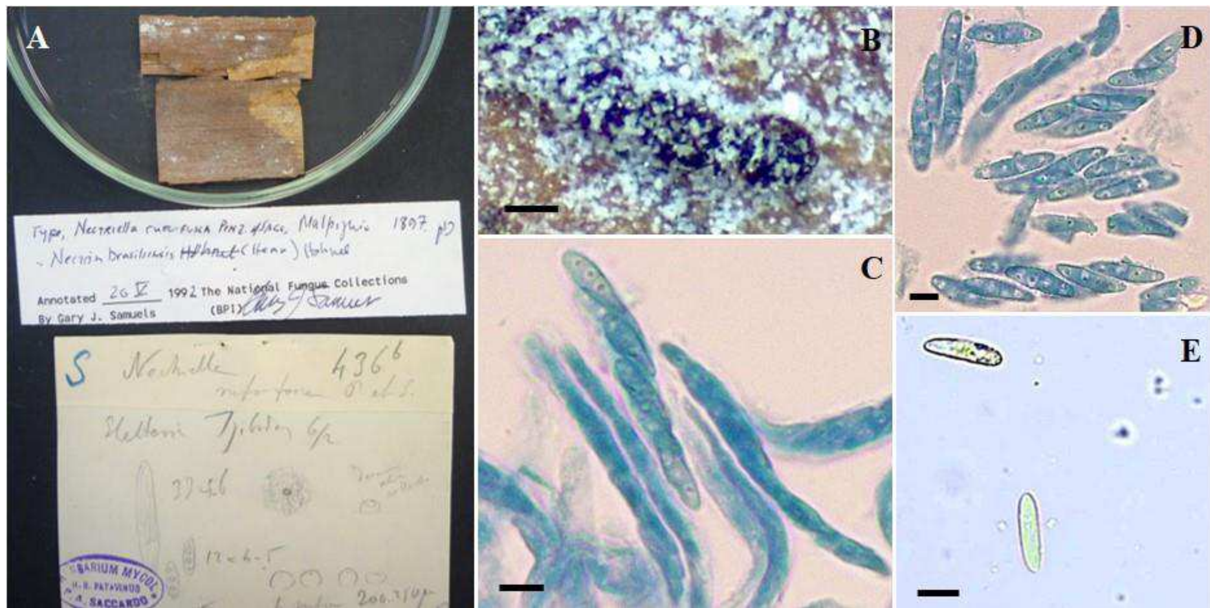


Figure 22. A *Nectriella rufofusca*, specimen and original drawings; **B** Perithecia on natural substrate; **C** Asci with ascospores; **D** Ascospores; **E** Conidia. Scale bars: **B**= 500 µm; **C**-**D**-**E**= 5 µm.

Pleonectria affinis Saccardo, Boletim da Sociedade Broteriana 1: 139. 1922. **Figure 23**

Specimen examined:

- Type specimen of *Pleonectria affinis* from Africa (San Thomè), on bark, 1917.

Perithecia crowed in groups, on an erumpent stroma from bark, globose, warted, red brownish, 335-437 µm diameter (n= 8); dark-red in 3% KOH (KOH+) and yellow 100% lactic acid (LA+). **Asci** clavate, 84-*91.4*-102 × 14-*15.2*-16 µm (n= 5), 8-spored, ascospores biseriate above and uniseriate below. **Ascospores** muriform, narrowly ellipsoidal, 21-*30.6*-40 × 5-*8.6*-11 µm (n= 20), 5 transverse septa and 1 longitudinal septum, slightly constricted at each septum, hyaline, warted.

NOTES - This specimen has never been morphologically revised.

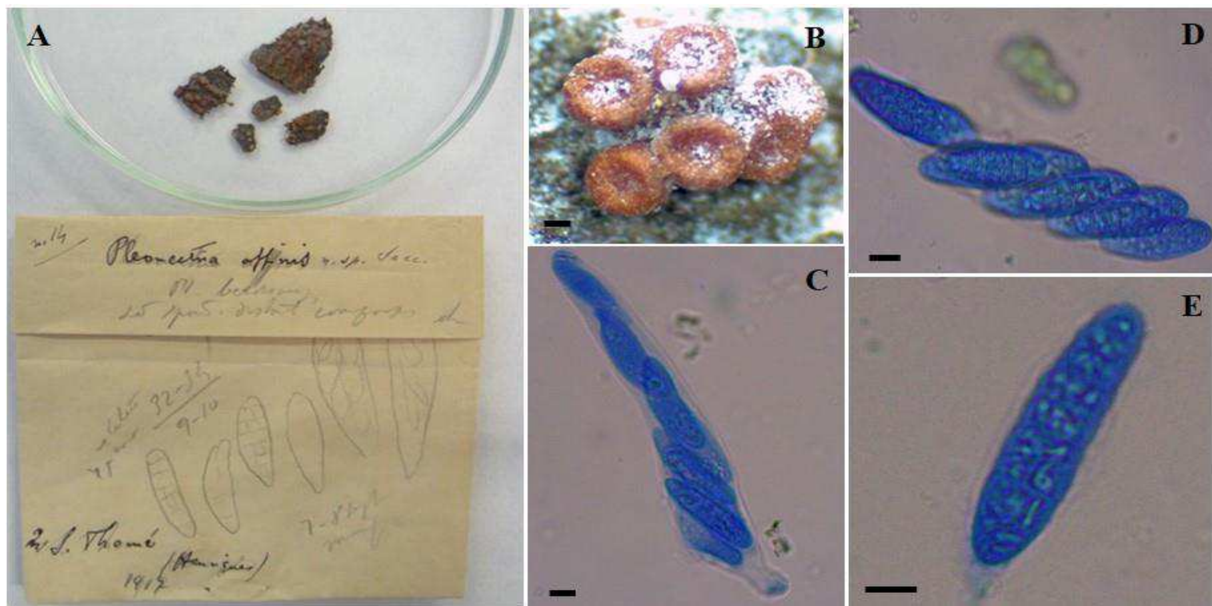


Figure 23. **A** *Pleonectria affinis*, specimen and original drawings; **B** Perithecia on natural substrate; **C**-**D** Asci with ascospores; **E** Ascospore. Scale bars: B= 100 µm; **C**-**D**-**E**= 5 µm.

Pleonectria berolinensis Saccardo, Michelia 1: 123. 1878. **Figure 24**

≡*Thyronectria berolinensis* (Sacc.) Seaver, Mycologia 1 (5): 205 (1909).

Specimen examined:

- Holotype specimen of *Pleonectria berolinensis* (number 352) from Germany (Berlin Botanical Garden), on dead branch of *Ribes aureum* (Hirooka et al., 2012).

Perithecia crowed in groups, on an erumpent stroma from bark, globose with a depressed apical region, smooth, red brownish, apical region slightly darker, 301-533 µm diameter (n= 10); dark-red in 3% KOH (KOH+) and yellow 100% lactic acid (LA+). **Asci** narrowly clavate, 87-*104.5*-115 × 9-*11*-13µm (n= 4), 8-spored, ascospores uniseriate. **Ascospores** muriform, narrowly ellipsoidal, 13-*17.3*-21 × 5-*6.6*-9 µm (n= 30), 5 transverse septa and 1 longitudinal septum, slightly constricted at each septum, hyaline, smooth.

NOTES - This specimen was morphologically revised in 2010, as reported in the papery label associated with the sample. This species should be a member of the Nectriaceae genus *Thyronectria* (*Pleonectria* is considered a synonym of *Thyronectria*) with the name *T. berolinensis* (Lombard et al., 2015).

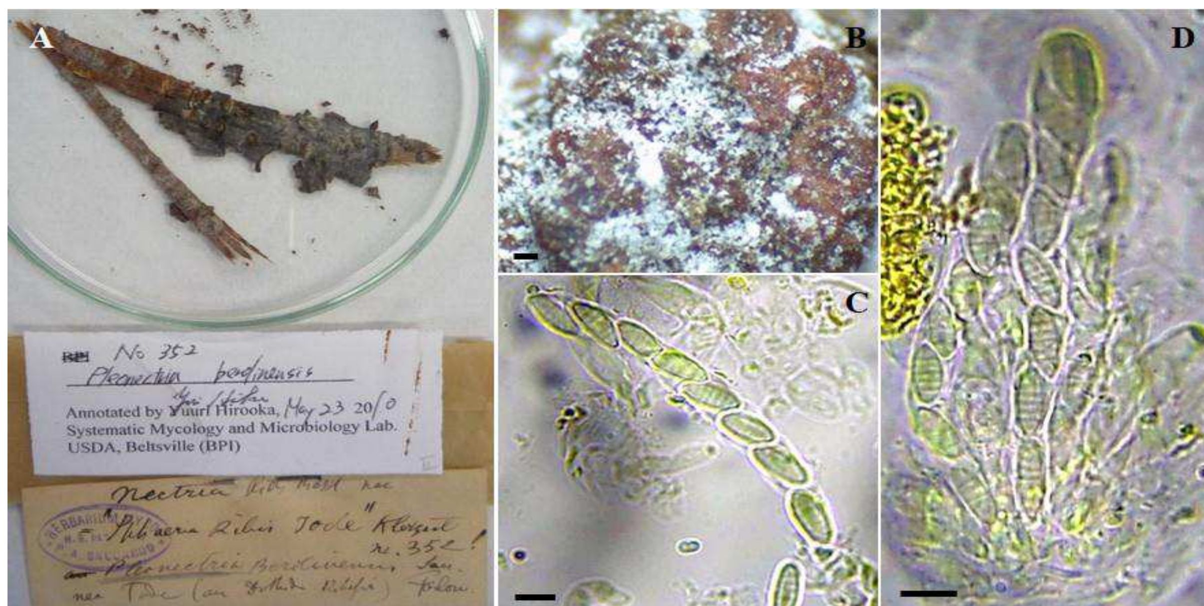


Figure 24. **A** *Pleonectria berolinensis*; **B** Perithecia on natural substrate; **C**-**D** Asci with ascospores. Scale bars: **B**= 100 μm; **C**-**D**= 10 μm.

DNA extraction, amplification and sequencing

A total of 56 *Nectria* specimens and 20 *Nectria*-like specimens were investigated. DNA was successfully extracted from 48 *Nectria* and 16 *Nectria*-like specimens. Both ITS1 and ITS2 were amplified from 39 *Nectria* and six *Nectria*-like specimens; the ITS1 region only was amplified from four *Nectria* and from seven *Nectria*-like specimens, while the ITS2 region only was not amplified from any specimens. For five *Nectria* and three *Nectria*-like specimens the amplifications were completely unsuccessful (**Figures 25-26**). Without considering multiple samples, the ITS1 and/or ITS2 sequences were amplified from 34 *Nectria* and 12 *Nectria*-like type specimens.

