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Postgraduate Interdisciplinary Doctoral Study in
Protection of Nature and Environment

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CONTRIBUTION OF „VITAL TAXONOMY“ METHODS TO THE *ASCOMYCOTA* TAXONOMY

PhD Thesis

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CONTRIBUTION OF „VITAL TAXONOMY“ METHODS TO THE *ASCOMYCOTA* TAXONOMY

Ivana Kušan, mag. biol.

Thesis performed at: Division for Marine and Environmental Research, Ruđer Bošković Institute in Zagreb

Supervisor: Armin Mešić, PhD, Senior Research Associate, Ruđer Bošković Institute in Zagreb

Summary:
Traditional laboratory methods in mycology include light microscopy and examination of dried material. Working with fungi from the phylum *Ascomycota*, these methods are insufficiently informative because large number of important taxonomic characters are irretrievably lost or changed during the material conservation. A set of laboratory methods called „vital taxonomy“ include systematic collection and exhaustive analysis of morphological, cytological, cytochemical, histochemical and physiological data sets on all types of living cells and tissues. This thesis provides a contribution of „vital taxonomy“ methods in establishing clearer and some new taxonomic features important to distinguish closely related species from several different taxonomic groups in the phylum *Ascomycota*. *Thecotheus platyapiculatus* and *Tricharina tophiseda* are proposed as new species. For the purpose of easier identification and differentiation from similar species, a re-description of *Hamatocathoscypha rotundispora* and *Tricharina japonica* is given. The genus *Ascorhizoctonia* is proposed for emendation to include only two species, *A. praecox* and *A. intermedia*. A worldwide key to the species with apiculate spores in the genus *Thecotheus* is provided along with a guide for the study of spore apiculi. Nomenclatural standard for the ascospore shapes is elaborated regarding the prolate symmetrical spores.

(95 pages, 19 figures, 8 tables, 145 references)
Original in: English
Key words: anamorph, *Ascorhizoctonia*, ascospore morphology and morphometry, axenic cultures, biodiversity, biogeography, cell inclusions, character states, ecology, *Hamatocanthoscypha rotundispora*, key to the species, microscopy, taxonomic characters, taxonomic procedures, teleomorph, *Thecotheus platyapiculatus* nom. prov., *Tricharina tophiseda* nom. prov., *Tricharina japonica*

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DOPRINOS METODA „VITALNE TAKSONOMIJE“ TAKSONOMIJI GLJIVA ODJELJKA *ASCOMYCOTA*

Ivana Kušan, mag. biol.

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Mentor: Dr. sc. Armin Mešić, viši znanstveni suradnik, Institut Ruđer Bošković u Zagrebu

Kratki sažetak doktorske disertacije:
Tradicionalne laboratorijske metode u mikologiji uključuju pregled suhog materijala uz pomoć svjetlosne mikroskopije. U radu s gljivama iz odjeljka *Ascomycota* ove su metode nedovoljno informativne jer se brojni važni taksonomski karakteri nepovratno gube ili mijenjaju tijekom konzerviranja uzoraka. Skup laboratorijskih metoda „vitalne taksonomije“ uključuje sustavno prikupljanje i iscrpno analiziranje morfoloških, citoloških, citokemijskih, histokemijskih te fizioloških podataka o obilježjima svih tipova živih stanica i tkiva gljiva. U ovome radu metode „vitalne taksonomije“ doprinose utvrđivanju jasnijih i nekih potpuno novih taksonomskih značajki važnih za razlikovanje blisko srodnih vrsta iz nekoliko različitih taksonomskih grupa u odjeljku *Ascomycota*. *Thecotheus platyapiculatus* i *Tricharina tophiseda* predlažu se kao nove vrste za znanost. Za vrste *Hamatocathoscypha rotundispora* i *Tricharina japonica* dan je proširen opis zbog lakše identifikacije i razlikovanja od sličnih vrsta. Predlaže se nova taksonomska koncepcija roda *Ascorhizoctonia* s uključivanjem samo dviju vrsta, *A. praecox* i *A. intermedia*. Izrađen je svjetski ključ za identifikaciju vrsta iz roda *Thecotheus* s apikulatnim sporama zajedno sa smjernicama za proučavanje takvog tipa spora. Detaljno je izložen nomenklaturni standard za imenovanje oblika prolatnih simetričnih spora.

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Contents

1. INTRODUCTION

1.1. Generalities about fungi and its constituent group *Ascomycota*

Fungi are eukaryotic, heterotrophic organisms, devoid of chlorophyll, that obtain their nutrients by adsorption, store carbohydrates in a form of glycogen and reproduce by sexually and/or asexually produced spores. Most fungi lack flagella and have mycelium-forming bodies composed of hyphae with distinctive cell wall carbohydrates (chitin, α- and β-glucans). Thanks to their numerous adaptations and various life strategies, members of the kingdom *Fungi* inhabit large number of different habitats and microhabitats and interact with all major groups of organisms. Fungi and animals evolved from a common ancestor about a billion (± 500 million) years ago (Berbee & Taylor 2010) and nowadays constitute a major eukaryotic lineage equal in numbers to animals and exceeding the plants. Because of their key role in ecosystem functions and influence on humans and human-related activities, fungi are often considered to be the most important organisms in the world (Mueller & Bills 2004). Even though their importance is undeniable, the lack of basic information on taxonomical diversity in this group is huge and fungi are considered one of the most underexplored groups of living organisms on Earth (Hawksworth & Kallin-Arroyo 1995). Only about 100,000 fungal species is currently known to science (Kirk *et al*. 2008) representing only 2–7% of total fungal diversity. The most conservative estimates were set up to 1,500,000 fungal species, based on the 1:6 ratio of plant species to fungal species (Hawksworth 1991). This estimate was soon re-examined because numerous potential fungal habitats/substrates and localities remain understudied (Hawksworth 2001). More recent estimate, based mostly on high-throughput molecular methods, suggest that 5,100,000 fungal species could live on Earth (Blackwell 2011). Within the kingdom *Fungi*, the majority of described fungal species (~98%) are members of dikarya clade (James *et al*. 2006) which includes only two phyla: *Ascomycota* Caval.-Sm. (~65,000 species) and *Basidiomycota* R.T. Moore (~32,000 species).

Members of the phylum *Ascomycota* are characterized by the production of meiospores (ascospores) in specialized sac-shaped sporangia (asci), which may or may not be produced within a fruitbody (ascoma). Species of this group are ecologically the most versatile organisms within fungi. Recent phylogenetic classification (Lutzoni *et al*. 2004, James *et al*. 2006, Hibbett *et al*. 2007) divided this phylum into three monophyletic subphyla: *Taphrinomycotina*, *Saccharomycotina* and

Pezizomycotina. *Taphrinomycotina* is the earliest diverging clade and it includes species that exhibit yeast-like and dimorphic (yeast-like and filamentous) growth forms. The subphylum *Saccharomycotina* consists of "true yeasts", e.g. *Saccharomyces cerevisiae* and *Candida albicans*. The largest subphylum is *Pezizomycotina*, comprising filamentous and fruitbody-forming species, both lichenized and non-lichenized (Lumbsch & Huhndorf 2010). The genera and the species groups treated in this work are members of the subphylum *Pezizomycotina*, orders *Helotiales* Nannf. and *Pezizales* J. Schröt. According to Mueller & Schmit (2007) the order *Helotiales* is among the poorest explored major groups of fungi having only 3% described species (2,036) out of a 70,000 estimated species. The order *Pezizales* is explored up to 34% (1,029 species out of 3,000 estimated ones), probably due to the larger fruitbodies development.

As on the global level, fungal biodiversity in Croatia is also very high. Only 4,500 species of fungi is currently recorded in Croatia out of the estimated 20,000 species (Tkalčec *et al*. 2008). The phylum *Ascomycota* is represented with ca. 1,500 non-lichenized (pers. data) and 1,019 lichenized species (Radović *et al*. 2009). Therefore, inventory projects are necessary to ascertain the mycobiota diversity at the best possible extent. Research of ascomycetous fungi in two Croatian National Parks, as well as on the south Dalmatian island of Korčula, uncovered the astonishing diversity of *Ascomycota* (pers. data). A certain number of collections could not be attributed to any of the described taxon. One of the reasons for this is existence of the vast number of fungal species that are still inadequately described, often lacking some important differential taxonomic characters. On the other hand, some specific microhabitats were neglected in previous research or were never explored before (e.g. tufa barriers). This research showed that those habitats could carry a large number of unique species, with some of them still unknown to science.

1.2. Taxonomy

Because of the globally low level of biodiversity exploration, there is an evidence of a strong need for further extensive mycological taxonomic research. Numerous species yet unknown to science should be discovered, described and properly taxonomically evaluated. This would greatly improve our understanding of this important and large group of organisms and the functioning of the living world in general.

According to Stuessy (2009), taxonomy is a synthetic science, drawing up data from diverse fields such as morphology, anatomy, cytology, genetics, cytogenetics, chemistry and molecular biology. It has no data of its own. Every new technical development in these fields offers promise and brings new light into improved description of relationship of organisms. The accumulation of data and their interpretation never cease. Not only do new techniques of data-gathering provide more information that must be brought to bear on understnding relationships, but also these new interpretations reveal new taxonomic groups that must be understood and utilized. In the modern systematics (Fig. 1) data are gathered from organisms and their interactions with the environment and used to answer questions about classification, phylogeny and the process of evolution. Evolutionary taxonomist who seeks to cluster the organisms of his particular interest using a combination of phylogenetic relationship, a relationship between predecessor and descendant(s) and degree of evolutionary change, differ two aspects when dealing with taxa: taxonomic characters and character states (Fig. 19, Table 8). When we make the scheme of phylogenetic relationships of any particular group of the organisms its branches may be superimposed by corresponding taxonomic character change. Thus, some separate phylogenetic branch may be characterized for instance by appearance of the spore polar lipid bodies that have been evolved from the ancestor that had spores devoid of lipids. In such case spore lipid bodies represent the taxonomic character while both absence and presence of spore lipid bodies are character states.

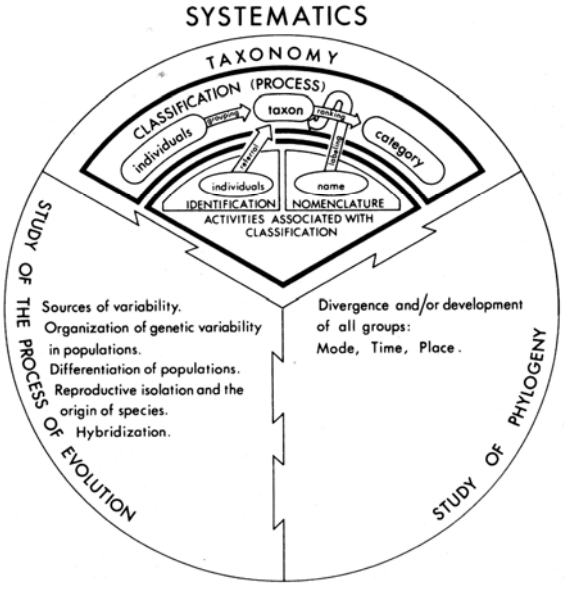


FIGURE 1. Diagram of conceptual and procedural relationships among and within areas of systematics (Stuessy 1979).