Despite the high level of fungal contaminants, represented mainly by *Aspergillus* P. Micheli ex Haller and *Penicillium* Link (Ascomycota, Eurotiomycetes, Eurotiales, Aspergillaceae), *Alternaria* Nees and *Cladosporium* Link (Ascomycota, Dothideomycetes, Pleosporales, Didymellaceae), *Wallemia* Johan-Olsen (Basidiomycota, Wallemiomycetes, Wallemiales, Wallemiaceae) and *Malassezia* Baill. (Basidiomycota, Ustilaginomycotina, Exobasidiomycetes, *incertae sedis*) species, and the presence, in some samples analysed, of non-target sequences belonging to different species of the Nectriaceae and/or Bionectriaceae families, the Illumina sequencing allowed to assign ITS1 and ITS2 sequences of 17 different *Nectria* (22 in total when considering multiple specimens) and two *Nectria*-like (*Nectriella*) types; while for two *Nectria*, one *Hypocrea*, one *Nectriella*, and two *Pleonectria* types only

the ITS1 sequences were recovered (**Figures 25-26**; **Table 3**). The obtained sequences were then investigated by a phylogenetic analysis which permitted to obtain a taxonomic re-evaluation of the types analysed.

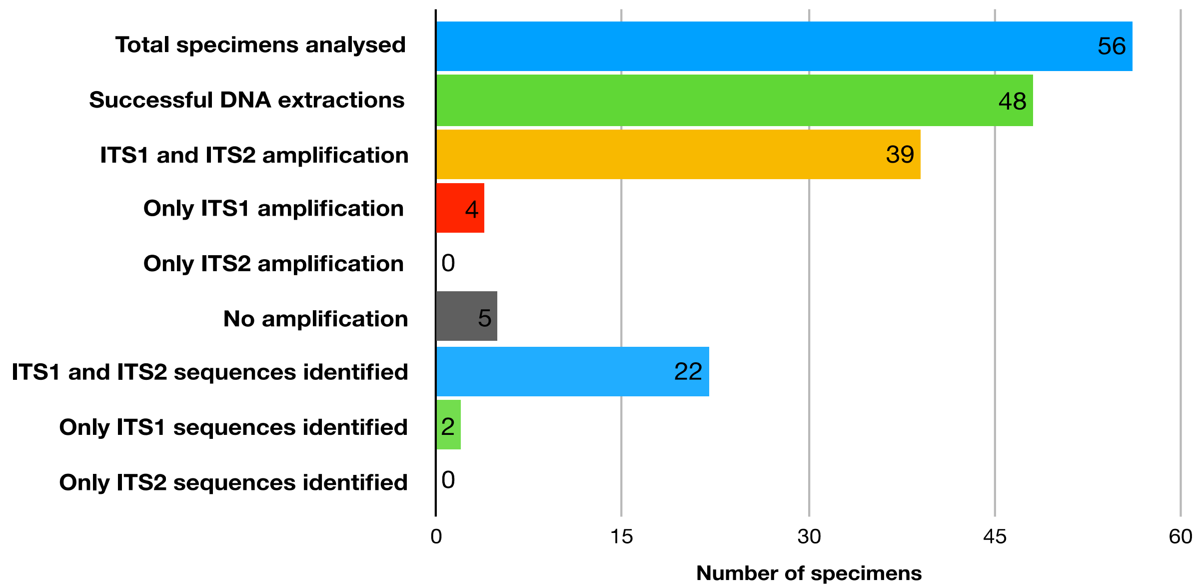


Figure 25. Success of DNA extraction, amplification and sequencing for the 56 *Nectria* specimens analysed in this work.

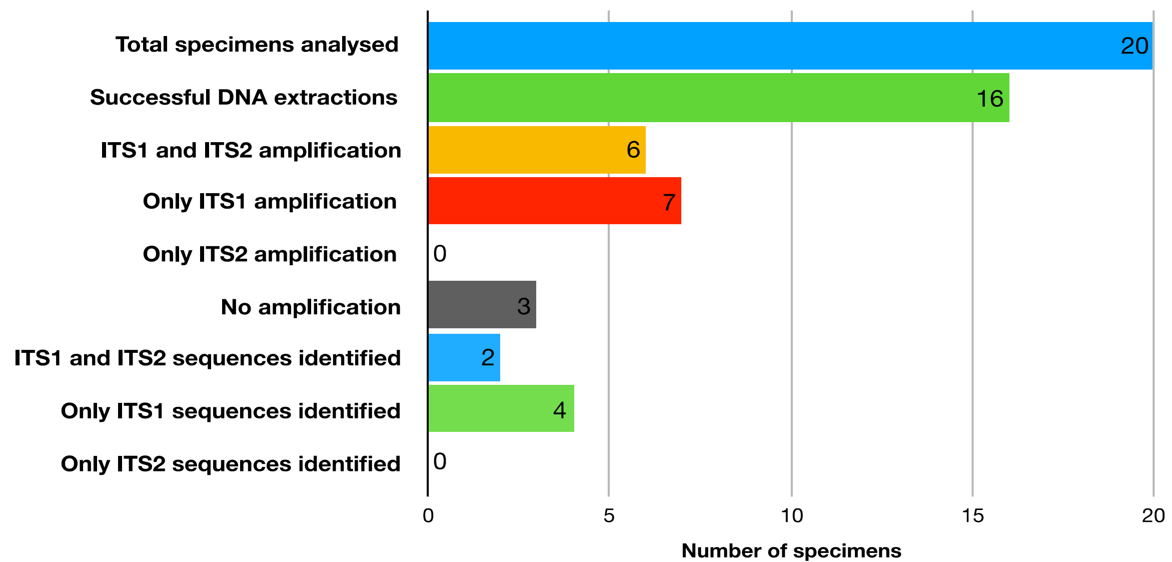


Figure 26. Success of DNA extraction, amplification and sequencing for the 20 *Nectria*-like specimens analysed in this work.

Table 3. List of the type specimens for which ITS1 and/or ITS2 sequences were identified. The number of target sequences out of the total number of sequences per specimen is also reported.

	Sequences of the target fungus	
Herbarium name	**ITS1**	**ITS2**
Nectria albofimbriata 1	4130 out of 72176	50 out of 499
Nectria albofimbriata 2	63598 out of 192715	1215 out of 9239
Nectria ambigua	756 out of 62845	
Nectria ambigua var. *pallens*	5472 out of 134915*	9900 out of 276220*
Nectria congesta	67114 out of 98767	4428 out of 8812
Nectria coronata	62206 out of 134915*	4907 out of 276220*
Nectria cyanostoma	171 out of 52108	1015 out of 178872
Nectria dolichospora 1	198 out of 32882	3543 out of 12063
Nectria dolichospora 2	35105 out of 96248	6297 of 69326
Nectria flageoletiana	53398 out of 67729	8374 out of 11722
Nectria granuligera 1	190680 out of 192079	39566 out of 51573
Nectria granuligera 2	117332 out of 123586	3525 out of 10750
Nectria illudens	40538 out of 102859	85 out of 6772
Nectria leucotricha	3885 out of 145263	682 out of 61769
Nectria mantuana	2744 out of 11907	39339 out of 76857
Nectria pezicula	11763 out of 170117	1369 out of 28211
Nectria peziza subsp. *reyesiana*	22606 out of 67712	38784 out of 60733
Nectria phyllostachydis	19673 out of 74837	252 out of 22383
Nectria raripila	32991 out of 99066	42117 out of 68125
Nectria squamuligera 1	164212 out of 225057	2068 out of 5194
Nectria squamuligera 2	3931 out of 4556	1986 out of 4412
Nectria squamuligera 3	2434 out of 99019	132 out of 34850
Nectria tjibodensis	3533 out of 168342	
Nectria tjibodensis var. *crebrior*	7331 out of 7555	34351 out of 54532
Hypocrea discolor	469 out of 36598	
Nectriella bacillispora	300110 out of 341438	
Nectriella maquilingica	287543 out of 301572	301525 out of 317980
Nectriella rufofusca	145197 out of 233005	158666 out of 308396
Pleonectria affinis	51527 out of 95489	
Pleonectria berolinensis	460 out of 106751	

**Nectria coronata* and *Nectria ambigua* var. *pallens* are closely associated on the same substrate, an observation also reported in Samuels et al. (1990). The sequences identified for the two different types coming from the same ITS1 and ITS2 amplification.

Taxonomic re-evaluation

The taxonomic assignment has been obtained by combining sequence, phylogenetic and morphological analysis.

Nectria peziza subsp. *reyesiana*

N. peziza subsp. *reyesiana* was placed in synonymy with *N. peziza* and, as consequence, with *Hydropisphaera peziza* (see introduction section). However, since its original description this type specimen has never been revised. The target ITS1 and the ITS2 sequences identified after the sequencing data analysis have pointed out a different scenario with respect to the current "name status" of this species. In fact, the BLASTN analysis showed that the ITS1 sequence has a high similarity value with ITS sequences annotated as *Fusicolla* sp. or *Fusicolla violacea* Gräfenhan & Seifert, while ITS2 with sequences annotated as *Fusarium dimerum* Penz., *F. acquaeductum* (Radlk. & Rabenh.) Lagerh. & Rabenh. or *F. merismoides* Corda in the NCBI database. All these *Fusarium* species are members of the Nectriaceae genus *Fusicolla* Bonord. (Gräfenhan et al., 2011; Lombard et al., 2015). Based on the BLASTN results and on the latest published works regarding the phylogeny of nectriaceous fungi (Gräfenhan et al., 2011; Lombard et al., 2015), well-annotated sequences belonging to species of *Fusicolla* and related nectriaceous genera, such as *Cosmospora* Rabenh., *Dialonectria* (Sacc.) Cooke, *Microcera* Desm., *Mariannaea* G. Arnaud ex Samson, *Volutella* Tode, *Coccinonectria* L. Lombard & Crous and *Stylonectria* Höhn., have been downloaded from the NCBI and used for a phylogenetic analysis in order to obtain a better taxonomic identification of *N. peziza* subsp. *reyesiana*. The phylogenetic analysis, reported in **Figure 27**, confirms the taxonomic assignment based on BLASTN results, placing the ITS sequence of the analysed type in a clade which encompasses *Fusicolla* ITS sequences and segregates from the other genera in the phylogram.