Generally, the taxonomy could be considered as "the world's oldest profession" (Knapp 2010). One of our everlasting historical requisites is the need in naming and in classification of all the objects in our surrounding so that we could be able to use them properly. According to Manktelow (2010) the history of taxonomy dates back to the origin of human language. The author quotes pharmacopoeia that was written by Shen Nung, Emperor of China around 3000 BC and medicinal plants that were illustrated on wall paintings in Egypt approximatively 1500 BC as the earliest known testimonies of early taxonomists in pre-Linnean time. Mayr (1982) points that the Aristotle (384–322 BC) was first known person who started to classify all living things in the ancient Greece. Some of his terms are still frequently in use today. Continuing to Aristotle's work, his student Theophrastus wrote Historia Plantarum (370–285 BC) in which he treat 500 plant species of which some generic names are still in current use. Pliny the Elder (Roman Empire, 23–79 AD) was the last taxonomist of the ancient history. His 160 volumes of Naturalis Historia contain descriptions of many plant species. Medieval era practically passes without a significant taxonomic work. Several pre-Linnean taxonomists appeared only after about 1.500 years. One of the most important furhter achievements in taxonomy was that of Italian botanist Andrea Cesalpino (sometimes called "the first taxonomist") who published *De plantis libri XVI* (1583), a milestone of botany, where he described 1,500 plant species and established some families still recognized today. A century later, the English botanist John Ray published over 18,000 plant species and in his work *Methodus Plantarum Nova* (1682) taxa are based on a combination of large number of characters.

The Swedish naturalist Carl Linnaeus established a new era of taxonomy. He was at first influenced by an older French botanist Tournefort. Linnaeus' most important works are *Systema Naturae*, first edition (1735) where he established the major groups of living organisms (see Table 1) and minerals, *Species Plantarum* (1753) where he ushered nomenclatural binominal naming in taxonomy that is still in use today and compiled the first global flora (8,530 flowering plants), and *Systema Naturae*, 10th edition (1758) with global fauna. *Species Plantarum* is determinated as a starting-point in mycological and botanical taxonomy, while the latter is a starting-point in zoological taxonomy. Names published before these two works are regarded as "pre-Linnean" and are considered as nomenclaturally invalid. One of the biggest impacts of Linnaeus' work was uprising of botany and zoology as newborn sciences of their own. First who emphasized the importance of creation rules in taxonomy, such as name priority, was French botanist De Candolle who introduced the term taxonomy (De Candolle 1813). Pan-european decision about creation of a

Code of botanical nomenclature was finaly made in Vienna 1905 but unified global Code (ICBN) was not accomplished until 1935.

Its origin and development, the modern taxonomy owe to Darwin-Wallace evolutionary theory exposed in Linnean Society in 1858 where both naturalsist were recognized as "proponents of a novel hypothesis on the driving force of organismic evolution" (Kutschera 2003). Soon after, a number of scientists sustained the view that Darwinian principles of common ancestry and evolutionary lineages should be included in taxonomy. Ernst Haeckel (1866) was a German biologist who started the construction of evolutionary trees and revising major taxa (Table 1) and he introduced the term phylogeny. The German biologist Willi Hennig invented cladistics in 1966, stating that only those characters that are shared by two or more taxa derived through evolution from a last common ancestor (synapomorphies) should be used in classification. Any taxon should thus include all descendants from only one single ancestor (the rule of monophyly). In this way every hypothesis on systematics could now be tested through cladistic methods for the first time. The invention of the polymerase chain reaction (PCR) that enabled rapid amplification of DNA-sequences for use in systematics together with accelerated development of computer aplications capable to operate with very large data sets enabled progress of molecular phylogeny. In this way cladistic principles became more or less the rule for further systematic work. However, circumstances and some events in a recent time science brought a number of obstacles and problems to further development of taxonomy as summarized in Wheeler (2004) and Korf (2005).

TABLE 1. Historical overview of selected biological classifications using kingdoms as taxonomic units with a highest rank.

<table>
<tr><th>Linnaeus (1735)</th><th>Haeckel (1866)</th><th>Copeland (1938)</th><th>Whittaker (1969)</th><th>Cavalier-Smith (1998)</th></tr>
<tr><td rowspan="3">-</td><td rowspan="3">Protista</td><td>Monera</td><td>Monera</td><td>Bacteria</td></tr>
<tr><td rowspan="2">Protoctista</td><td rowspan="2">Protista</td><td>Protozoa</td></tr>
<tr><td>Chromista</td></tr>
<tr><td rowspan="2">Vegetabilia</td><td rowspan="2">Plantae</td><td rowspan="2">Plantae</td><td>Plantae</td><td>Plantae</td></tr>
<tr><td>Fungi</td><td>Fungi</td></tr>
<tr><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td></tr>
</table>

Before the Linnaean time fungi were considered as plants that lack chlorophyll and were treated as “saprophytes”. Whittaker (1969) was first to emphasize the necessity of recognizing fungi as a separate kingdom (Table 1), a view which was more or less sustained ever since. In one of the most recent classification, the fungi represent one of the six recognized kingdoms (Cavalier-Smith 1998). However, Woese *et al*. (1990) elaborate the necessity of recognizing a taxonomic rank higher than a kingdom on the basis of collected phylogenetic data-sets and they introduced three-domain system of the living world: Archaea, Bacteria and Eucarya where fungi are considered as one of the kingdoms within the domain Eucarya.

1.3. Traditional methods in the taxonomy of fungi

The most important research tool in fungal taxonomy today, as it was in the beginnings of mycology, is transmission light microscope that provides a variety of light microscopical methods. Molecular phylogenetic analyses that are developing for the last 25 years, achieved essential importance in our understanding about true phylogenetic patterns in all existing groups of fungi, an issue that had been constantly vague in all history of mycology before they have developed. Nevertheless, all taxonomically relevant non-molecular features (e.g. morphology, cytology, cyto- and histochemistry, ecophysiology, life history etc.) will continue to have great value in fungal taxonomy in the future, not only for practical reasons when handling with particular organism and/or its derivates of our interest but also for fundamental (taxonomic and ecological) issues. Therefore, microscopy remains the central research method in mycotaxonomists hands.

When comparing the material of various collections only of a single species, the microscopist could be able to document different kinds of variations during every thorough microscopical observation. Thus, some taxonomically unimportant variations can be visible even within a single fruitbody as well as when comparing different fruitbodies of the same species within a single population. On the other hand, constant variation that is ascertained between different populations in the same species should draw the taxonomist attention. Ontogenetic variations (together with the life history) are also often important in composing a detailed profile of a certain species and for delimiting two hardly distinguishable species. However, in many groups of ascomycetous fungi variations caused by using different mounting media and variations caused by dehydration and chemical fixation are tremendously important, and if handled without awareness could result in

severe errors in taxonomical conclusions. The latter two kinds of variations are the main subject of this thesis.

Traditional microscopical processing of the fungal collection involves making microscopical preparation by rehydration of dried material and by isolating and mounting the certain parts of the fruitbody containing taxonomically important tissues and cell types in a mounting medium (or mounting the entire fruitbody in fungi with minute fruitbodies, <0.5 mm in diam.). After fieldwork, macroscopic features of the fresh fruitbodies are documented and then, in the traditional procedure, fruitbodies are immediately preserved by drying under the stream of warm and dry air in order to be permanently conserved and stored (exsiccata) for further analyses. In this procedure cells and tissues in dried fruitbodies lose nearly all chemically unbound water (dehydration), while resistant chitinous cell walls are structurally preserved thanks to which the configuration of the cells and tissues can be more or less recovered by the subsequent rehydration, usually with the aid of potassium hydroxide aqueous solution. In addition to the loss of water, the living fungal cell irreversibly loses almost all of its cellular inclusions, while those that endure dehydration (e.g. lipid bodies) irrevocably change their shape and conformation specific to the living cell of a certain fungal species. Also, after tissue rehydration, particularly in ascomycetous species containing larger cells (> 20 μm), such cells never fully regain its original form and dimensions.

1.4. Thesis goal

The aim of this study was to test the morphological, cytological, cytochemical and histochemical characteristics as well as ecophysiological features of living cells and tissues obtained by "vital taxonomy" methods in studied species groups from the phylum *Ascomycota* and to evaluate their use in taxonomy. I have tested the hypothesis that microscopic analysis of living structures and broader use of vital taxonomy methods (the propagation of living material in axenic culture) could bring to a discovery of a larger number of reliable taxonomic characters that have well-defined variability, including some novel characters that could help in clarification of the taxonomic relationships at the species and generic levels (in the genera *Hamatocanthoscypha* Svrček, *Thecotheus* Boud., *Tricharina* Eckblad, *Ascorhizoctonia* Chin S. Yang & Korf).

2. GENERAL PART

2.1. The history and definition of "vital taxonomy"

French mycologist Émile Boudier was the first author in the history of mycological research who emphasized the advantages of working with living material of ascomycete fungi (*in statu vivo*). In his work on the classification of ascomycete fungi with apothecial fruitbodies, Boudier (1885) pointed out that he never worked on dried material when compiling descriptions of types because he was assured that such material almost always gave "inaccurate" results. Such advance in the research was made possible only after German physicist Ernst Abbe (1879 a, b) accomplished the compound light microscope equipped with oil-immersion lense in 1877. Boudier believed that the results obtained by working on dried material are "obstacle to good classification". In Boudier's "Icones Mycologicae" (1905–1910) original drawings in colour display 600 species of fungi at the current classification of that time. All drawings have inestimable scientific value and faithfully represent the structure of cells and tissues of the given species in the living condition. Thanks to his Icones which clearly point out taxonomically important characters (present only in the living material), today we can easily recognize majority of species depicted by Boudier including some that were not seen by specialists during more than one hundred years. Apart from his clearly depicted species features, today we can interpret in some issues also the characters attributable as generic markers that are now confirmed by molecular phylogenetic analysis (e.g. Van Vooren *et al.* 2015). Another French mycologist, Lagarde (1906) wrote that "in the process of drying delicate parts of apothecial fruitbodies in ascomycetous fungi are undergoing irreversible and profound changes" and that work with the dried herbarium specimens "always gives mediocre results that are subjected to misinterpretation". Studying the anatomy of the apothecia Corner (1929) concluded that the best method of examining the growth of hyphae and the origin of tissues is by means of freehand sections of living material. In her study on the ontogeny of ascospore ornamentation in *Pezizales*, Le Gal (1947) wrote that "only vital observations could give us satisfactory results". Quite often taxonomists tend to describe either living or (more frequently) dead elements without taking possible alterations into consideration and rarely give detailed indications of their preparative treatments (living vs. dead). Yet some authors were aware of the alterations between living and dead elements and were trying not to mix data (Graddon 1951, Scheuer 1988, Huhtinen 1990).

At the end of the 70's of the 20th century a German mycologist Hans-Otto Baral was the first to continue with systematical work on living material after more than a half century. A set of microscopical methods applied for advances in taxonomy by treating the living material and studying the living structures Baral (1989) defines as "vital taxonomy". Using transmission light microscopy, Baral (1992) analyses significant differences in the microscopic structures comparing features and behavior of the cells and cellular inclusions in preparations made from fresh (living) material and rehydrated dry (dead) herbarium material mounted in tap water medium (Figs. 2, 3). He ascertained the most important differences: A) the volume of the spores, asci and paraphyses is substantially reduced in dead state (Fig. 3), B) the spores in living asci are located at the top, while the dead asci contain spores distributed along their entire length (Fig. 2b, 2e), C) in living cells with lipid content, lipid bodies are not irregularly shaped, coalesced with wide variety of conformations as in dead cells but are organized and arranged in a specific and a consistent manner (Fig. 2a, 2d), D) the living paraphyses often contain refractive vacuolar bodies as opposed to dead ones where such inclusions are completely lacking (Fig. 2c, 2f). In this monographic treatment Baral gives a systematic review of "vital taxonomy" methods and a detailed comparison with the classic "herbarium taxonomy". He systematically applies these methods to the taxonomy of two ascomycetous orders: *Helotiales* and *Orbiliales* Baral, O.E. Eriksson, G. Marson & E. Weber. The methods of "vital taxonomy" have been hitherto used almost exclusively in the taxonomy of ascomycetous fungi. The number of mycologists who apply them is still low even though described methods demonstrate numerous advantages in a number of papers giving effective solutions to taxonomic problems with high applicability in discrimination / delimitation of cryptic species or in recognition of hardly distinguishable taxa (e.g. Baral 1984, Zhang *et al*. 2007, Baral & Bemmann 2014, etc.).