The species of the genus *Fusicolla* are characterized by perithecia that do not change colour in KOH and by the formation of a slimy, pale orange sheet of hyphae over the substratum, where perithecia are fully or partially immersed (Lombard et al., 2015). These characters have been observed in the *N. peziza* subsp. *reyesiana* specimen. Indeed, the sexual reproductive structures are partially immersed in a sort of pillow of pale hyphae developed above the substrate.

In conclusion, these results consistently suggest that this species might be reclassified as a new member of the genus *Fusicolla*.

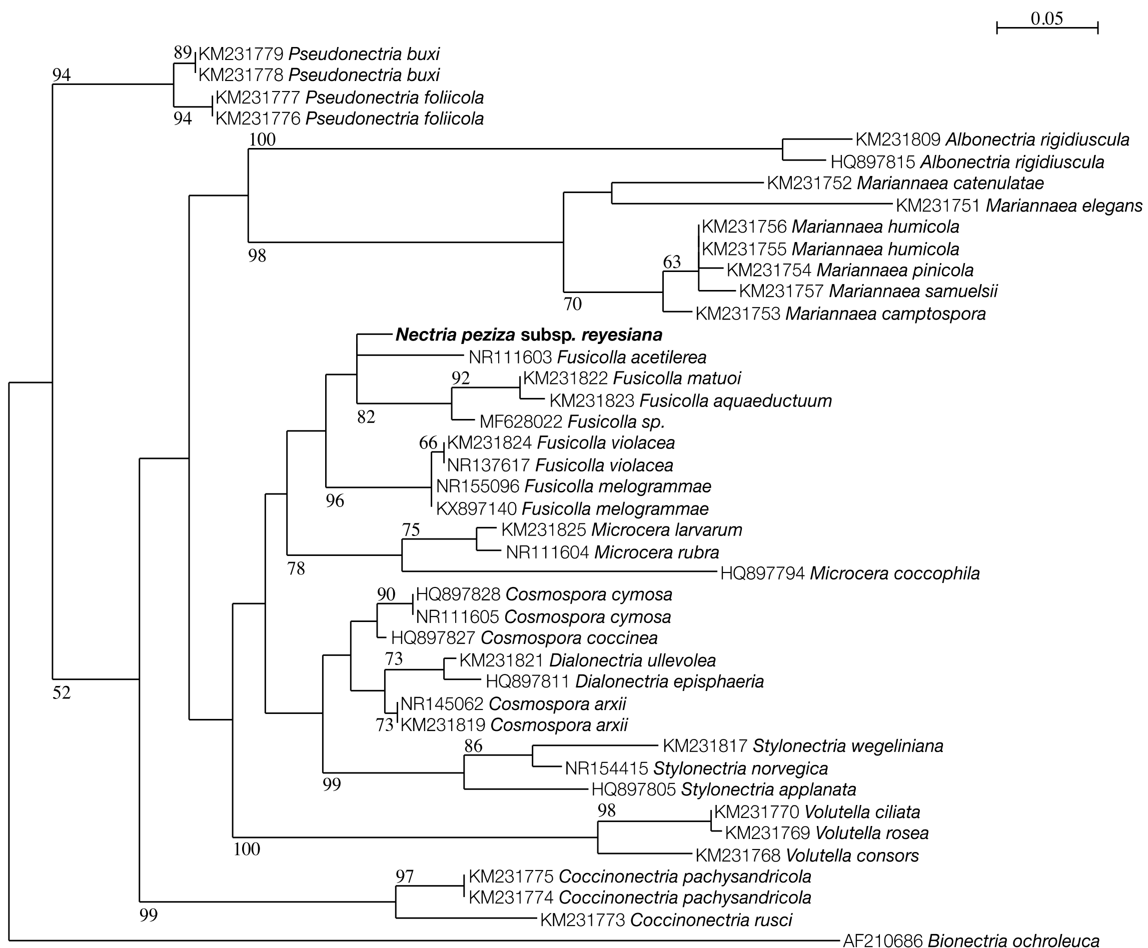


Figure 27. Phylogram generated from maximum likelihood analysis based on combined ITS1 and ITS2 sequences of species belonging to different genera of the family Nectriaceae. Maximum likelihood bootstrap values ≥ 50 are given above the nodes. Accession numbers are reported for the sequences retrieved from public databases. The tree was rooted with *Bionectria ochroleuca* (Ascomycota, Sordariomycetes, Hypocreales, Bionectriaceae). The Saccardo specimen is marked in bold**.**

Sarcopodium group

Nectria raripila, *Nectria tjibodensis* and *Nectria tjibodensis* var. *crebrior* are considered members of the Nectriaceae genus *Sarcopodium* (Lombard et al., 2015). After former morphological revisions, *N. tjibodensis* and *N. tjibodensis* var. *crebrior* were placed in synonymy with already described *Sarcopodium* species, while *N. raripila* was reclassified as a new species of the genus *Sarcopodium*. The molecular analysis, using the ITS1 and/or ITS2 sequences obtained with the Illumina sequencing, has confirmed that these three type specimens belong to the genus *Sarcopodium*. However, when considering the phylogram reported in **Figure 28**, it seems that the reclassifications of *N. tjibodensis* and *N. tjibodensis* var. *crebrior* done in the past should be reconsidered.

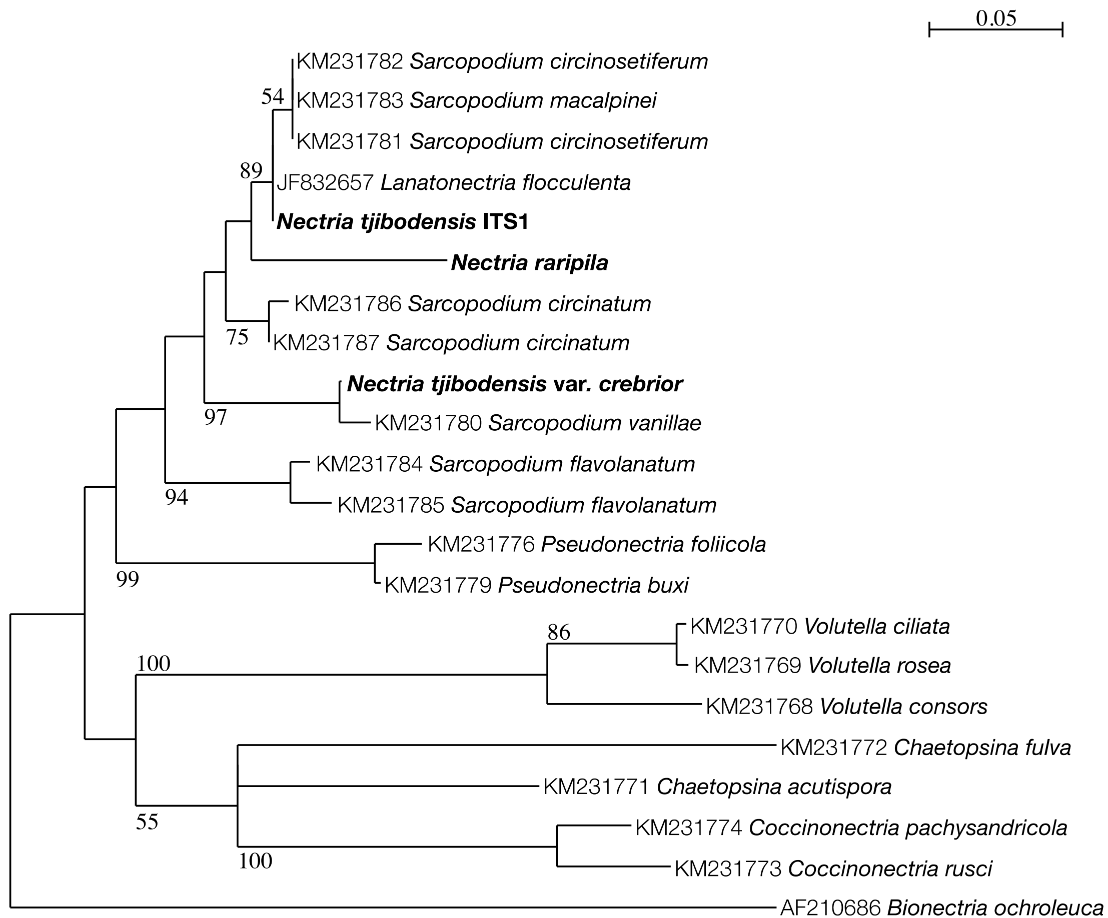


Figure 28. Phylogram generated from maximum likelihood analysis based on combined ITS1 and ITS2 sequences of species belonging to the Nectriaceae genera *Sarcopodium*, *Pseudonectria*, *Volutella*, *Chaetopsina* and *Coccinonectria*. Maximum likelihood bootstrap values ≥ 50 are given above the nodes. Accession numbers are reported for the sequences retrieved from public databases. The tree was rooted with *Bionectria ochroleuca* (Ascomycota, Sordariomycetes, Hypocreales, Bionectriaceae). The Saccardo specimens are marked in bold**.**

N. tjibodensis was placed in synonymy with *Lanatonectria flavolanata* (Berk. &Broome) Samuels & Rossman (Rossman et al., 1999), but recently the sexual genus *Lanatonectria* Samuels and Rossman has been recombined as a synonym of the asexual genus *Sarcopodium* (Lombard et al., 2015). *L. flavolanata* became *S. flavolanatum* and, as consequence, *N. tjibodensis* a synonym of *S. flavolanatum*. However, the phylogenetic analysis has placed the ITS1 sequence of *N. tjibodensis* close to the ITS sequence of *L. flocculenta*, now considered a synonym of *S. macalpinei* (Lombard et al., 2015), as well as to *S. macalpinei* sequence, and far from the ITS sequences of *S. flavolanatum* (**Figure 28**). In addition, the new morphological observations of the type specimen *N. tjibodensis* fit well the detailed morphological description of *L. flocculenta* reported in Rossman et al. (1999). Therefore, considering the morphological and molecular analyses, *N. tjibodensis* might be reclassified as synonym of *S. macalpinei* and not of *S. flavolanatum* as previously supposed.