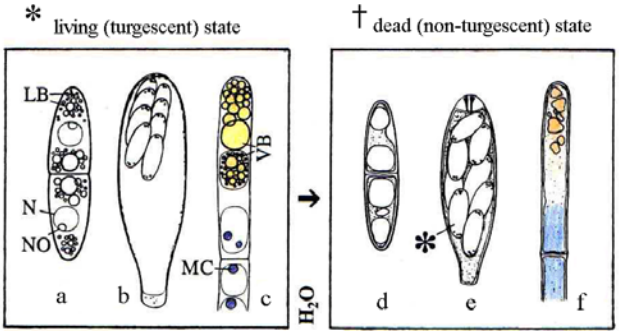


FIGURE 2. Hymenial elements (ascospore, ascus, paraphysis) of fictitious species of *Helotiales* in the living (**a-c**) versus dead state (**d-f**); the dead ascus contains still living ascospores (Baral 1992).

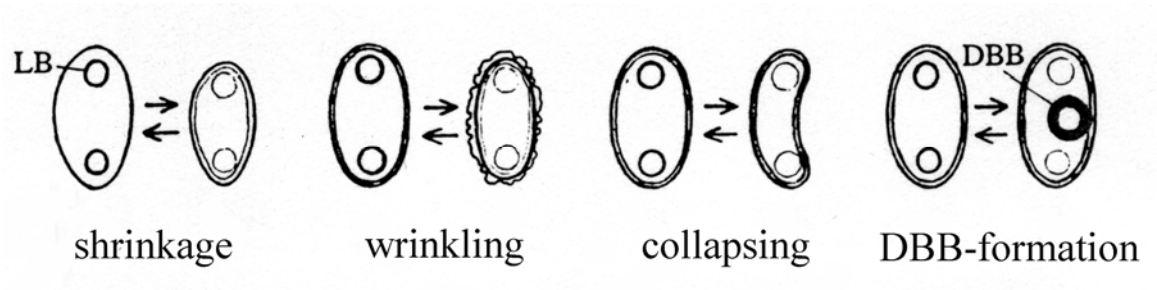


FIGURE 3. Four reactions of a living cell on water loss, induced either by desiccation, or by a medium with a high osmosity (Baral 1992).

"Vital taxonomy" is the set of taxonomical research methods centred with microscopical treatment of the organism or its taxonomically important organs (in fungi it is typically a fruitbody) while it still contain living cells and tissues. Because it can contain a number of additional research aspects that could be added to the core method, it is considered as a set of methods. Most often, the central method is combined with the comparative study on the alterations detectable when the observed living cells die (Fig. 3). Various valuable cytological and cyto- and histochemical data can be obtained by an application of certain lethal media (reagents and stains) to the living material. Such data cannot be obtained through application of the same media on the thoroughly dried (dead) material. The comparative study of dried material (dead state) on the same collection treated previously in the living state is also very much advisable so that given collection could be compared to the type material of the species that is considered as conspecific to our collection (Fig. 4). If the two are matching, we could conclude the identification and assign wide variety of new data obtained on fresh (living) material to the corresponding species. Note that some taxonomically essential cytochemical and histochemical characters could be irreversibly lost in an old conserved material, i.e. hemiamiloidity in the genus *Pezicula* Tul. & C. Tulasne could be permanently lost in a 40–50 year old exsiccata (Baral 2009). The taxonomic importance of hemiamyloidity is elaborated in Baral (1987).

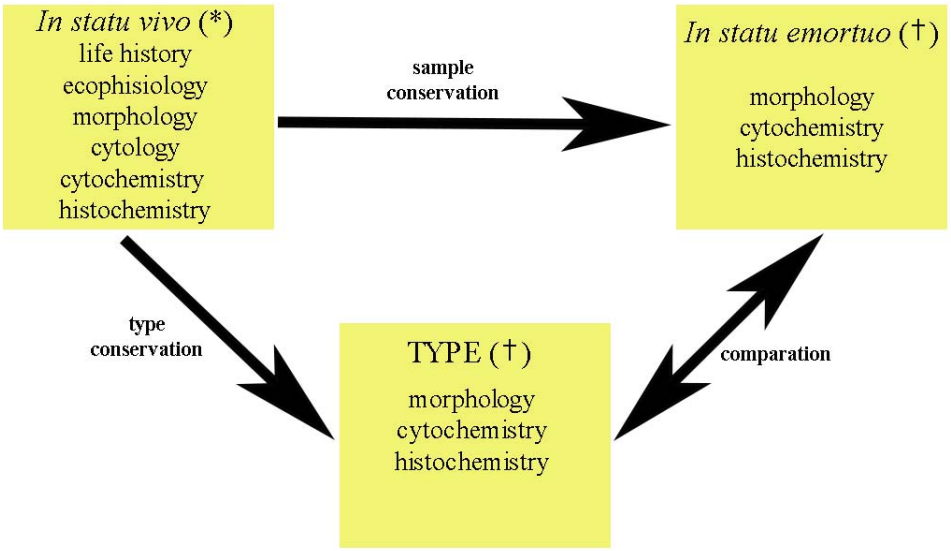


FIGURE 4. A scheme showing the relations between taxonomical data sets obtained *in statu vivo* with the data sets obtained from the same collection *in statu emortuo* and the corresponding type material. Del. I. Kušan.

Another important extension to the core "vital" method is a propagation of a given species to axenic cultures by means of inoculation of still viable spores to the agar plate with minimal contamination risk. In this way a taxonomist is able to continue the study of an organism and obtain data on its ecophysiology and life history. One more method could be considered as an extension to "vital taxonomy": sampling of still living tissues for the subsequent molecular analysis by placing it into a DNA lysis buffer directly on the field. This method has been proven to give good results in preserving and later extraction of DNA (Landvik *et al*. 1998b). Consequently, we may say that "vital taxonomy" methods represent a polyphasic taxonomy focused on research of the living structures. Such research can provide incomparably more taxonomically (and phylogenetically) informative data than „traditional" research methods based on study of dead structures, thus giving the priority of polyphasic documentation (species data sets or holistic iconotype) about the organism over the type specimen represented by an exsiccatum. Apart from its value at the species level, these methods are capable to reveal valuable markers and reliable recognition at generic level and higher taxa, often demonstrating high overlapping with the phylogenetic trees obtained by multigene molecular analysis (Pfister *et al*. 2009, Matočec *et al*. 2014, Van Vooren *et al*. 2015). Thus, species as well as genera and higher taxa are very often clearly delimited from each other using solely the

data (characters) obtained by light microscopy of the living cells. The following example depicts differences among four European species of the genus *Sarcoscypha* solely on the basis of living cells in the hymenial layer (fertile part of an apothecium, Fig. 5). Those species are almost unrecognizable from each other by "traditional" microscopical methods.

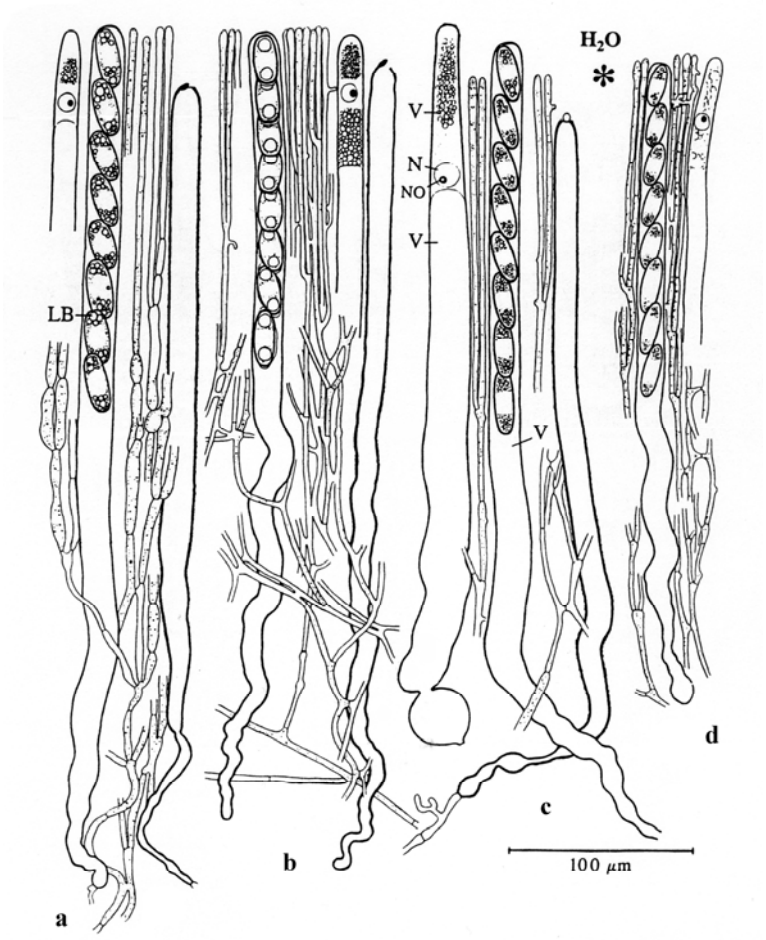


FIGURE 5. The four European species of *Sarcoscypha* (Fr.) Boud. distinguished, besides other features, by the size of the lipid bodies in the ascospores (Baral 1984): **a.** *S. austriaca* (Beck ex Sacc.) Boud. **b.** *S. jurana* (Boud.) Baral. **c.** *S. coccinea* (Scop.) Lambotte s. str. **d.** *S. macaronesica* Baral & Korf.

Wholeheartedly supporting "vital taxonomy" methods, experienced American mycologist Richard P. Korf (2005) encouraged the young students with the following words: "I close with this advice to young fungal taxonomists: (a) forget the lure of instant fame in following the latest technological fad, but instead (b) go out into the field and collect, take ample notes, culture if

possible, study—if you can take along a field microscope—while your specimens are alive (Baral 1992) and while you simultaneously swat mosquitoes in a tropical rain forest, (c) learn to love sleuthing in the stacks of a good library as well as on the internet, (d) scour the world's herbaria, (e) publish even if you feel you are only 95% correct, much preferable to being a perfectionist that never publishes—one whose data dies with himself or herself, (f) never be the graduate student who emails a scientist asking for cultures if you are incapable of doing your own fieldwork, of identifying specimens, and of using the taxonomic literature, and, (g) *above all,* leave a luxurious legacy of data for future taxonomists to build upon."

2.2. Three cases of taxonomical considerations elaborated in this thesis

2.2.1. *Hamatocanthoscypha rotundispora* Raitv. & R. Galán (*Hyaloscyphaceae*, *Helotiales*)

The genus *Uncinia* Velen. was introduced by Velenovský (1934) for a group of species different from *Hyaloscypha* Boud. by fleshy apothecia having thin-walled, septate, basally slender and curved hairs. Since no type was designated, Raitviir (1970) in his treatement of the genus *Uncinia* selected *U. laricionis* Velen. as lectotype and connected the genus with tiny hyaloscyphaceous species having cylindrical, tapering but not obtuse and curved / hooked hairs. *Uncinia* Velen. is a later homonym of *Uncinia* Pers. and therefore the generic name *Unciniella* K. Holm & L. Holm was proposed (Holm & Holm 1977). In the same year, but five months earlier than work of Holms appeared, Svrček (1977) established the genus *Hamatocanthoscypha* and *Unciniella* thus falled into the synonymy of *Hamatocanthoscypha*. Svrček (1977) circumscribed *Hamatocanthoscypha* to embrace small, sessile to shortly stipitate apothecia with short hairs. The hairs were characterized as being tapering to narrowly cylindrical, clearly curved to hooked, thin-walled, smooth, and aseptate to 3-septate. The paraphyses were described as not protruding above the ascal tips and not hooked. According to Raitviir (2004), the genus contains 13 species living as saprotrophs on forest litter and strongly decayed wood in the northern hemisphere (Asia, Europe, North America and Cuba).