N. tjibodensis var. *crebrior* was placed in synonymy with *L. flocculenta* (= *S. macalpinei*). The phylogenetic analysis (**Figure 28**) has placed *N. tjibodensis* var. *crebrior* in a highly supported clade (MLB= 97%) with the ITS sequence of *Sarcopodium vanillae* (Petch) B. Sutton and far from the ITS sequences of *L. flocculenta* and *S. macalpinei*. *Sarcopodium vanillae* was morphologically described by Sutton (1891), which reported only a description based on characters linked to an asexual morph; as consequence, a direct comparison between the morphologies of *N. tjibodensis* var. *crebrior* and *S. vanillae* is not possible. However, the high similarity value between the *N. tjibodensis* var. *crebrior* and *S. vanillae* sequences (99%) suggests that *N. tjibodensis* var. *crebrior* could be considered as the teleomorph stage of *S. vanillae.*

Hydropisphaera group

The genus *Hydropisphaera*, based on the type species *H. peziza*, was introduced by Dumortier (1822) and for a long time it was considered a synonym of *Nectria* Fr. Rossman et al. (1999) reintroduced *Hydropisphaera* as a distinct genus of the family Bionectriaceae, recognizing 18 different species. The sexual reproductive structures of *Hydropisphaera* species are yellow to brown, and do not change colour in KOH or LA (as it is characteristic of the Bionectriaceae) and the species of this genus are characterized by and an *Acremonium*-like or *Gliomastix*-like asexual morph (Lechat and Fournier, 2016). Nowadays, 29 species are recognised in this genus (*Index Fungorum;* http://www.indexfungorum.org).

The Saccardo collection contains four *Nectria* and one *Nectria*-like type specimens that nowadays are considered as species of the genus *Hydropisphaera*: *N. dolichospora* (*H. dolichospora*), *N. leucotricha* (*H. leucotricha*), *N. hypoxantha* Penz. & Sacc. (*H. hypoxantha* (Penz. & Sacc.) Rossman & Samuels), *N. pezicula* (synonym of *H. peziza*) and *Nectriella rufofusca* (*H. rufofusca*). Except for *N. hypoxantha*, ITS1 and ITS2 sequences were identified with the Illumina sequencing and used in the phylogenetic analysis reported in **Figure 29**.

The molecular analysis, obtained using *Hydropisphaera* ITS sequences deposited in GenBank, has confirmed that *N. dolichospora* (two specimens), *N. leucotricha* and *Nectriella rufofusca* are distinct species of the genus *Hydropisphaera*, as reported by Rossman et al. (1999), but it has also showed that *N. pezicula* should be taxonomically reclassified (**Figure 29**). Indeed, although it is considered as a synonym of *H. peziza* and the morphological traits observed in *N. pezicula* are similar to those of *H. peziza* (Rossman et al., 1999), the molecular analysis has placed the *N. pezicula* ITS sequence separated from that of *H. peziza*. This result

suggests that *N. pezicula* should be reconsidered as a distinct species of the *Hydropisphaera* genus and not a synonym of *H. peziza*.

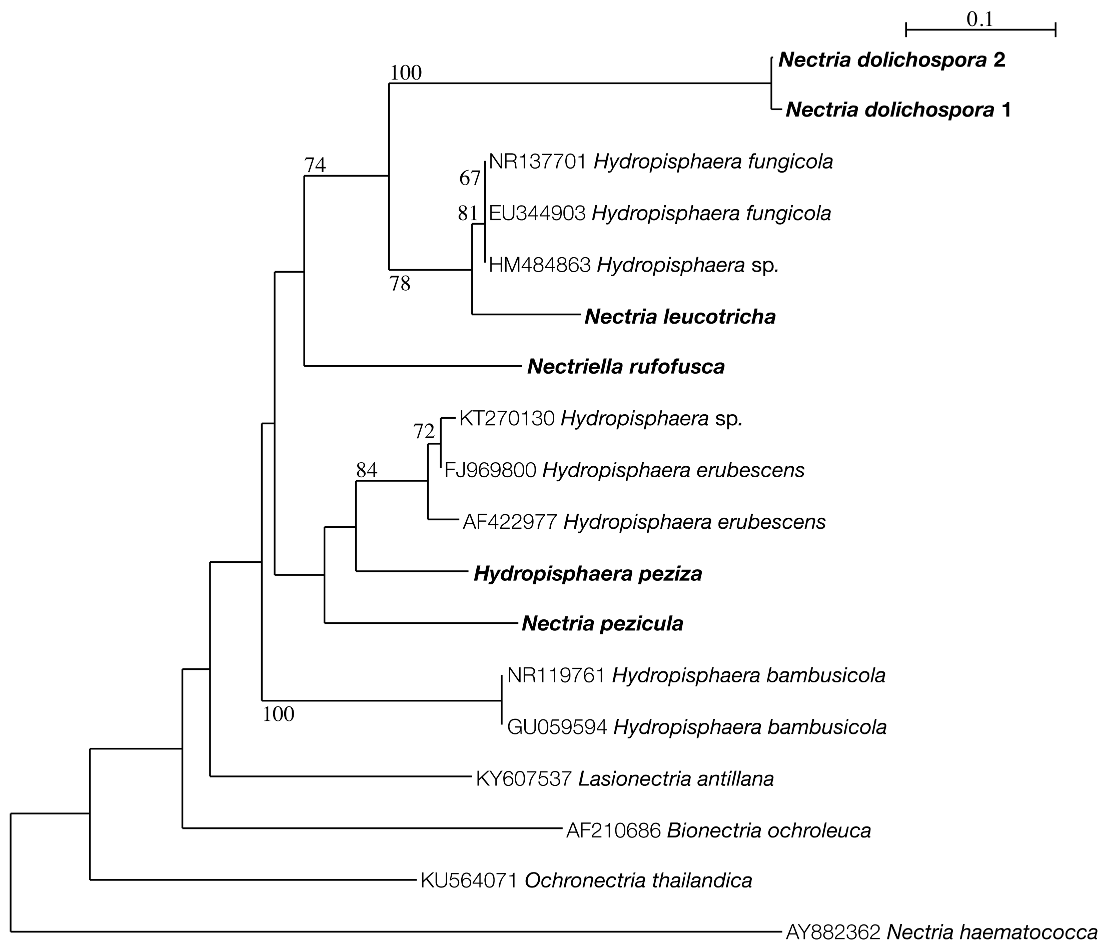


Figure 29. Phylogram generated from maximum likelihood analysis based on combined ITS1 and ITS2 sequences of species belonging to the Bionectriaceae genera *Hydropisphaera*, *Bionectria*, *Lasionectria* and *Ochronectria*. Maximum likelihood bootstrap values ≥ 50 are given above the nodes. Accession numbers are reported for the sequences retrieved from public databases. The tree was rooted with *Nectria haematococca* (Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae). The Saccardo specimens and the new sequence of *Hydropisphaera peziza* are marked in bold.

Bionectria group

The sequence analysis using BLASTN placed *Nectria ambigua*, *N. ambigua* var. *pallens*, *N. carneoflavida*, *N. congesta*, *N. flageoletiana, N. granuligera* (two specimens), *N. phyllostachydis*, *N. squamuligera* (three specimens) and *Nectriella maquilingica* type specimens in the Bionectriaceae genus *Bionectria* (anamorph *Clonostachys*). Morphologically *Bionectria* is broadly circumscribed and the use of teleomorphic characters only is impractical for the identifications of the species belonging to this genus. Based on combined molecular information about ITS regions and *tub2* this genus was divided into five different subgenera:

Bionectria, *Zebrinella*, *Astromata, Myronectria* and *Epiphloea* (Schroers, 2001). Although the use of the ITS sequences alone does not seem to be sufficient to completely support this subdivision (Schroers, 2001), and this can be also observed in the phylogram reported in **Figure 30**. The phylogenetic analysis done in this study has allowed to reclassify some types within the genus *Bionectria* (**Figure 30**).

The type specimen *N. ambigua* var. *pallens* was revised from a morphological point of view more than once, suggesting a synonymy with two different *Bionectria* species belonging to the subgenus *Zebrinella*: *B. subquaternata* and *B. grammicospora*. The species grouped in this subgenus are characterized by the presence of striate ascospores (Schroers, 2001), a trait observed also in the type specimen of *N. ambigua* var. *pallens*. However, the ITS sequence of *N. ambigua* var. *pallens* appears to be extremely different from the newly generated ITS sequence of *B. subquaternata* (similarity value= 89%) and from the ITS sequences of *B. grammicospora* (similarity value= 90%). The molecular analysis has placed this type in a medium supported clade (MLB= 68%) with *B. subquaternata*. This result suggests that *N. ambigua* var. *pallens* might be considered as a distinct *Bionectria* species belonging to the subgenus *Zebrinella* (**Figure 30)**.

The ITS sequence of *N. flageoletiana* clusters with *Bionectria compactiuscula* Schroers sequences in a highly supported clade (MLB= 97%) separated by the others. Combining this result with the high morphological similarity between *N. flageoletiana* and *B. compactiuscula* (Schroers, 2001), it is plausible to suppose that *N. flageoletiana* and *B. compactiuscula* are the same species (**Figure 30**).

The phylogenetic analysis has placed the types classified in the Saccardo collection as *N. congesta*, *N. phyllostachidis, N. granuligera* (two specimens), *N. squamuligera* (three specimens) and *Nectriella maquilingica* within a not supported clade composed by *Bionectria* species belonging to the subgenus *Bionectria* (Schroers, 2001) (**Figure 30)**. *Nectria congesta* and *N. phyllostachidis* form a non-supported clade with ITS sequences of *B. ochroleuca* suggesting a synonymy with this species, but the presence of other *B. ochroleuca* sequences far from this group does not allow to infer the synonymy. The multiple specimens of *N. granuligera* and *N. squamuligera* and the type specimen of *Nectriella maquilingica* form a non-supported clade with the only one ITS sequence of *Bionectria apocyni* deposited in NCBI (**Figure 30**). Morphologically these type specimens are very similar, but the dimensions of their asci and ascospores are smaller than those of *B. apocyni* and more similar to *B. byssicola* (Schroers, 2001), an observation that agrees with the synonymy proposed by Rossman et al. (1993) between *N. maquilingica* and *B. byssicola*. However, the ITS sequence of *B. byssicola*

is far from the group of the type sequences. For these types, without further molecular information it is therefore impossible to define a more precise taxonomic assignment below the genus level.

For the type *N. ambigua* only the ITS1 sequence has been obtained and included in the phylogenetic analysis (**Figure 30**). The position of the ITS1 sequence within the phylogram suggests that this type is a distinct *Bionectria* species, excluding the supposed synonymy with *B. apocyni* reported by Rossman et al. (1999).