A *Hamatocanthoscypha* species was collected in the Mediterranean region of Croatia on fallen *Juniperus phoenicea* twigs beset with leaves in litter. After its study in both living and dead state and comparison to the relevant literature (Huhtinen 1990, Galán & Raitviir 1994, Raitviir 2004) it seemed that this collection did not represent any of the known *Hamatocanthoscypha* species. The

only species with similar spore dimensions and nonamyloid asci arising from croziers was *H. rotundispora* Raitv. & R. Galán. However, after re-examination of the type material our collection appeared to fit quite well *H. rotundispora*. The protologue gives erroneously broadly ellipsoid to subglobose spore shapes with a too narrowly defined dimensional range for this species. In this thesis, a detailed re-description of *H. rotundispora* is provided. It is based on data obtained from the type material as well as recently collected material in both living and dead states from several European localities.

2.2.2. *Thecotheus platyapiculatus* nom. prov. (*Ascobolaceae*, *Pezizales*)

The genus *Thecotheus* was erected by Boudier (1869) for only one species *Thecotheus pelletieri* (P. Crouan & H. Crouan) Boud. having 32-spored asci. Subsequent studies of numerous authors working with fimicolous "discomycetes" have firmly established that *Thecotheus* is a valid genus, containing both multispored and 8-spored species (i.e. Chenantais 1918; Le Gal 1960, 1963; Kimbrough 1966, 1969; Pfister 1972, 1981; Aas 1992). Asci in this genus are diffusely blueing in iodine solutions, ascospores are thick-walled, hyaline and can possess cyanophilic markings and apiculi. There are currently 22 species recognized in this primary fimicolous genus of cosmopolitan distribution (Aas 1992, Gené *et al*. 1993, Wang & Kimbrough 1993, Wang 1994, Nagao *et al*. 2003, Doveri & Coué 2008). Among domestic animals most common substrate is a dung of a cow, horse, sheep and goat (Kimbrough 1969, Aas 1992), and red deer and roe deer within wild animals (Aas 1992). Horse dung is reported as a substrate for seven *Thecotheus* species. However, four non-fimicolous species occur on herbaceous, lignicolous or terricolous substrata (*Thecotheus pallens* (Boud.) Kimbr., *T. phycophilus* Pfister, *T. rivicola* (Vacek) Kimbr. & Pfister and *T. urinamans* Nagao, Udagawa & Bougher).

During systematic research on fimicolous fungi in the northern part of the Mt. Velebit, a new species of *Thecotheus* was revealed. It is presented in this thesis as *T. platyapiculatus* I. Kušan & Matočec, *nomen provisorium*. Taxonomic characters are studied in detail on both living and dead material. The most important differential characters of the new species related to apiculi, spore symmetry and ascal strangulation are discussed and compared to the most similar *T. keithii* (W. Phillips) Aas. Guidelines to the standardised procedure of treating *Thecotheus* collections in order to efficiently characterise and differentiate species in the genus are recommended.

2.2.3. *Tricharina tophiseda* nom. prov., *T. japonica* Chin S. Yang & Korf and *Ascorhizoctonia* Chin S. Yang & Korf emend. prov. (*Pyronemataceae*, *Pezizales*)

The genus *Tricharina* Eckblad (1968) was established to replace *Tricharia* Boudier (1885), which was a homonym of *Tricharia* Fée (1824) (a genus of lichenised fungi) and therefore illegitimate. *Tricharina* was later emended and monographed by Yang & Korf (1985a), who recognized 12 taxa in this genus, but transferred several species to the new genus *Wilcoxina* Chin S. Yang & Korf (1985a) based on differences in characters of apothecial morphologies, mycelial growth in pure culture, anamorphs and life strategies. Since then, only two new combinations have been added to *Tricharina*: *T. flava* (Fuckel) J. Moravec by Moravec (1990) and *T. herinkii* (Svrček) Benkert by Benkert (2010). The genus *Tricharina* is pleomorphic, possessing *Tricharina* apothecial teleomorphic states, and sporodochioid anamorphic states described as *Ascorhizoctonia* Chin S. Yang & Korf by Yang & Korf (1985b). Recent molecular phylogenetic studies (Egger 1996, Stielow *et al.* 2013, Hansen *et al*. 2013, Van Vooren *et al*. 2015) have shown that this small genus is apparently polyphyletic, and thus in need of thorough taxonomic revision.

In the first systematic research of fungal biodiversity on the territory of Krka National Park (Croatia) during 2007 and 2008, an effort was put into exploring both non-forest terrestrial and aquatic habitats. River Krka is a natural fluviokarstic phenomenon with preserved large travertine waterfalls (tufa barriers) containing various microhabitats. These habitats are rich in ascomycetous fungi, including a *Tricharina* species discovered twice in 2008 growing on tufa barriers (a novel substrate for this genus). Microscopical observations revealed that two *Tricharina* collections were conspecific and similar, but not identical, to descriptions of *Tricharina japonica* Chin S. Yang & Korf (1985a), and were therefore considered to be a species new to science. The holotype of *T. japonica* was re-examined and studied in detail, together with a recent European collection, to clarify differences between these species. Additional tests with pure cultures were also conducted to investigate whether an ascorhizoctonia-type anamorph similar to those reported for other *Tricharina* species was produced (Yang & Korf 1985b, 1985a; Yang & Kristiansen 1989, Barrera & Romero 2001). The examination of the holotype of *T. japonica* (along with the recent European collection) showed that the new *Tricharina* species differs in several important aspects. The proposed new species is presented in this thesis as *Tricharina tophiseda* Matočec & I. Kušan, *nomen provisorium*.

Following the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress in Melbourne, Australia, July 2011 (McNeill & Turland 2011, McNeill *et al*. 2012), anamorphic genus *Ascorhizoctonia* should fall into the synonymy with *Tricharina* because it is a younger name (Yang & Korf 1985b) for teleomorphs described within the genus *Tricharina*. Since *Ascorhizoctonia* is typified with *A. praecox* Chin S. Yang & Korf and *Tricharina* with *T. gilva* (Boud.) Eckblad, the genus *Ascorhizoctonia* can be remodelled and used in a restricted sense for *Tricharina praecox* which is consistently positioned at a distance from the core *Tricharina* clade comprising *T. gilva* and other *Tricharina* species in a number of recent phylogenetic studies involving *Tricharina* (e.g. Egger 1996, Wei *et al*. 2010, Hansen *et al*. 2013, Stielow *et al*. 2013, Van Vooren *et al*. 2015). Therefore, it is proposed in this thesis to emend the anamorphic genus *Ascorhizoctonia* to include only two species, *A. praecox* and closely related *A. intermedia* Egger, Chin S. Yang & Korf.

3. MATERIALS AND METHODS

3.1. Fieldwork

In the frame of biodiversity research of ascomycetous fungi from Alpine to Adriatic area, several fieldwork sessions produced collections that were selected for this thesis. These are: (1) research of fimicolous (i.e. coprophilous) mycobiota on excrements of wild animals and horses in the Sjeverni Velebit National Park, Croatia, (2) research of both non-forest terrestrial and aquatic habitats, especially large travertine waterfalls (tufa barriers) that contain various microhabitats, in the Krka National Park, Croatia, and (3) research of the sclerophyllous evergreen Mediterranean vegetation on the island of Korčula, Croatia.

The standard fieldwork procedure included different elements as follows:

1. searching for suitable substrates in the targeted specific microhabitats,
2. spotting the fruitbodies on the substrate,
3. making macrophotographs of the whole fructification (assemblage of fruitbodies) *in situ*, as well as of fruitbody details when neccesary,
4. collecting and labeling suitable fruitbodies that constitute an individual collection (representative of the presumed individuum), together with a part of a substrate or with entire substratal unit(s) in a plastic box in a way to avoid dehydration, drying, mechanical damage and deterioration,
5. recording basic ecological data related to each collection and a general habitat type at the sampling site,
6. recording of geographical position using a GPS device and
7. storing collected material in a living state in a cool (approx. 4–8 ºC) and humid environment in a way to prevent any mechanical tissue damage at all time until microscopical examination takes place (within at most week or two, depending on the species).

3.2. Macroscopy and microscopy

Before preparing microscopical sections, all collected fruitbodies undergo the macroscopical treatment which includes measurements, meristics and textual description of gross morphology. Microscopic characters based on living cells and tissues were recorded using "vital taxonomy" methods (Baral 1992) while those based on dead cells and tissues were obtained both from fixed fresh material and rehydrated dried specimens. Microscopic structures were observed in tap water (H_2O), and also in potassium hydroxide (KOH) as 5% (wt/wt) aqueous solution; Lugol's solution (IKI) after Baral (1987); Brilliant Cresyl Blue (CRB) aqueous solution (Baral 1992); Congo Red (CR) aqueous solution (Pfister *et al*. 2009); and Cotton Blue (CB) lactic acid solution (Erb & Matheis 1983), all applied individually to water mounted sections. Acetocarmine (AC) for staining nuclei (Harmaja 1974b) and Melzer's reagent (MLZ) for testing dextrinoidity (Huhtinen 1990) were applied to sections pretreated with KOH. *Hamatocanthoscypha rotundispora* was observed only in H_2O, KOH, CRB and MLZ. Its air dried apothecia were rehydrated by spraying with a water mist, and were subsequently examined in H_2O and KOH.

Microscopic features were observed with transmission light microscopes (bright field) under magnification up to 1000×. All described microscopic elements were observed in H_2O unless otherwise stated. Drawings were made free hand to scale and microphotographs were taken with a camera mounted directly on the microscope. Qualitative and quantitative characters were based on apothecial texture and hymenial elements of a minimum of two ascomata in each collection. In the case of the proposed new *Thecotheus* species ascospore measurements do not include apiculi. Randomly selected ascospores (freshly ejected from asci in living material) were measured directly using an ocular micrometer scale. Spore measurements are based on samples of 25–100 (depending on the given collection) fully mature and randomly selected ascospores (in a living material ejected from asci). In the descriptions of the proposed new *Thecotheus* and *Tricharina* species and *Tricharina japonica* length, width and length/width ratio ("Q" value) are given as: (min.) stat. min. – arith. mean – stat. max. (max.) where "min." = minimum (lowest measured value), "stat. min." = statistical minimum (arithmetic mean minus two times standard deviation), arith. mean = arithmetic mean, "stat. max." = statistical maximum (arithmetic mean plus two times standard deviation), "max." = maximum (highest measured value). Standard deviations (StD) of spore length and width are also given. Spore measurements (length, width) and "Q" value in the re-description of

Hamatocanthoscypha rotundispora are given as minimal and maximal values. Spore wall layers are described following Heim (1962) except for the exospore which is here termed as perispore after Harmaja (1974a). Colour descriptions are based on Kornerup & Wanscher (1967) with the colour code(s) in parentheses.

3.3. Pure cultures

Ascospore germination of the proposed new *Thecotheus* species was tested on dung agar (DA) which was prepared according to Conway (1975) using 100% horse dung and dung-oatmeal agar (DOA) prepared after Benedict & Tyler (1962). Both media were acidified to pH ca. 5 by adding sterilized 10% (wt/wt) aqueous solution of tartaric acid after agar sterilization. Three years old apothecia (CNF 2/8950) dried at the room temperature were checked for living ascospores by evaluating the refractivity and overall agglutination of sporoplasm to spore wall in H_2O and additional dying in CRB (homogenously/slightly stained sporoplasm) and IKI (nuclear structures visibility). Before inoculation on DA and DOA each apothecial fragment was briefly rehydrated in sterile tap water and then pretreated in hydrochloric acid (pH=3.3; 0.0005 M HCl) for two hours at 25 °C and 60 °C and tap water for two hours at 25 °C and 60 °C. Additionally, two apothecial fragments were placed directly on DA in prepared minute depressions containing 2% (wt/wt) aqueous solution of KOH for four hours at 25 °C and 60 °C (Conway 1975). All apothecial fragments were placed with the hymenium down to achieve spore contact with agar and then incubated at 24 °C.

Petri dishes (90 mm diam.) containing Czapek yeast autolysate (CYA) agar were inverted and exposed to living apothecia of the proposed new *Tricharina* species for two hours to allow inoculation by freshly discharged spores. After six days, colonies from germinating ascospores were transferred to axenic culture on malt extract agar (MEA) using aseptic technique. Both media were prepared according to Frisvad & Samson (2004). Cultures were grown in the dark at 20°C (±1 °C) for one week. Petri dishes were observed for germination using a stereomicroscope at magnification up to 64×, and anamorphic structures produced in both media were analysed in the same manner as apothecial structures. Additionally, six year old apothecia of the proposed new *Tricharina* species (CNF 2/8079) and three year old apothecia of *T. japonica* (R.D. 31.01.245.11) were tested for spore

germination, cultural characters and anamorph production after Yang & Korf (1985b), modified by using 10% (wt/wt) tartaric acid instead of antibiotics to suppress bacterial growth.

3.4. Spore shape nomenclature

Regarding the common basic discrepancies between the protologues, other species descriptions and my own observations of spore shapes in a number of cases, I propose here a standardised tool for determining the ascospore shape - revised and supplemented mathematical delimitation of radially symmetric spore shapes by relying on Bas (1969), with the addition of categories with higher elongation factor as a standard when describing collections and species (Table 2, 3). In cases of more complex spore shapes (heteropolar and/or bilaterally or biradially symmetric spores) it is necessary to precisely define spore shape regarding its polarity, symmetry and different symmetry planes (Domínguez de Toledo 1994) (Table 3). Homopolar bilateral symmetric spores and spores with complex shapes are not treated here, whilst biradial symmetric spore projections may be recognized here by separately viewing the two planes of symmetry (Table 3). In spore shape naming I have adopted all adequate available names and introduced new additional names to cover remaining spore shapes (Largent *et al.* 1977, Royal Botanic Gardens Kew 2013). For the “ellipsoid” spore shape I have taken an ellipse as a projection whose distance between two focal points (the foci) is equal to minor axis. Spore shapes in this thesis are named after this newly proposed standard.

TABLE 2. Geometric criteria for defining the simple spore shape regarding the Q value, polarity and symmetry in prolate straight symmetric homopolar (isopolar) spores derived from Bas (1969) and Domínguez de Toledo (1994). Drawings represent spores with ± mean Q value within each group. Del. N. Matočec.

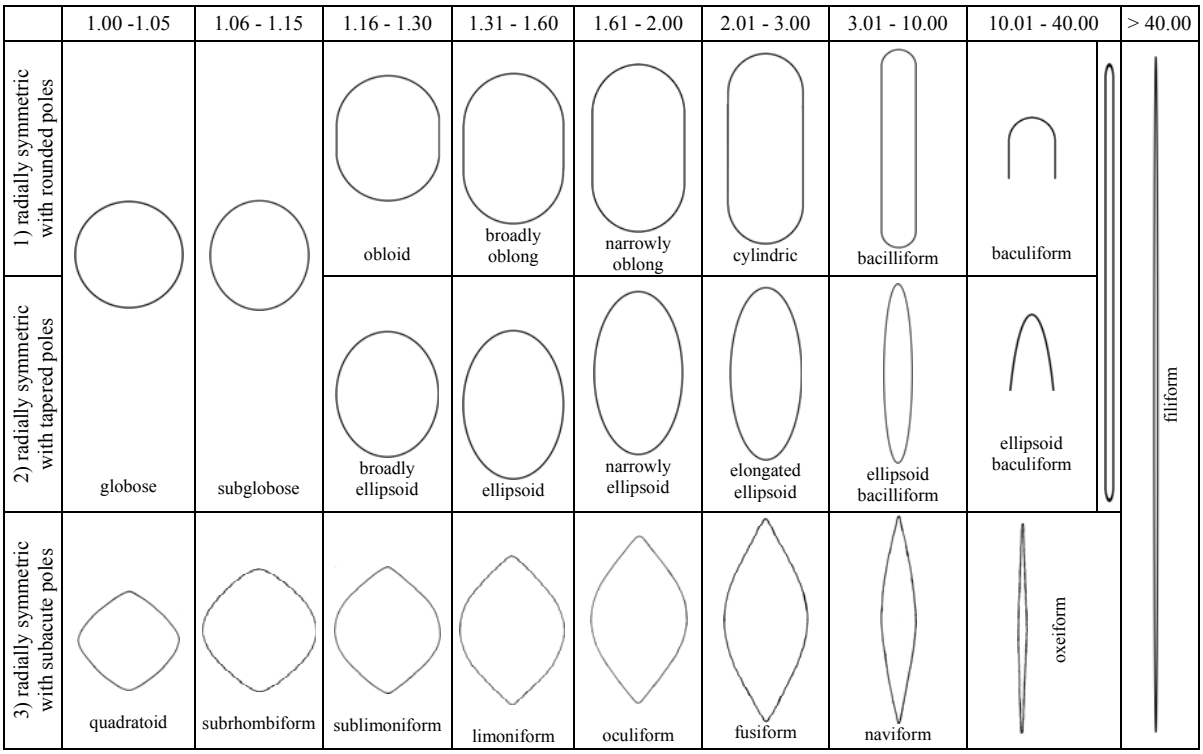
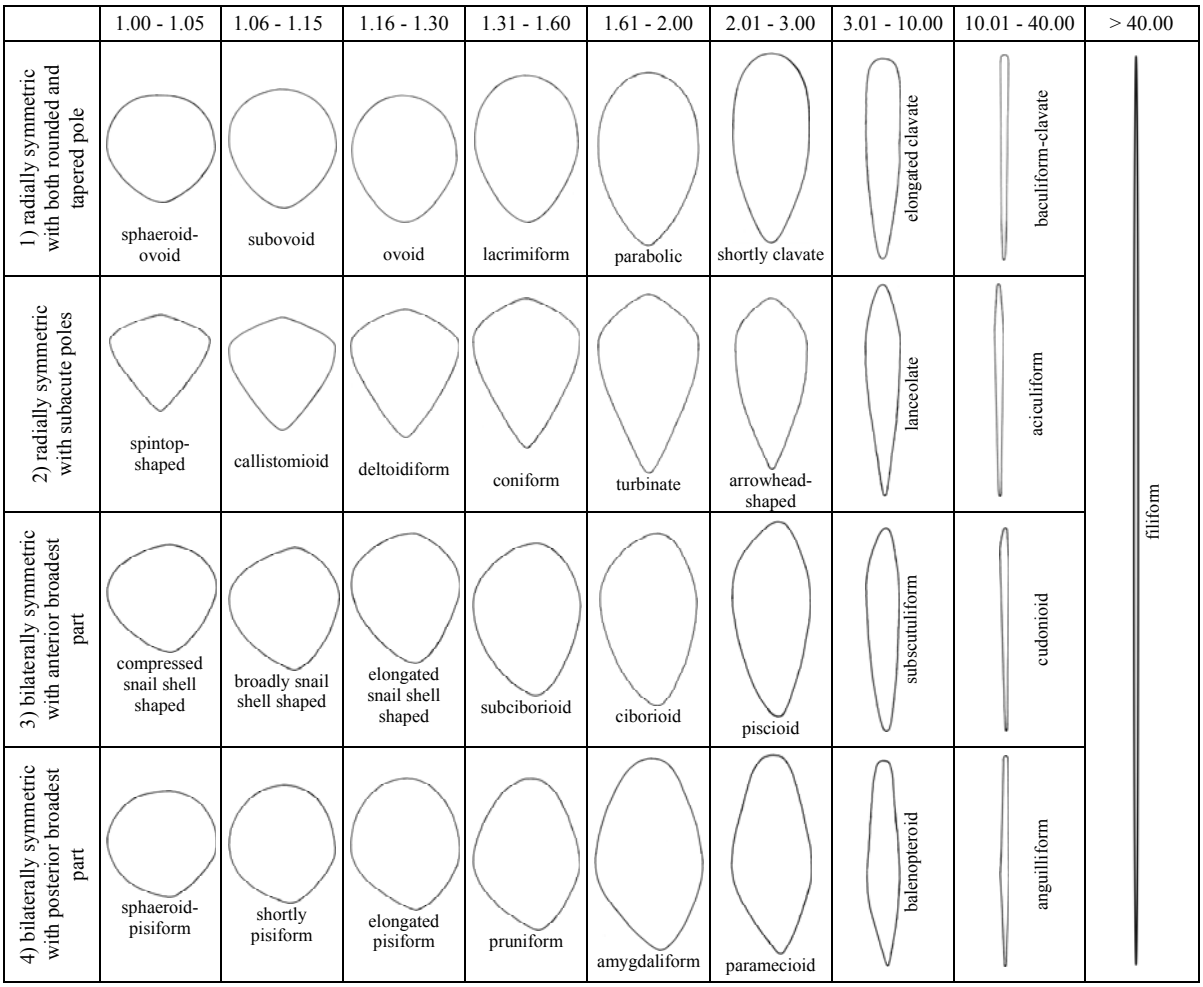


TABLE 3. Geometric criteria for defining the simple spore shape regarding the Q value, polarity and symmetry in prolate straight symmetric heteropolar spores derived from Bas (1969) and Domínguez de Toledo (1994). Drawings represent spores with ± mean Q value within each group. Del. N. Matočec.



3.5. Ascospore morphometry analysis

In the case of *Hamatocanthoscypha rotundispora*, ascospore morphometry analysis was performed. Spore length and width were measured on six independent statistical samples (each of 50 spores) as follows: (1) CNF 2/8847 and (2) CNF 2/9110 both in living state (tap water) and freshly fixed with KOH, (3) CNF 2/8889 and (4) AH 6696 rehydrated from the exsiccata with KOH. Length and width values were used for calculation of Q value and volume (according to a formula for a

perfect ellipsoid body where the third axis is equal to width) of each particular ascospore. Descriptive statistics were calculated and a test for normality (Shapiro & Wilk 1965) was performed for each of four morphometrical variables (L, W, Q and V) and for each of six statistical samples separately. Finally, non-parametrical Mann-Whitney test (Lehmann 2006) was used for pairwise comparisons of: (1) statistical samples on living and dead spores of the same collection (in order to estimate influence of shrinkage to ascospore morphometry), (2) statistical samples on living spores of different collections (in order to estimate intraspecific ascospore morphometrical variability using "vital taxonomy") and (3) statistical samples obtained on dead spores of different collections (in order to estimate intraspecific variability based on fixed material).

3.6. Material examined

All material collected during this study as well as collections of the newly proposed taxa from the genera *Thecotheus* and *Tricharina* are deposited in the Croatian National Fungarium (CNF), Zagreb, Croatia. Detailed data about all loaned and examined collections are listed in the Results chapter, below taxa descriptions.

Additional material for taxonomic analyses was loaned from institutional collections (herbaria/fungaria) and private fungaria. Type material of *Hamatocanthoscypha rotundispora* was loaned from Universidad de Alcalá Herbario (AH) and the type of *Tricharina japonica* from Cornell University Plant Pathology Herbarium (CUP). Private fungaria of Michel Hairaud (M.H.) and Brigitte Capoen (B.C.) were consulted for French specimens of *Hamatocanthoscypha rotundispora* and René Dougoud's private fungarium (R.D.) for the only known collection of *Tricharina japonica* beside the type collection. Duplicates of all French collections of *Hamatocanthoscypha rotundispora* are deposited in CNF.