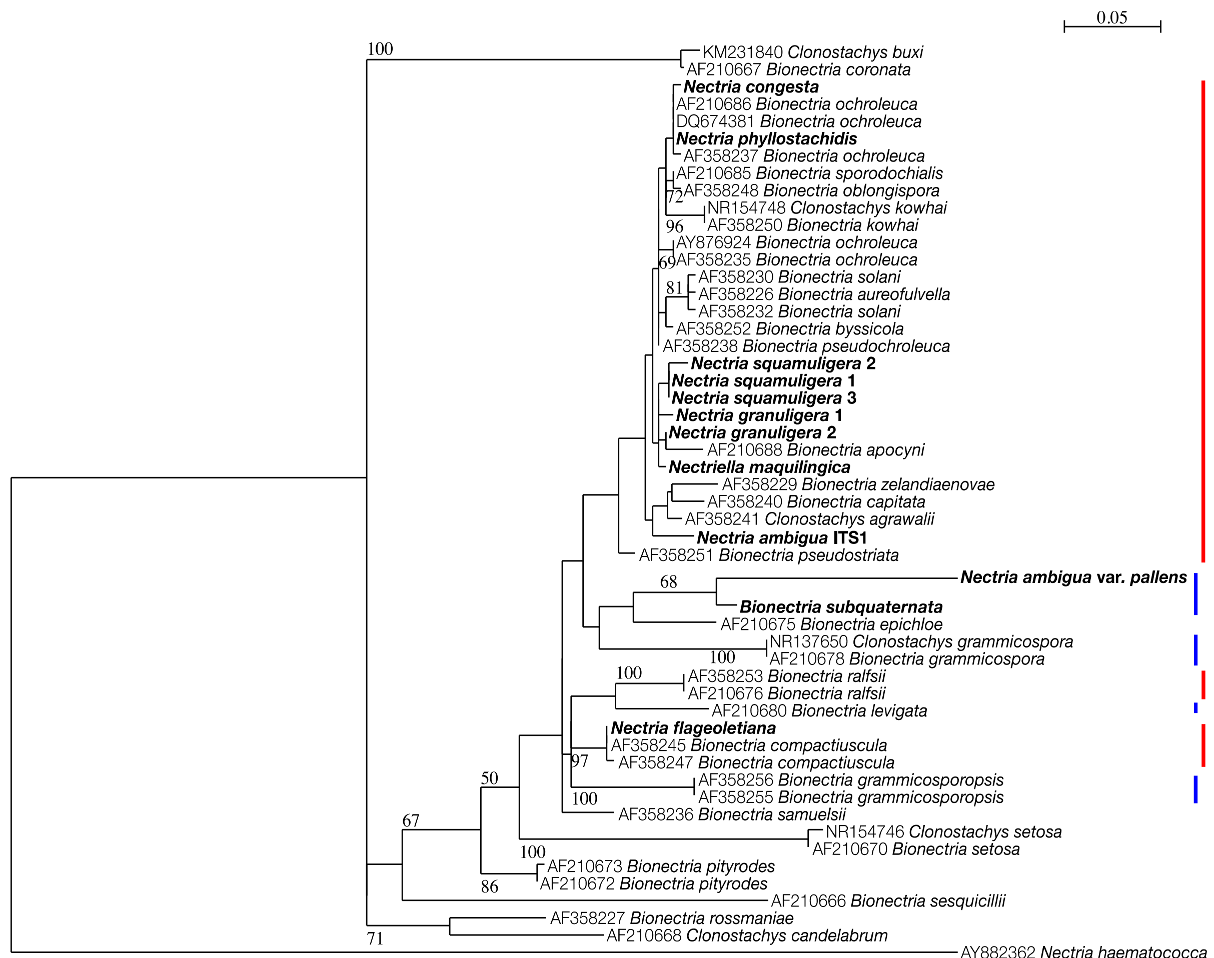


Figure 30. Phylogram generated from maximum likelihood analysis based on combined ITS1 and ITS2 sequences of species belonging to the genus *Bionectria*. Maximum likelihood bootstrap values ≥ 50 are given above the nodes. Accession numbers are reported for the sequences retrieved from public databases. The tree was rooted with *Nectria haematococca* (Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae). The Saccardo specimens and the new sequence of *Bionectria subquaternata* are marked in bold**.** Red bars indicate members of the *Bionectria* subgenus *Bionectria* while blue bars members of the *Bionectria* subgenus *Zebrinella.*

***Protocreopsis* and *Lasionectria* group**

N. albofimbriata is considered a species of the Bionectriaceae genus *Protocreopsis*, with the name *P. albofimbriata*. This genus consists of 16 species (*Index Fungorum*;

http://www.indexfungorum.org), that can be recognized by means of the ascomata completely enclosed in long, white to tan hyphae. Species of this genus generally grow on monocotyledonous leaves (palms or *Musaceae*) in tropical areas and are characterized by pale perithecia surrounded by a hyphal stroma, striate ascospores and an *Acremonium* Link anamorph (Rossman et al., 1999).

The ITS1 and ITS2 sequences for two out of three different *N. albofimbriata* specimens of the Saccardo collection have been identified by considering the information about the anamorph stage (*Acremonium* sp.) and comparing the BLASTN results for one specimen with those for the other. As reported in the phylogram (**Figure 32**), the ITS sequences of the two *N. albofimbriata* specimens cluster with ITS sequences of species belonging to the Bionectriaceae genus *Lasionectria,* as well as the ITS sequence obtained from the holotype specimen *Nectria mantuana*, showing that it is difficult to discriminate the two genera on the basis of the ITS region. In addition, the recovered ITS sequence from the holotype *N. mantuana* appears to be very different from the single *Lasionectria mantuana* ITS sequence deposited in GenBank (similarity value= 92%), thus suggesting that the ITS sequence deposited in the public database does not belong to *L. mantuana*.

The phylogram also includes the ITS1 sequence found for the type specimen *Hypocrea discolor* Penzig & Saccardo (1897) because, during the sampling for the analysis, a morphological similarity with *N. albofimbriata* showed up even if a detailed description was not possible (**Figure 31**). This observation has been confirmed by the molecular analysis, indicating that this species may be a synonym of *P. albofimbriata* (**Figure 32**).

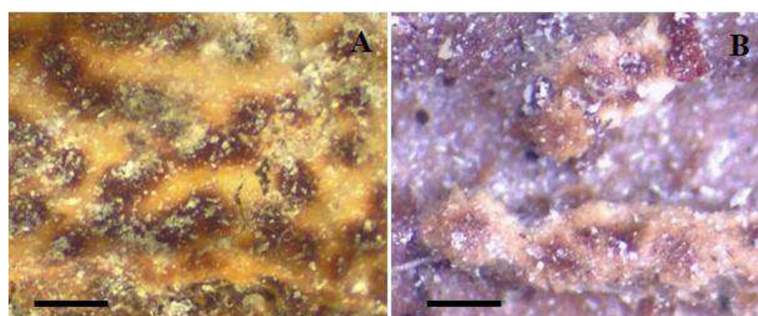


Figure 31. A *Hypocrea discolor* perithecia on natural substrate; **B** *Nectria albofimbriata* perithecia on natural substrate. Scale bars: **A**-**B**= 500 µm.

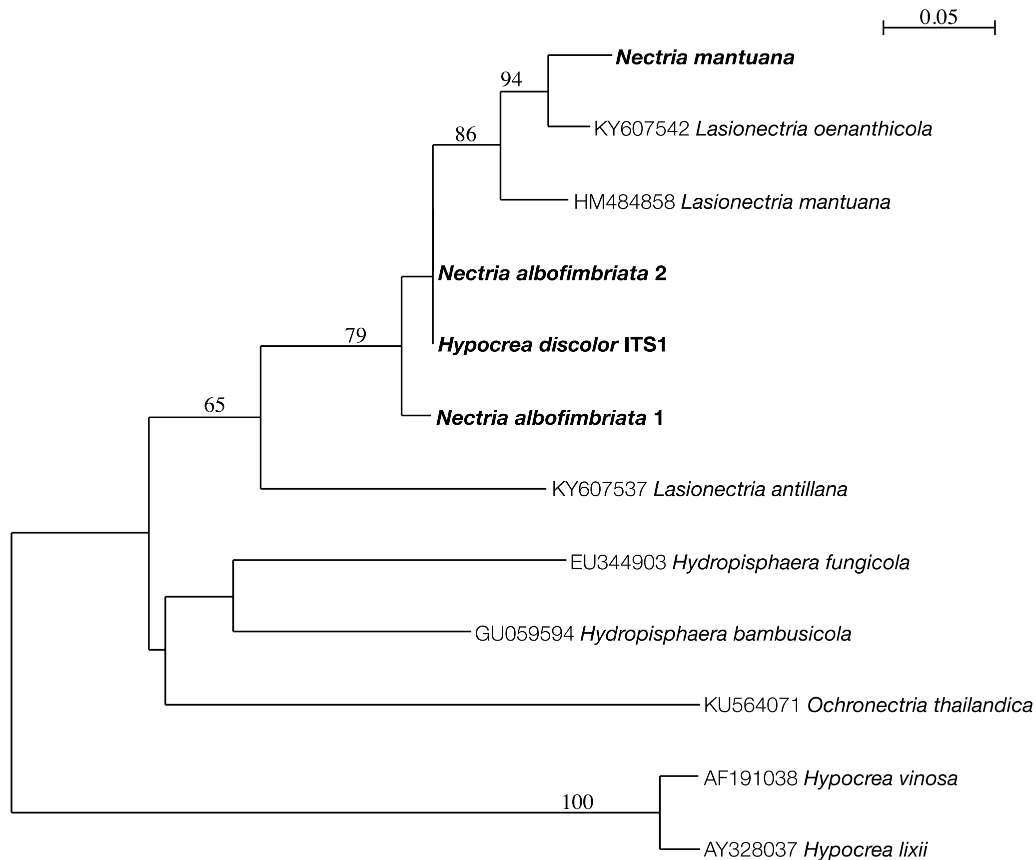


Figure 32. Phylogram generated from maximum likelihood analysis based on combined ITS1 and ITS2 sequences of species belonging to the genus *Lasionectria* and *Protocreopsis*. Maximum likelihood bootstrap values ≥ 50 are given above the nodes. Accession numbers are reported for the sequences retrieved from public databases. The tree was rooted with *Hypocrea vinosa* and *Hypocrea lixii* (Ascomycota, Sordariomycetes, Hypocreales, Hypocreaceae). The Saccardo specimens are marked in bold**.**

Nectriaceae group

The BLASTN and the subsequent phylogenetic analysis using the ITS1 and/or ITS2 sequences identified for the types *Pleonectria berolinensis*, *Nectriella bacillispora*, *Nectria coronata*, *Nectria cyanostoma* and *Nectria illudens* (**Figure 33**) confirmed the reclassification of these specimens reported in **Table 1** and **2** (*Nectria berolinensis* is a synonym of *Thyronectria berolinensis*; Jaklitsch and Voglmayr, 2014).