3.7. Abbreviations and symbols

* = living state of cell/tissue

† = dead state of cell/tissue

AC = Acetocarmine

AH = Universidad de Alcalá Herbario, Alcalá de Henares, Madrid, Spain

BSG = bipolar spore granules

CB = Cotton blue lactic solution

CMA = corn meal agar

CNF = Croatian National Fungarium, Croatian Mycological Society, Zagreb, Croatia

CR = Congo red aqueous solution

CRB = Brilliant cresyl blue aqueous solution

CRB-KOH = KOH added to CRB mount

CUP = Cornell University Plant Pathology Herbarium, Ithaca, New York, USA

CYA = Czapek yeast autolysate agar

DBB = de Bary bubble

ES = Spain

FR = France

HR = Croatia

H_2O = tap water

IKI = Lugol's solution

KOH = potassium hydroxide aqueous solution

L = ascospore length

LB = lipid body

MC = metachromatic corpuscle

MEA = malt extract agar

MLZ = Melzer's reagent

N/NO = nucleus/nucleolus

Q = ascospore length/width ratio

RBI = refractive bodies content index

RCG = refractive cytoplasmic globule

SCB = KOH-soluble cytoplasmic body

SVB = semi-resistant vacuolar body

V = ascospore volume

VB = vacuolar body

W = ascospore width

4. RESULTS

4.1. Taxonomical considerations on *Hamatocanthoscypha rotundispora*

4.1.1. Re-description

Hamatocanthoscypha rotundispora **Raitv. & R. Galán in Galán & Raitviir (1994: 459)**
Figs. 6–8

Ascomata apothecial, superficial, solitary, gregarious, cup-shaped, stipitate. When fresh, disc 0.1–0.8 mm, *in statu sicco* up to 0.3 mm in diameter, concave to plane, white (1A1), stipe cylindrical, 0.1–0.3 mm high, up to 0.1 mm wide, base greyish yellow (4B4). Hymenium white (1A1), smooth, unwrinkled, normally not translucent. Margin sharp, slightly elevated above hymenium, with dense white (1A1) shortly protruding hairs, ground white (1A1). Excipular and stipe surface concolourous; very sparsely beset with shorter hairs. Subiculum, associated asexual states or stromatic tissues not observed.

Hymenial elements: **Hymenium** [*]38–45 µm, [†]33–42 µm thick. **Asci** [*]30–43.2 × 4.9–5.8 µm, [†]26–39.5 × 3.7–5.4 µm, [*]asci protruding above paraphyses for 4.8–9.1 µm, *pars sporifera* [*]10–14.3(20.2) µm, 8-spored, spore arrangement biseriate in living but uniseriate in dead asci, cylindric-subventricose, with conical-truncate apex and ± truncate base, apex seemingly without apical ring and barely thickened in [*]asci, but considerably thickened and with faintly visible apical ring in [†]IKI, arising from croziers, often 2–4 asci arise from a single crozier cell, in [*/†]IKI completely inamyloid, in [*]CRB walls not stained. **Ascospores** [*]3.3–5.2(5.7) × (1.9)2–3(3.2) µm (n = 150), [†](2.2)2.4–4(4.2) × (1.1)1.4–2.3(2.6) µm (n = 200), descriptive statistics of ascospore morphometrical variables (L, W, Q and V) is given in Table 3; subciborioid to ciborioid or rarely amygdaliform in lateral (side) view and limoniform to oculiform in dorsiventral (front) view, also fusiform and piscioid in dead spores, bilateral symmetric and slightly heteropolar, 1-celled, wall hyaline and strongly refractive, 0.2–0.3 µm thick, smooth; containing 1–3(5) LBs that are positioned eccentric or near poles, [*]0.2–0.8 µm in diam., rarely eguttulate, after ejection spores are aggregated together and each enveloped by a delicate sheath; in [*]CRB with one to a few globose greyish violet (19C7) MCs, [*]0.3–2 µm in diam. that are dissolving in [†]CRB-KOH; in [*]CRB sporoplasm and walls not stained, in [*]IKI sporoplasm light yellow (3A5) to

yellow (2A7), without glycogen accumulations. **Paraphyses** cylindric-obtuse to subclavate, sparse, simple, not branching, hyaline, sometimes with thin, hyaline, moderately refractive exudate in apical part, apical cells *15–35 × 2–3.2 µm, †1.4–2.1 µm wide, each apical cell with a single cylindrical, hyaline, very slightly refractive VB which is either completely absent in dead cells or present as irregular remnants; in *CRB VBs turquoise (24A8), but instantly vanish in †CRB-KOH; in *IKI cytoplasm not notably stained. **Subhymenium** scarcely distinguishable from medulla, composed of hyaline, thin-walled hyphae and ascogenous cells, layer *7–11.8 µm, †4–6 µm thick.

Margin: **submarginal tissue** composed of hyaline *textura prismatica*, cells ± parallel to surface, *7.6–13.2 × 2–2.6 µm, thin-walled, not gelatinised, outer cells of margin abundantly beset with **hairs**, these strongly curved, uncinate to helical, partly intertwined, with outwardly bent tips, tapering toward apex but not pointed, 2–3(4)-celled, *28–55 × 2–4.1 µm, at apex *0.9–1.6 µm wide, †(20)37–56(62) × 2.9–4.1 µm, at apex †0.9–1.3(1.5) µm wide, base sometimes swollen at lower positioned hairs, basal cells occasionally with hyaline and moderately refractive RCGs, apical cell wall rather sparsely covered with flat to granular, hyaline, highly refractive exudate; exudate in *CRB turquoise (24A8), in †CRB-KOH stable and becoming light lilac (15A5), RCGs in *CRB greyish violet (19C7), disappearing in †CRB-KOH; in */†IKI exudate pastel yellow to light yellow (3A4-3A5) or rarely reddish orange (7B8) and cytoplasm granular yellow (2A6); in *IKI RCGs highly contrasted, in MLZ exudate not stained and partly dissolved.

Excipular flank: **Medullary excipulum** reduced, hyaline, layer *6.2–9 µm, †5–8(12) µm thick, composed of sparse, narrow, cylindrical hyphae running parallel to receptacle surface (*textura porrecta-prismatica*), at apothecial centre *textura porrecta-intricata*, hyphae *(0.9)1.4–2.7 µm, †1.1–2.2 µm wide, with thin and not gelatinised walls; *IKI-, *CRB-, †MLZ-. **Ectal excipulum** composed of hyaline *textura prismatica* with cells oriented parallel to the surface, layer *8.2–12.5 µm, †6–10 µm thick at middle flank, cells *5.3–14.1 × 2.4–6.5 µm, †5.3–16.5 × 1.7–6 µm, ±thin-walled, not gelatinised, some outermost cells contain sparse LBs but more often RCGs that are hyaline and of moderate to high refractivity; in *CRB cytoplasm greyish blue (20B4), cell walls not stained, RCGs greyish violet (19C7) and instantly vanishing in †CRB-KOH, LBs stable; in *IKI RCGs and LBs not stained. Outermost cells give rise to hyaline hairs from upper flank to the stipe, 1(2)-celled, *5–26 × 2.2–3.7 µm, at apices *1.1–2.5 µm wide, †5.1–23.4(33) × 2–4.1(5) µm, at apices †0.9–2 µm wide, thin-walled, smooth or faintly encrusted, bent outwards, apex rounded to slightly tapered, not pointed,

shorter and less bent than marginal hairs, forming pustulate aggregations (2–5 hairs per aggregation); *IKI-, *CRB-, †MLZ-.

Stipe composed of a medullar (central) and an ectal layer and of a histologically differentiated basal part. Medullar part hyaline, thin-walled *textura porrecta*, hyphae ±vertically oriented, †1.5–2.1 µm wide. Ectal layer hyaline, thin-walled *textura prismatica*, †6–9 µm thick, cells oriented at a very high angle to the stipe surface, †1.8–3.5 µm wide, with sparse RCGs; outermost cells giving rise to hyaline hairs, 1(2)-celled, †7.1–22.6 × 2–3.5 µm, at apices †1.6–2.1 µm wide, cylindric-obtuse to very slightly tapered, apices curved to uncinate and bent downwards, with very sparse, granular, hyaline, moderately refractive exudate on their surface. Basal part of densely packed, thick-walled, pigmented *textura prismatica-angularis*, this part *40–65 × 70–80 µm, cells ±vertically oriented, prismatic cells *6–8.8 × 2–3.5 µm, more isodiametric cells *4.2–5.1 µm wide, walls glassy, light yellow (2A5), highly refractive, not gelatinised, *cytoplasm subhyaline; surface of basal part covered with scanty greyish yellow (4B4) cylindrical basal hyphae, individual cells †5–10.1 × 2.3–4.8 µm, moderately thick-walled, wall †0.3–0.6 µm thick.

Material examined:

—CROATIA. Dalmatia: Island of Korčula, SE from Lumbarda, Pržina cove, elev. 8 m, 42°55'06" N, 17°11'13" E, habitat: degraded thermo-mediterranean fully evergreen maquis on sandy soil with *Juniperus phoenicea*, *Pistacia lentiscus*, *Olea sylvestris*, *Smilax aspera*, *Arundo donax*, *Agave americana* and *Olea europaea*, substrate: rotten twigs and leaves of *Juniperus phoenicea* in litter, associated with *Pithya cupressina* (Batsch) Fuckel, 31 December 2010, *leg.* M. Vojinović (CNF 2/8847).

—FRANCE. Poitou-Charentes: Charente-Maritime, Island of Ré, Forêt Domaniale de la Combe à l'Eau (Ars-en-Ré), elev. 7 m, 46°12'55" N, 1°32'14" E, habitat: 25 years old plantation of *Cupressus macrocarpa* on sandy ground, substrate: *C. macrocarpa* leaves and branchlets still attached to cut branches lying on ground, 2 collections: (1) associated with *Chloroscypha alutipes* (W. Phillips) Dennis and *Boubovia ascoboloides* (Korf & W.Y. Zhuang) Y.J. Yao & Spooner, 10 December 2011, *leg.* M. Hairaud (M.H. 071211), duplex (CNF 2/9110) and (2) associated with *Cistella acuum* (Alb. & Schwein.) Svrček, *C. alutipes* and *B. ascoboloides*, 31 December 2011, *leg.* M. Hairaud (M.H. 231111), duplex (CNF 2/9113).

—FRANCE. Bretagne: Côtes d'Armor, Tourony (Trégastel), elev. 4 m, 48°49'34" N, 3°29'36" E, habitat: public park, substrate: branches of *C. macrocarpa* lying on soil, 13 November 2009, ***leg.*** B. Capoen (B.C. 141109), duplex (CNF 2/8889).

—SPAIN. Guadalajara: Tamajón, Ermita de los Enebrales, on fallen decaying leaves of *Juniperus thurifera*, 3 December 1991, ***leg.*** R. Galán & A. Raitviir (AH 6696 = R.G. 6696, isotype, designated by Galán & Raitviir 1994).

Notes: The protologue of *H. rotundispora* (Galán & Raitviir 1994) designates TAA-136197 as holotype and R.G. 6696 [= AH 6696] as isotype, although on the exsiccata label that I received from AH, R.G. 6696 is clearly indicated as holotype, with an isotype deposited in Ain Raitviir's herbarium (A.R., in TAA). I was informed by Ilmi Parmasto (*pers. comm.*) that the specimen TAA-136197 is inaccessible / misplaced. Therefore I rely here on the study of the isotype material which consists of about 30 mature apothecia and also includes a slide containing a completely dried section.

—SPAIN. Navarra, Rada, Laguna de Rada, elev. 365 m, 42º18'09" N, 1º34'52' W, substrate: on rotten leaves of *Cupressus* sp., 6 January 2013, ***leg.*** M. Tapia (ERD-5751).

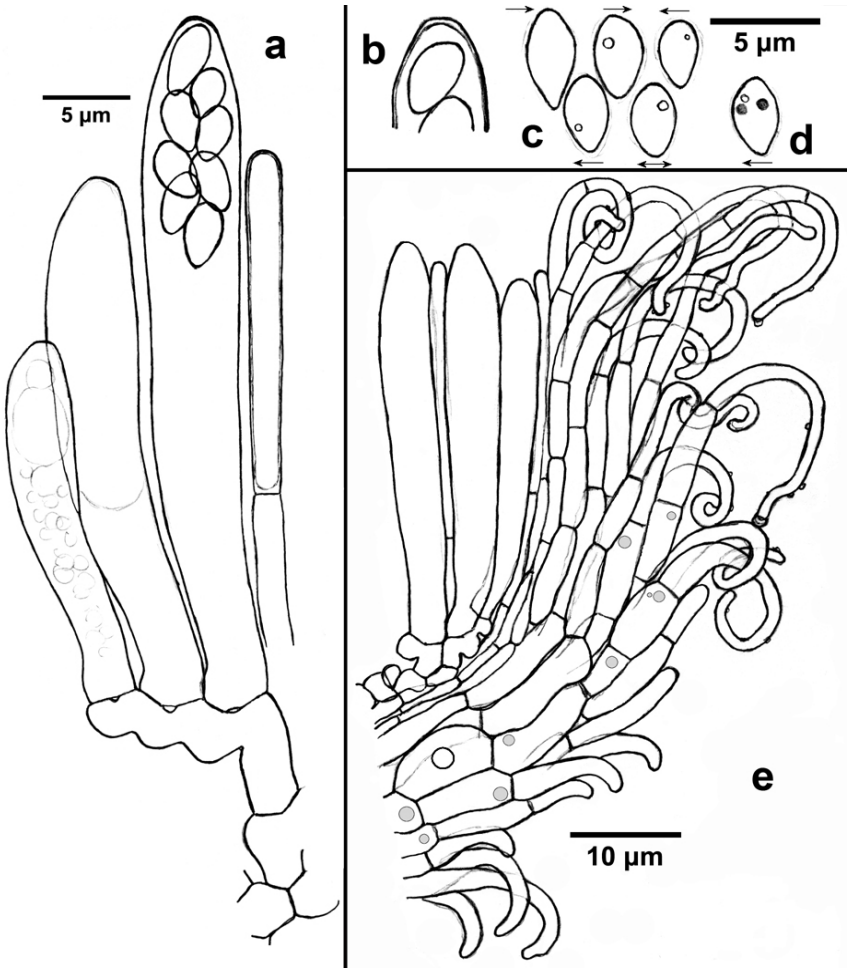


FIGURE 6. *Hamatocanthoscypha rotundispora* (CNF 2/8847): **a.** *Asci with a part of ascogenous system and paraphysis in H_2O. **b.** *Ascus tip in H_2O. **c.** *Ascospores in H_2O, some with LBs (transparent circle). **d.** *Ascospore in CRB, with LB (transparent circle) and MCs (shaded circles). **e.** *Apothecial tissues and hairs at margin, in H_2O, with LB (transparent circle) and RCGs (shaded circles). ↔ display dorsiventral (front) spore view, → display lateral (side) spore view (arrow directed towards dorsal side). Del. N. Matočec.

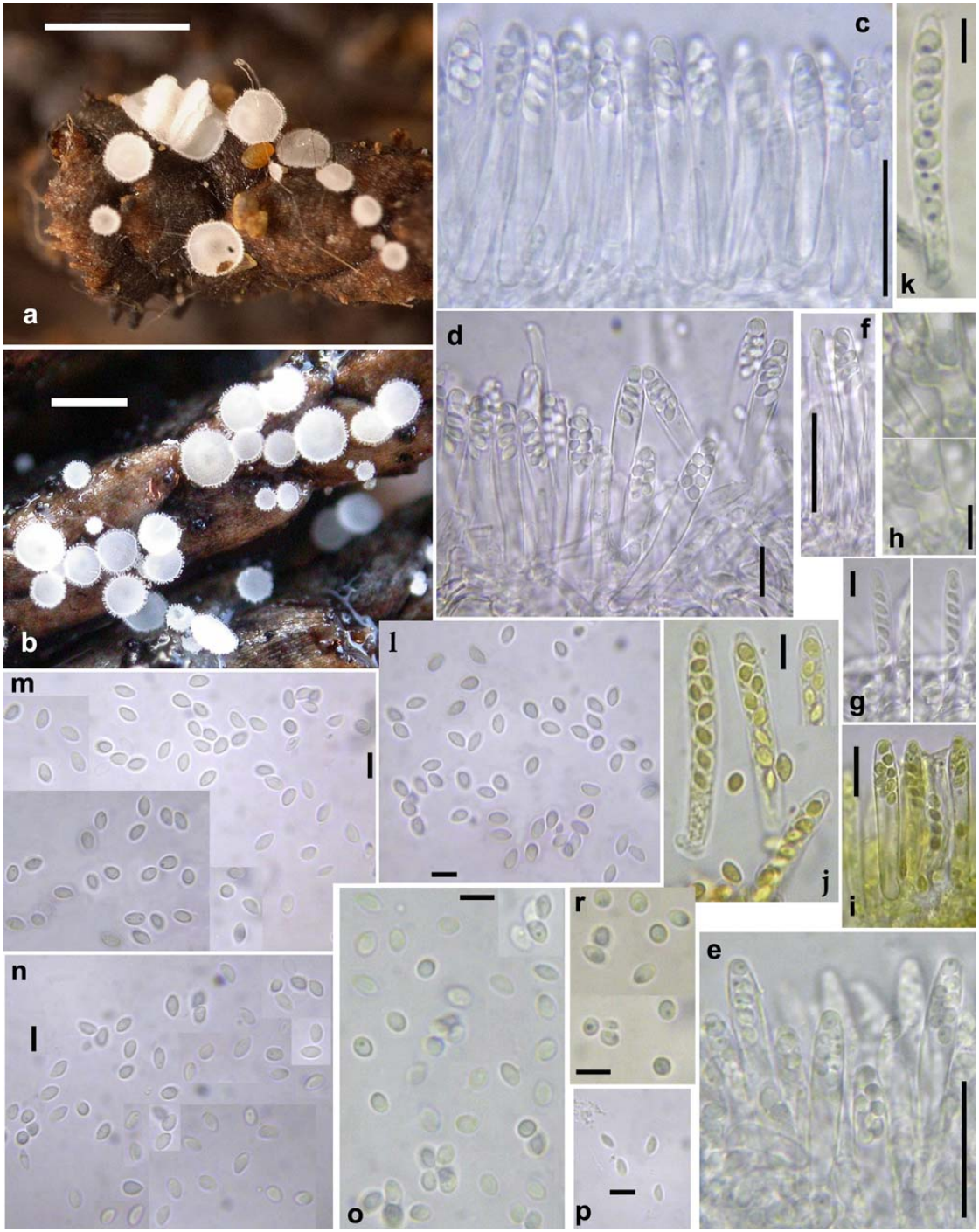


FIGURE 7. *Hamatocanthoscypha rotundispora*—apothecia and hymenial elements: **a–b.** Living apothecia. **c–e.** *Asci in H_2O. **f.** *Asci and paraphysis in H_2O. **g.** †Asci in KOH. **h.** *Croziers in H_2O. **i.** *Asci in IKI. **j.** †Asci in IKI. **k.** *Asci in CRB. **l–o.** *Ascospores in H_2O. **p.** †Ascospores in H_2O. **r.** *Ascospores in CRB. g and p from AH 6696 (holotype), a, c, e, h, j, k, o and r from CNF 2/8847; b from CNF 2/9110; d, f, i, l, m and n from CNF 2/9113. Scale bars: g, h, j–r = 5 µm; d, i = 10 µm; c,

e, f=20 µm; a=0.5 mm; b=1 mm. All phot. N. Matočec & I. Kušan except b which is taken by M. Hairaud.

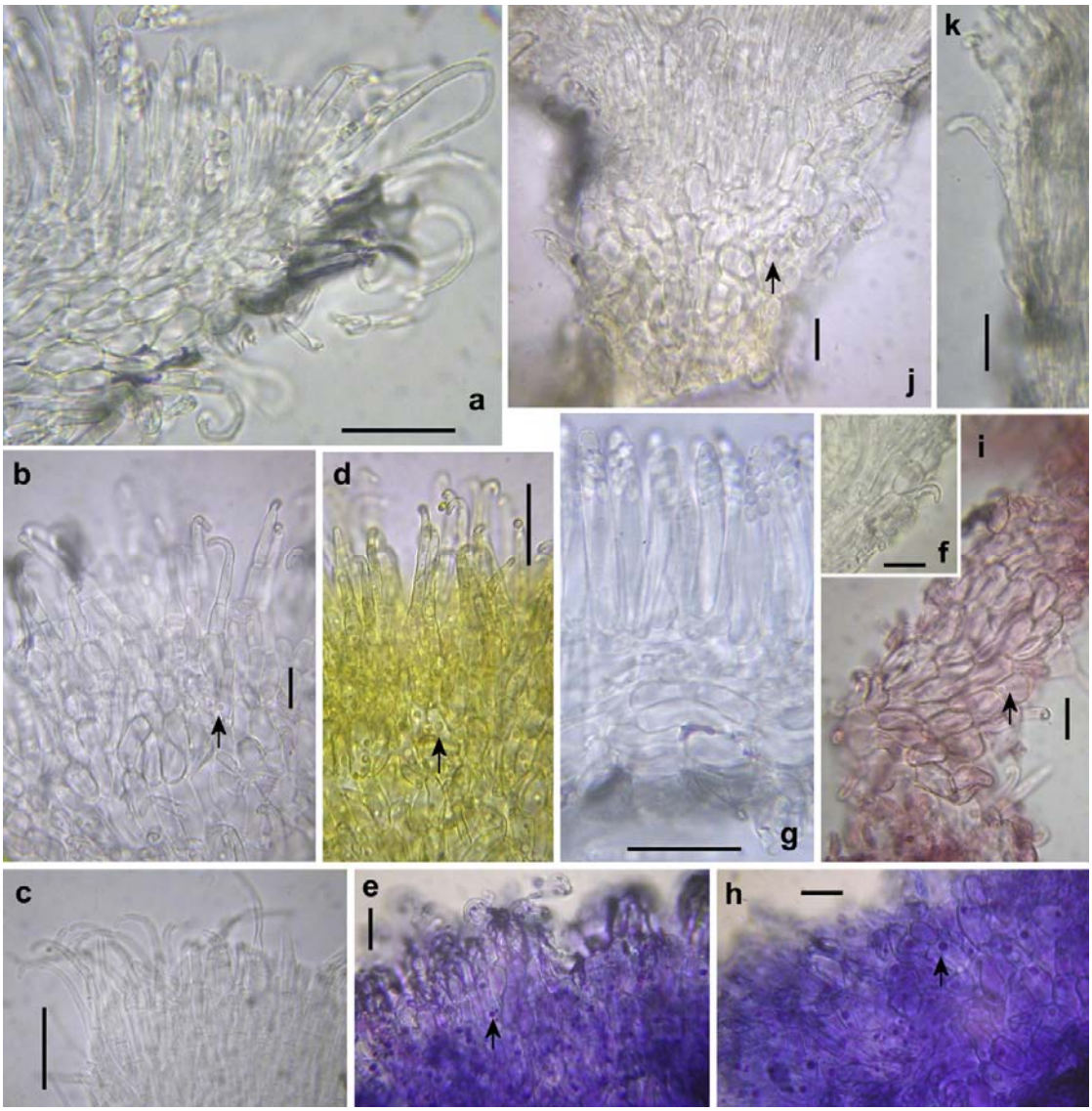


FIGURE 8. *Hamatocanthoscypha rotundispora*—sterile apothecial tissue: **a.** *Apothecial tissues and hairs in H_2O. **b.** *Marginal hairs in H_2O. **c.** †Marginal hairs in KOH. **d.** *Marginal hairs in IKI. **e.** *Marginal hairs in CRB. **f.** *Surface of excipular flank in H_2O. **g.** *Excipular flank with hymenium in H_2O. **h.** *Squashed ectal excipulum in CRB. **i.** Ectal excipulum in CRB-KOH. **j.** *Stipe basal part in H_2O. **k.** *Stipe surface in H_2O. Arrows indicate RCGs except in i where it shows LB. c from AH 6696 (holotype); a, g and k from CNF 2/8847; b, d–f and h–j from CNF 2/9113. Scale bars: b, e, f, h–k = 10 µm; a, c, d, g = 20 µm. Phot. N. Matočec & I. Kušan.