No recent information about *Pleonectria affinis* is available and this species was not mentioned by Hirooka et al. (2012) when they revised the Nectriaceae genus *Pleonectria*. However, *P. affinis* was considered to be similar to the species *Thyronectria berolinensis* (Jaklitsch and Voglmayr, 2014). The BLASTN result of the identified target ITS1 sequence has highlighted a high similarity value (97%) with sequences of *Nectria pseudotrichia* Berk. & M.A. Curtis. In the phylogram (**Figure 33**), the ITS1 sequence of the type *P. affinis* clusters with *Nectria pseudotrichia* sequences in a highly supported clade (MLB= 97%).

Nectria pseudotrichia along with *N. antarctica* (Speg.) Rossman are the only two species belonging to the genus *Nectria* with muriform ascospores (Jaklitsch and Voglmayr, 2014), a particular morphological character of the ascospores which also observed for the type *P. affinis* (**Figure 23**). The results obtained from molecular and morphological analysis proved that *P. affinis* is a synonym of *N. pseudotrichia*.

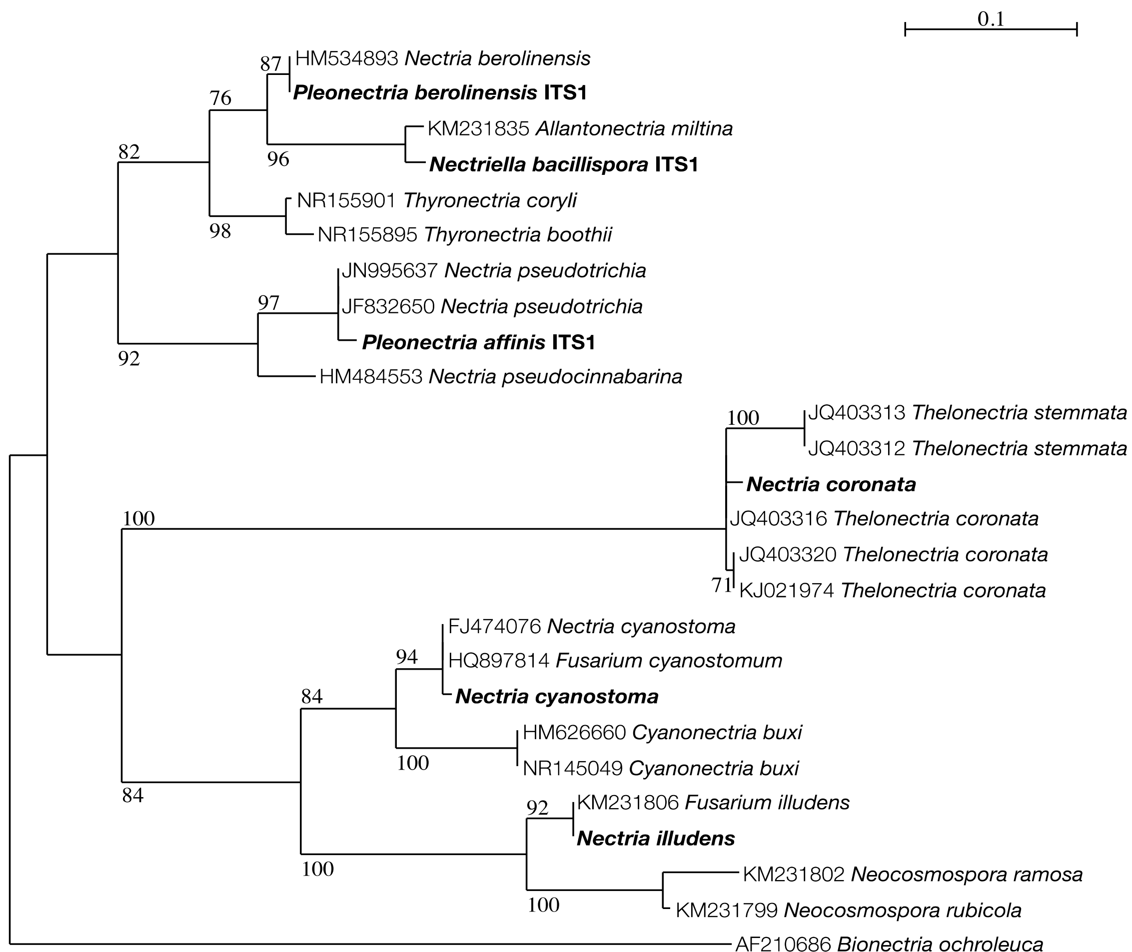


Figure 33. Phylogram generated from maximum likelihood analysis based on combined ITS1 and ITS2 sequences of species belonging to different genera of the family Nectriaceae. Maximum likelihood bootstrap values ≥ 50 are given above the nodes. Accession numbers are reported for the sequences retrieved from public databases. The tree was rooted with *Bionectria ochroleuca* (Ascomycota, Sordariomycetes, Hypocreales, Bionectriaceae). The Saccardo specimens are marked in bold**.**

CONCLUSION

In the present work an Illumina MiSeq sequencing approach has been applied to recover barcode sequences from ancient *Nectria* and *Nectria*-like type specimens stored in the Saccardo mycological herbarium. The sequencing method, already tested on other specimens of the Saccardo collection (Forin et al., 2018), has allowed to recover ITS1 and/or ITS2 sequences for 25 type specimens (30 specimens in total when considering multiple samples). The combined morphological and molecular data analysis suggests that there is a need to reclassify 1) some *Nectria*/*Nectria*-like types previously reclassified only on a morphological basis and 2) some types never considered for revisions (**Figure 34**). Indeed, five *Nectria*/*Nectria*-like types should be reclassified as synonyms of existing species (one can be considered as the teleomorph stage of a morphologically already described anamorphic species), three *Nectria* types previously placed in synonymy with other species should be actually considered as distinct species inside the same genus where they were reclassified and one never revised *Nectria* type should be considered a nectriaceous new species. In addition, for 11 types the original species name has been confirmed, while for five *Nectria*/*Nectria*-like types a taxonomic assignment has been possible only at the genus level (**Table 4**). This study has permitted not only to completely reclassify some species but also to generate new DNA sequences that will be a significant resource for future phylogenetic studies because types represent the only certain link to a specific Latin binomial name and the molecular information coming from this material is essential for taxonomic studies, in particular when synonymies are suggested on the grounds of morphological characters alone.

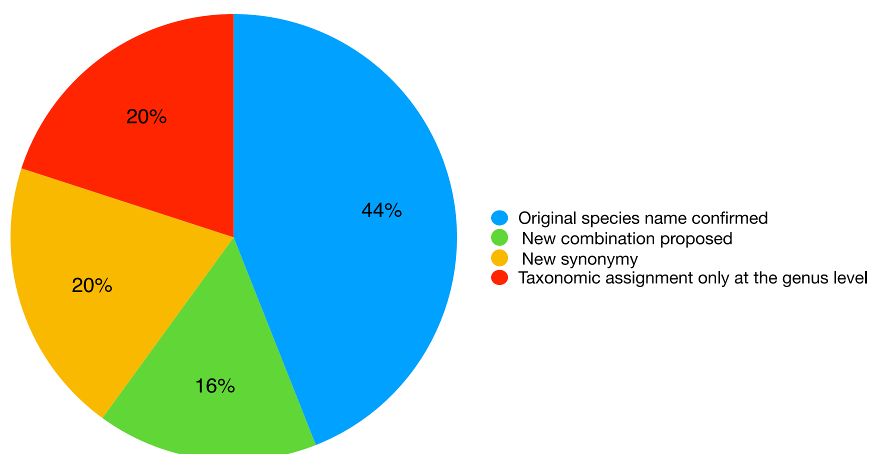


Figure 34. Taxonomic re-evaluation of the 25 type specimens for which ITS1 and/or ITS2 sequences were identified.

Table 4. List of the type specimens for which ITS1 and/or ITS2 sequences were identified. The herbarium name, the current name of the species recovered from (*Index Fungorum*, MycoBank (http://www.mycobank.org/) and literature), and the taxonomic assignment resulting from this work are reported.

Herbarium name	Current name	Taxonomic assignment
Nectria albofimbriata	*Protocreopsis albofimbriata*	*Protocreopsis albofimbriata*
Nectria ambigua	*Bionectria apocyni*	*Bionectria ambigua* comb. nov. *ad interim*
Nectria ambigua var. *pallens*	*Pezicula livida*	*Bionectria pallens* comb. nov. *ad interim*
Nectria congesta		*Bionectria* sp.
Nectria coronata	*Thelonectria coronata*	*Thelonectria coronata*
Nectria cyanostoma	*Cyanonectria cyanostoma*	*Cyanonectria cyanostoma*
Nectria dolichospora	*Hydropisphaera dolichospora*	*Hydropisphaera dolichospora*
Nectria flageoletiana		Synonym of *Bionectria compactiuscula*
Nectria granuligera	*Creonectria granuligera*	*Bionectria* sp.
Nectria illudens	*Neocosmospora illudens*	*Neocosmospora illudens*
Nectria leucotricha	*Hydropisphaera leucotricha*	*Hydropisphaera leucotricha*
Nectria mantuana	*Lasionectria mantuana*	*Lasionectria mantuana*
Nectria pezicula	*Hydropisphaera peziza*	*Hydropisphaera pezicula* comb. nov. *ad interim*
Nectria peziza subsp. *reyesiana*	*Hydrophisphaera peziza*	*Fusicolla reyesiana* comb. nov. *ad interim*
Nectria phyllostachydis		*Bionectria* sp.
Nectria raripila	*Sarcopodium raripilum*	*Sarcopodium raripilum*
Nectria squamuligera		*Bionectria* sp.
Nectria tjibodensis	*Sarcopodium flavolanatum*	Synonym of *Sarcopodium macalpinei*
Nectria tjibodensis var. *crebrior*	*Actinostilbe flocculenta*	Synonym/Teleomorph stage of *Sarcopodium vanillae*
Hypocrea discolor		Synonym of *Protocreopsis albofimbriata*
Nectriella bacillispora	*Allantonectria miltina*	*Allantonectria miltina*
Nectriella maquilingica	*Bionectria byssicola*	*Bionectria* sp.
Nectriella rufofusca	*Hydropisphaera rufofusca*	*Hydropisphaera rufofusca*
Pleonectria affinis		Synonym of *Nectria pseudotrichia*
Pleonectria berolinensis	*Thyronectria berolinensis*	*Thyronectria berolinensis*

GENERAL CONCLUSIONS AND PERSPECTIVES

The fungal specimens stored in fungaria worldwide represent a huge source of molecular information that may be exploited to obtain important molecular data. The adding of DNA-barcodes from reference herbarium samples is useful to reduce the gap between the number of described fungal species and their molecular information, presently available only for a fraction of them, and to facilitate a better taxonomic identification of the fungal species (Osmundson et al., 2013; Hawksworth and Lücking, 2017). In addition, many specimens preserved in these collections, name-bearing type specimens, have an extraordinary scientific value because they represent the only link between a taxonomic hypothesis and a scientific name (Puillandre et al., 2012); therefore, the recovery of DNA barcodes from these important samples may help to solve any taxonomic ambiguities (Larsson et al., 2004; Liimatainen et al., 2014) or to identify "unidentified" environmental DNA sequences (Nagy et al., 2011).

In this work, a next generation sequencing (NGS) approach was applied to recover ITS sequences, the consensus barcode for the identification of fungal species (Schoch et al., 2012), from ancient fungal specimens preserved in the Pier Andrea Saccardo mycological collection of the University of Padua. The Illumina MiSeq sequencing method was chosen to try to overcome the combination of two main problems: 1) the high level of DNA degradation of the Saccardo herbarium samples (fragments shorter than 500 bp) and 2) the presence of contaminations by exogenous fungal DNA. These issues are typical of historical herbaria/fungaria and represent the main reasons why these collections are scarcely exploited resources to obtain DNA information. The specimen preparation practices and storage conditions of the herbarium samples are the main cause of the DNA degradation; thus, they can negatively affect the ability to extract, amplify and sequence DNA (Staats et al., 2011). Moreover, the presence of fungal contaminations is critical for mycological collections because the universal primers for the ITS amplification cannot discriminate between the contaminating DNA and the target DNA, therefore a mix of fungal sequences can be generated that enhances the risk of sequencing failure. The *Nectria* study case reported in Chapter 2 represents an extreme case under this respect, as Illumina sequencing of some specimens yielded, in addition to the sequence of the target fungus, sequences of other species belonging to the Nectriaceae and/or Bionectriaceae families, probably due to the coexistence of different species on the same substrate or to the parasitic lifestyle of some of these species on other fungi (mycoparasitism) (e.g. Tsuneda and Skoropad, 1980).

The direct Sanger sequencing, after the PCR amplification, cannot be used to obtain the target ITS sequence and a method capable of producing a large number of sequences simultaneously is necessary. The Illumina method, commonly used in metabarcoding studies that involve fungal community analysis (Lindahl et al., 2013), allows to sequence DNA fragments in the size range of the ITS1/ITS2 regions (about 300 bp; Tedersoo et al., 2015), more easily amplifiable from degraded DNA compared to the entire ITS region (about 600 bp) and it yields millions of sequences, thus increasing the probability to obtain the target sequence of the analysed sample within a mixture of different DNA. Moreover, the possibility to pool different tagged PCR-amplicons coming from different fungal specimens, in order to recognize the sequences of each sample, makes Illumina sequencing a time- and cost-effective method for DNA barcoding. This approach required a setup of a number of steps involved in the sample preparation for the sequencing and in the bioinformatic data analysis, and then its efficacy was first tested to obtain ITS2 sequences from 36 non-type *Peziza* specimens. Despite the presence of both external fungal contamination and cross-contamination between fungarium specimens, the high-throughput sequencing method used has permitted to recover ITS2 sequences from 23 out of the 36 specimens studied and, what is more, also a taxonomic re-evaluation of some samples at the species level and others at genus or higher level (Forin et al., 2018). Then, the method was used to retrieve ITS1 and ITS2 sequences from more important type specimens. In this part of the work, the ITS1 and/or ITS2 sequences were identified for 30 *Nectria* and *Nectria*-like specimens (25 type specimens without considering multiple specimens), out of 56 *Nectria* and *Nectria*-like specimens included for the Illumina sequencing (46 types without considering multiple specimens) and used for a re-evaluation of their current systematic position (Chapter 2). A chart illustrating the overall relative number of taxonomic re-evaluations is reported in **Figure 35**.

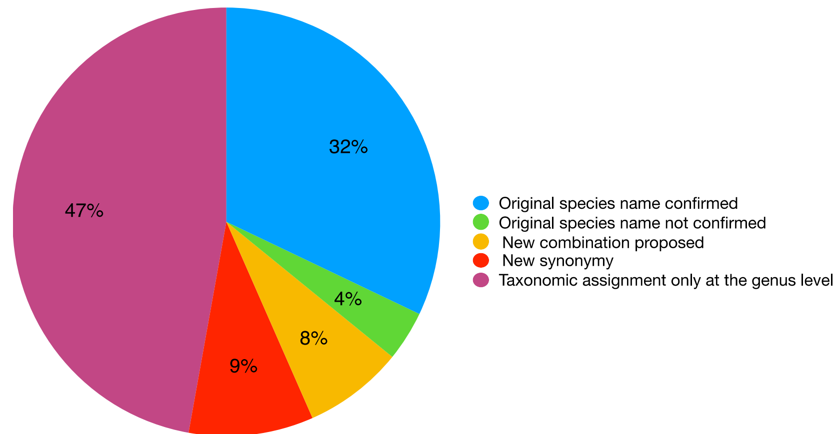


Figure 35. Taxonomic re-evaluation of the total 53 *Nectria*, *Nectria*-like and *Peziza* specimens (considering multiple specimens) for which ITS1 and/or ITS2 sequences were identified.

To our best knowledge, this work represents the first application of an Illumina sequencing method with the aim to obtain ITS sequences from problematic herbarium fungal material, even though the power of this approach has already been demonstrated by Staats et al. (2013), who applied a paired-end Illumina method for a genome-scale sequencing of dry-preserved plant, fungal and insect specimens. The results reported in this thesis show that the application of a high-throughput method can be useful to obtain molecular information from historical fungal specimens collected more than 100 years ago which have not experienced the best storage conditions for good DNA preservation and for preservation from possible exogenous DNA contaminations. Furthermore, it is also clear that the efficiency of this technique in the recovery of the target sequences strongly depends on the contamination levels. In fact, the presence of non-endogenous fungal DNA prevented from retrieving the target sequence from several specimens included in the Illumina sequencing libraries, despite the positive amplifications of the regions of interest. This is probably due to the fact that the fungal contaminants are more recent, and, as consequence, their less damaged DNA is more easily amplifiable than the degraded aDNA of the original fungarium specimens. To minimize this problem, the use of a more accurate method of DNA isolation from single ascospores within unbroken asci, if these structures are still visible and well preserved in ancient fungal specimens, could be a good solution to further increase the probability of obtaining the sequences of interest (Rubini et al., 2010), in particular when important type specimens are involved in the study. Despite the problems linked to the Saccardo fungal specimens, interesting results have been obtained from the study of type specimens. It is well known that the inclusion of DNA sequences in phylogenetic analysis coming from types is particularly important because it enables species names to be applied with absolute certainty. For

example, DNA sequences from the holotype of watermelon collected in 1773 showed that the type analysed is not the species now thought as watermelon (Chomicki and Renner, 2014). In the present research, ITS and/or ITS2 sequences from 25 different *Nectria* and *Nectria*-like type specimens have been obtained using the NGS approach. For 44% of these types the original species name has been confirmed, for 16% and 20% new nomenclature combinations and synonymies have been proposed, respectively, while for the remaining 20% the taxonomic assignment has been possible only at genus level (Chapter 2). The best example showing the importance of obtaining DNA sequences from type material is represented by the results obtained from the molecular analysis of three different *Nectria* types: *N. ambigua*, *N. ambigua* var. *pallens* and *N. pezicula*. These specimens were placed in synonymy with other existing species on the basis of morphological similarities, but the analysis of their ITS sequences suggests that they should be actually reconsidered as "new" distinct species (Chapter 2). This also demonstrates that in fungi the morphology alone does not always lead to a correct fungal systematic evaluation and therefore it is highly recommended to combine morphological and molecular analyses. In addition, this study has produced new ITS sequences that will be a significant resource for future phylogenetic studies and species identification. In view of these results, the application of this NGS method to recover crucial genetic information from as many as possible of the over 4,000 old type specimens stored in the Saccardo collection could result in a taxonomic reassessment of many fungal species.

In conclusion, this research has not only provided an additional scientific value to the Saccardo collection, but it sets up a method applicable to obtain important voucher-sequences from problematic herbarium material expanding the databases with well-annotated ITS barcode sequences. In addition, this technique might be extended, probably with further optimizations, to other molecular markers improving the taxonomic identification of some samples. Moreover, given the scientific importance of this collection and the concrete possibility of obtaining molecular information from important specimens, the maintenance and the conservation conditions of herbaria and the Saccardo fungarium in particular appear critical in order to preserve the samples and avoid further fungal contaminations.

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APPENDICES

Appendix 1. *Nectria* and *Nectria*-like type specimens included in the Illumina sequencing libraries. The numbers next to some species name indicate different fungal specimens.

Library 1 – ITS1

Tag	Specimen
1	*Nectria coronata/ambigua* var. *pallens*
2	*Nectria albofimbriata* 1
3	*Nectria granuligera* 1
4	*Nectria ambigua*
5	*Nectria ambigua*
6	*Nectria brassicae* 2
7	*Nectria brassicae* 1
8	*Nectria brassicae* 2
9	*Nectria brassicae* 3
10	*Nectria brassicae* 4
11	*Nectria carneoflavida*
12	*Nectria carneoflavida*
13	*Nectria congesta*
14	*Nectria cyanostoma* 1
15	*Nectria cyanostoma* 2
16	*Nectria dolichospora* 1
17	*Nectria dolichospora* 2
18	*Nectria eustoma*

Library 2 – ITS2

Tag	Specimen
1	*Nectria ambigua*
2	*Nectria congesta*
3	*Nectria carneoflavida*
4	*Nectria carneoflavida*
5	*Nectria coronata/ambigua* var. *pallens*
6	*Nectria albofimbriata* 1
7	*Nectria dolichospora* 1
8	*Nectria eustoma*
10	*Nectria flageoletiana*
12	*Nectria brassicae* 2
13	*Nectria hypoxantha*
14	*Nectria hypoxantha*
16	*Nectria granuligera* 2
17	*Nectria brassicae* 3
18	*Nectria mantuana*

Library 3 – ITS1

Tag	Specimen
1	*Nectria flageoletiana*
2	*Nectria flageoletiana*
3	*Nectria albofimbriata* 2
4	*Nectria granuligera* 2
5	*Nectria hypoxantha*
6	*Nectria hypoxantha*
7	*Nectria illudens*
8	*Nectria leucotricha* 1
9	*Nectria mantuana*
10	*Nectria nigella*
11	*Nectria peziculae* 2
12	*Nectria phyllostachidis*
13	*Nectria phyllostachidis*
14	*Nectria radians*
15	*Nectria raripila*
16	*Nectria reyesiana*
17	*Nectria rousseauna*
18	*Nectria sordescens*

Library 4 – ITS2

Tag	Specimen
2	*Nectria nigella*
4	*Nectria peziculae* 2
6	*Nectria phyllostachidis*
7	*Nectria cyanostoma* 2
8	*Nectria dolichospora* 1
9	*Nectria squamuligera* 2
10	*Nectria tasmanica*
11	*Nectria dolichospora* 2
12	*Nectria tjibodensis var. crebrior*
13	*Nectria eustoma*
14	*Nectria flageoletiana*
15	*Nectria granuligera* 1
16	*Nectria brassicae* 2

Library 5 – ITS1

Tag	Specimen
2	*Nectria squamuligera* 2
3	*Nectria squamuligera* 3
4	*Nectria tasmanica*
5	*Nectria tjibodensis*
6	*Nectria tjibodensis var. crebrior*
7	*Nectria xanthostroma* 1
8	*Nectria squamuligera* 1

Library 6 – ITS2

Tag	Specimen
2	*Nectria leucotricha* 1
4	*Nectria raripila*
5	*Nectria peziza* subsp. *reyesiana*
7	*Nectria sordescens*
8	*Nectria squamuligera* 1
9	*Nectria radians*
10	*Nectria tasmanica*
11	*Nectria tjibodensis*
12	*Nectria squamuligera* 3
15	*Nectria brassicae* 1

Library 7 – ITS1

Tag	Specimen
1	*Nectriella bacillispora*
2	*Nectria arundinella*
5	*Nectriella rufofusca*
6	*Nectria mantuana*
7	*Pleonectria affinis*
8	*Nectria tasmanica*
11	*Calonectria macrospora*
12	*Pleonectria berolinensis* 1
13	*Chilonectria macrospora*
15	*Hypocrea discolor*
17	*Nectria episphaeroides*
18	*Nectria theoprothala*

Library 8 – ITS2

Tag	Specimen
2	*Nectriella rufofusca*
3	*Nectria carneoflavida*
4	*Ophionectria trichia*
5	*Nectria albofimbriata* 2
6	*Nectria muscicola*
8	*Calonectria macrospora*
9	*Nectria sordescens*
11	*Nectriella maquilingica*
12	*Nectria tjibodensis*
13	*Chilonectria romana*
14	*Nectria mantuana*
16	*Nectria theoprothala*
18	*Nectria tasmanica*

Library 9 – ITS1

Tag	Specimen
2	*Nectria albofimbriata* 2
3	*Nectriella maquilingica*
4	*Nectria perpusilla*
5	*Nectria sordescens*
7	*Chilonectria rosellinii*
8	*Nectria muscicola*
11	*Nectria hypoxantha*
12	*Pleonectria berolinensis* 2
13	*Hypocrea fulva*
15	*Ophionectria trichia*
17	*Nectria tjibodensis*
18	*Chilonectria romana*

Library 10 – ITS2

Tag	Specimen
6	*Nectria tjibodensis*
7	*Nectria hypoxantha*
10	*Nectria perpusilla*
12	*Nectria episphaeroides*
14	*Chilonectria macrospora*
15	*Pleonectria berolinensis* 2
16	*Nectria illudens*
18	*Nectria arundinella*

Appendix 2. Illumina MiSeq pipeline used for the data analysis.

1) Software **PEAR**

- To assemble R1 and R2 fastq files (used for *Peziza* and *Nectria/Nectria*-like data analysis)

```
pear -f #path_R1.fastq# -r #path_R2.fastq# -o
#path_file_output.fastq# -q 28 -n 150 -t 150 -v 100
```

`-q` quality score threshold for trimming the low-quality part of a read; `-n` minimum length of the assembled sequences; `-t` minimum length of reads after trimming the low-quality part; `-v` minimum overlap size

2) Software **QIIME**

- To transform fastq file in two different fasta files (file.fna and file.qual) to use for the demultiplexing step (used for *Peziza* and *Nectria/Nectria*-like data analysis)

```
convert_fastaqual_fastq.py -c fastq_to_fastaqual -f
#path_output.fastq# -o #path_ouput#
```

`-f` fastq file obtained with PEAR (file with the assembled reads); `-o` output folder for .fna and .qual files

- Mapping file validation (used for *Peziza* data analysis)

```
validate_mapping_file.py -m #path_Mapping_file.txt# -o
#path_validate_mapping_file#
```

- Demultiplexing Forward, it subdivides the sequences considering the mapping file forward (used for *Peziza* data analysis)

```
split_libraries.py -m Mapping_fwd.txt -f #path_fasta# -q
#path_ file_qual# -o #path_file_fwd# -l 250 -s 28 -w 50 -H
13 -t -b 5 -M 3 -e 0 -a 0 --reverse_primer_mismatches 3 -z
truncate_only
```

`-l` minimum sequence length in nucleotides accepted; `-s` minimum average quality score allowed in read; `-w` enable sliding window test of quality scores; `-H` maximum length of homopolymer; `-t` calculate sequence lengths after trimming primers and barcodes, `-b` number representing the length of the barcode; `-M` maximum number of primer mismatches; `-e` maximum number of errors in barcode; `-a` maximum number of ambiguous bases; `--reverse_primer_mismatches` number of allowed mismatches for reverse primers; -z `truncate_only` remove the reverse primer

- Demultiplexing Reverse, it subdivides the sequences considering the mapping file reverse (used for *Peziza* data analysis)

```
split_libraries.py -m Mapping_rew.txt -f #path_fasta# - q
#path_ file_qual# -o #path_file_rew# -l 250 -s 28 -w 50 -H
13 -t -b 5 -M 3 -e 0 -a 0 --reverse_primer_mismatches 3 -z
truncate_only -n 5000000
```

`-n` id to use for the first sequence (using for example 500000 the reverse sequences will start with a high number avoiding in the next steps to have different sequences with the same number)

- Reverse complement of the sequences in the file rew obtained with the previous step (used for *Peziza* data analysis)

```
adjust_seq_orientation.py -i #path file_rew# -o
#output_file_rew_comp#
```

- To assemble the fwd and rew_comp files (used for *Peziza* data analysis)

```
cat file_fwd file_rev_comp > all_reads.fasta
```

3) Software **VSEARCH**

- Sequences dereplication, pre-cluster with 100% identity (used for *Peziza* and *Nectria/Nectria*-like data analysis)

```
vsearch --derep_fulllength #path_file_all_reads.fasta# --
output #path_file_derep.fasta# --uc #path_file_derep.uc# --
sizeout --threads 2 --minuniquesize 2
```

`--sizeout` in the output file is reported the number of sequences that form a cluster; `--threads 2` processors number; `--minuniquesize 2` minimum number of sequences to form a cluster (with 2 the singletons are removed)

4) Software **ITSx**

- ITS1/ITS2 sequences extraction (used for *Peziza* and *Nectria/Nectria*-like data analysis)

```
./ITSx -i #path_file_derep.fasta# -t F
```

`-t` set of profiles to use for the search (`F` = fungi)

5) Software **VSEARCH**

- OTUs creation (used for *Peziza* and *Nectria/Nectria*-like data analysis)

```
vsearch --cluster_fast #path_file_ITSx# --id 0.98 --sizein -
-sizeout --relabel OTU --centroid
#path_file_picked_OTUs.fasta# --threads 2
```

`--sizein` considers the OTU abundance information already present in the file; `--sizeout` writes the new OTU abundance information in the output file; `--id 0.98`

considers a 98% of identity to create an OTU; `--relabel` renames the OTU; `--centroid` creates the file with the sequence representing each OTU

- Chimera detection (used for *Peziza* and *Nectria/Nectria*-like data analysis)

```
vsearch --uchime_denovo #path_file_picked_OTUs# --
nonchimeras #path_file_picked_otus_nonchimeras.fasta# --
chimeras #path_file_picked_otus_chimeras.fasta# --borderline
#path_file_picked_otus_borderline.fasta# --threads 2
```

- Mapping OTUs on the total reads (all_reads.fasta) and creation of the OTU table (used for *Peziza* data analysis)

```
vsearch --usearch_global #path_file_total_reads.fasta# --db
#path_file_picked_otus_nonchimeras.fasta# --strand plus --id
0.98 --uc #path_file_otu98_table.uc# --threads 2
```

`--db` database to use as reference, in this case the file without chimera sequences (picked_otus_nonchimeras.fasta); `--uc` creates the file OTU_table.uc; `--id 0.98` considers a 98% of identity to create an OTU.

6) Software **PYTHON**

- Conversion OTU_table.uc in OTU_table.tsv (used for *Peziza* data analysis)

```
python                          #path_file_uc2otutab_mod.py#
#path_file_otu98_table.uc > #path_file_otu98_table.tsv#
```

dowload python_scripts.tar.gz from http://drive5.com/python/; use uc2otutab_mod.py in the folder drive5

7) Software **QIIME**

- Taxonomic assignment (used for *Peziza* data analysis)

```
assign_taxonomy.py -i
#path_file_picked_otus_nonchimeras.fasta# -t
#path_file_UNITE_public.taxonomy -r
#path_file_UNITE_public.edit.fasta#
```

`-i` input file (in this case picked_otus_nonchimera.fasta); `-t` file with the taxonomic annotation; `-r` file with the fasta sequences

download the last release of the UNITE dataset for QIIME (full “UNITE+INSD” dataset) to create the files: UNITE_public.taxonomy and UNITE_public.edit.fasta

- Taxonomy file

```
awk '{if($0 ~ /^>/){split($1,frame,"|"); print frame[1]"\t"frame[2]"|"frame[3]} }' UNITE_public.fasta | sed 's/>//g' > UNITE_public.taxonomy
```

- Fasta file without taxonomy

```
awk '{if($0 ~ /^>/){split($1,frame,"|"); print frame[1]} else {print $0}}' UNITE_public.fasta > UNITE_public.edit.fasta
```