4.1.2. Statistical analysis of the ascospore morphometrical variables

Descriptive statistics for each of four morphometrical variables (L, W, Q and V) and for each of six statistical samples are given in Table 4 and Fig. 9. Results of the Shapiro-Wilk test suggest that significant deviation of normal distribution exists in all morphometrical variables and also in all statistical samples (although not in the each variable-sample combination). Consequently, it can be concluded that non-parametric statistics (median, quartiles) is more appropriate for description of morphometrical variables distributions than parametric statistics. According to this, a non-parametric Mann-Whitney test was used for statistical sample comparison (see Table 5).

TABLE 4. Descriptive statistics of *Hamatocanthoscypha rotundispora* ascospore morphometric variables (n = 50 for each of six statistical samples). StD—standard deviation; S-W and p(S-W)—Shapiro-Wilk statistics for normality and respective probability (bold denotes statistical significance at probability level of p = 0.05); Min, L_Quart, U_Quart and Max—minimum, lower quartile, upper quartile and maximum.

	Statistical sample	Mean	StD	S-W	p(S-W)	Median	Min	L_Quart	U_Quart	Max
L (µm)	HR(CNF 2/8847)*	4.086	0.331	0.940	**0.013**	4.00	3.50	3.80	4.30	4.90
	FR(CNF 2/9110)*	4.596	0.413	0.974	0.348	4.55	3.90	4.30	4.80	5.70
	HR(CNF 2/8847)†	3.490	0.349	0.948	**0.029**	3.50	2.90	3.20	3.80	4.10
	FR(CNF 2/9110)†	3.012	0.313	0.978	0.480	3.00	2.30	2.90	3.20	3.70
	FR(CNF 2/8889)†	3.014	0.390	0.962	0.106	3.00	2.20	2.80	3.20	4.20
	ES(AH 6696)†	3.162	0.326	0.972	0.271	3.20	2.50	2.90	3.30	3.80
W (µm)	HR(CNF 2/8847)*	2.434	0.211	0.914	**0.002**	2.40	2.10	2.30	2.60	2.80
	FR(CNF 2/9110)*	2.598	0.264	0.968	0.194	2.60	2.10	2.40	2.80	3.20
	HR(CNF 2/8847)†	2.072	0.162	0.930	**0.006**	2.10	1.80	2.00	2.20	2.60
	FR(CNF 2/9110)†	1.772	0.195	0.955	0.057	1.80	1.40	1.60	1.90	2.20
	FR(CNF 2/8889)†	1.768	0.255	0.973	0.318	1.80	1.10	1.60	1.90	2.30
	ES(AH 6696)†	1.866	0.160	0.896	**0.000**	1.80	1.60	1.80	2.00	2.30
Q (L / W)	HR(CNF 2/8847)*	1.687	0.154	0.966	0.165	1.69	1.39	1.60	1.76	2.00
	FR(CNF 2/9110)*	1.777	0.144	0.983	0.665	1.77	1.44	1.67	1.88	2.09
	HR(CNF 2/8847)†	1.690	0.175	0.970	0.230	1.67	1.39	1.57	1.79	2.17
	FR(CNF 2/9110)†	1.710	0.186	0.976	0.406	1.68	1.37	1.59	1.88	2.13
	FR(CNF 2/8889)†	1.728	0.259	0.946	**0.024**	1.68	1.29	1.53	1.87	2.50
	ES(AH 6696)†	1.702	0.195	0.941	**0.015**	1.67	1.33	1.56	1.78	2.25
V (μm^3)	HR(CNF 2/8847)*	12.830	2.852	0.943	**0.018**	12.36	8.54	10.52	14.86	20.10
	FR(CNF 2/9110)*	16.587	4.495	0.941	**0.014**	15.04	9.23	13.29	19.28	26.15
	HR(CNF 2/8847)†	7.931	1.691	0.941	**0.015**	8.02	4.92	6.91	9.12	14.15
	FR(CNF 2/9110)†	5.067	1.483	0.953	**0.047**	4.88	2.71	4.02	6.05	8.87
	FR(CNF 2/8889)†	5.118	1.821	0.972	0.268	4.92	1.39	3.78	6.28	9.12
	ES(AH 6696)†	5.836	1.394	0.883	**0.000**	5.54	3.78	4.84	6.46	9.63

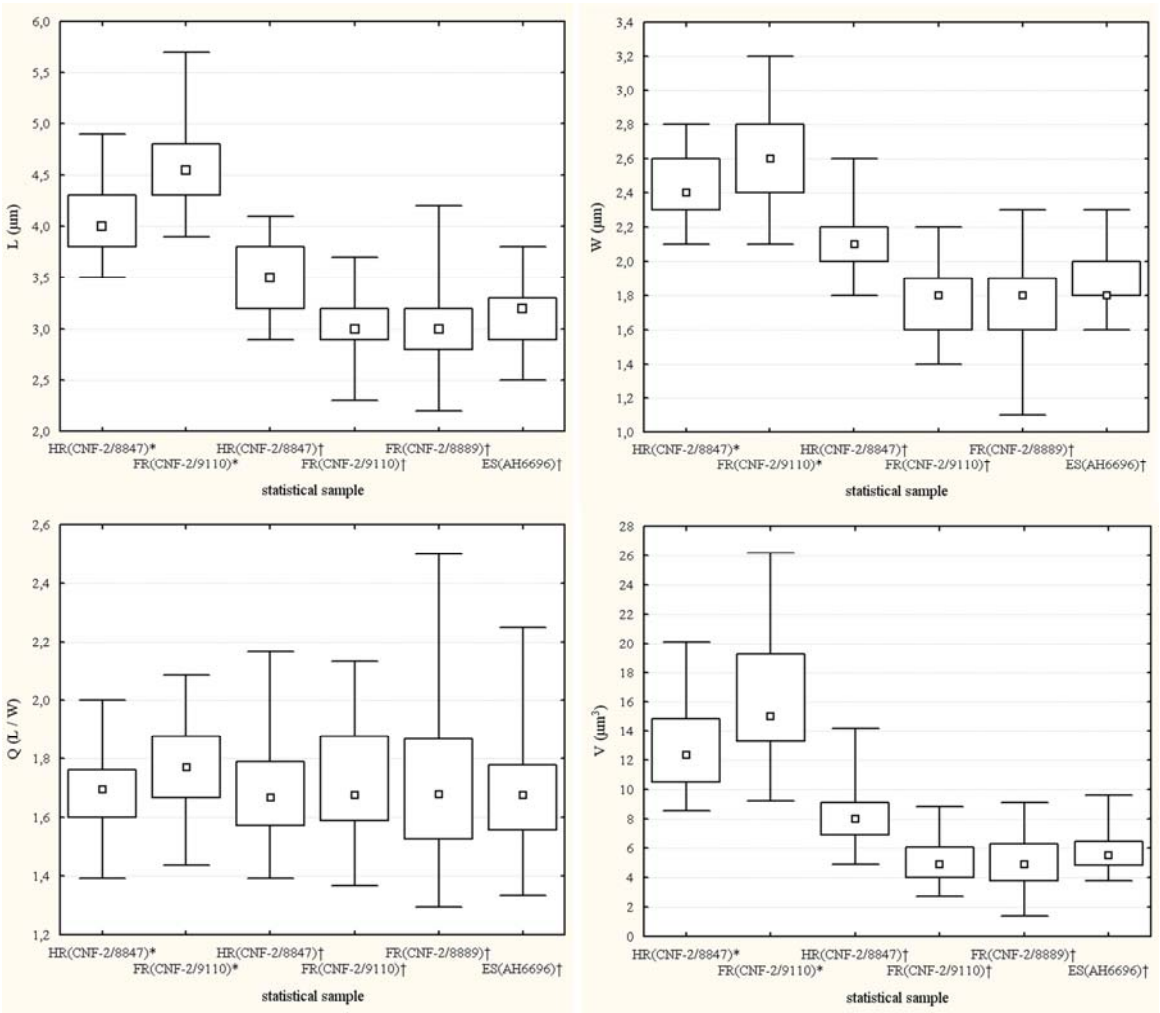


FIGURE 9. Distributions of *Hamatocanthoscypha rotundispora* ascospore morphometrical variables (n = 50 for each of six statistical samples). Point – median, box – quartiles, whisker – minimum and maximum.

Pairwise comparisons of living and dead statistical ascospore samples of the same collections [HR(CNF 2/8847)*/† and FR(CNF 2/9110)*/†] show (Table 5) significant differences in L, W and V (living ascospores are larger than dead). Moreover, in FR(CNF 2/9110)*/† significant difference was also found in Q, suggesting that treatment by KOH can result in nonhomogeneous spore shrinkage (i.e. L is disproportionally more reduced than W). Comparison of Croatian and French living statistical samples results in significant differences in all morphometrical variables (ascospores in French sample are larger and more elongated than that in Croatian sample) which suggest existence of intraspecific variability (Table 5). Similar results for L, W and V were also yielded in all pairwise

comparisons of dead samples, with exception of comparison of two French specimens (which did not differ in any morphometric variable). Significant differences in Q were not recorded in any comparison of dead samples (Table 5).

TABLE 5. A pairwise comparison of six statistical samples from four *Hamatocanthoscypha rotundispora* collections regarding ascospore morphometrical variables by Mann-Whitney test (n = 50 for each of six samples). Z and p(Z)—Mann-Whitney test statistics and respective probability (bold denotes statistical significance at probability level of p = 0.05).

	L		W		Q		V	
	Z	p(Z)	Z	p(Z)	Z	p(Z)	Z	p(Z)
HR(CNF 2/8847)* vs. HR(CNF 2/8847)†	6.815	**0.000**	7.307	**0.000**	0.396	0.692	7.966	**0.000**
FR(CNF 2/9110)* vs. FR(CNF 2/9110)†	8.617	**0.000**	8.586	**0.000**	1.985	**0.047**	8.617	**0.000**
HR(CNF 2/8847)* vs. FR(CNF 2/9110)*	-5.732	**0.000**	-3.078	**0.002**	-2.830	**0.005**	-4.295	**0.000**
HR(CNF 2/8847)† vs. FR(CNF 2/9110)†	5.756	**0.000**	6.480	**0.000**	-0.421	0.674	6.894	**0.000**
HR(CNF 2/8847)† vs. FR(CNF 2/8889)†	5.498	**0.000**	5.984	**0.000**	-0.455	0.649	6.411	**0.000**
HR(CNF 2/8847)† vs. ES(AH6696)†	4.288	**0.000**	5.453	**0.000**	-0.245	0.807	5.818	**0.000**
FR(CNF 2/9110)† vs. FR(CNF 2/8889)†	0.262	0.793	-0.234	0.815	0.031	0.975	-0.065	0.948
FR(CNF 2/9110)† vs. ES(AH6696)†	-1.982	**0.047**	-2.434	**0.015**	0.383	0.702	-2.758	**0.006**
FR(CNF 2/8889)† vs. ES(AH6696)†	-2.116	**0.034**	-1.889	0.059	0.196	0.844	-2.378	**0.017**

4.2. Taxonomical considerations on *Thecotheus platyapiculatus* nom. prov.

4.2.1. Description of the proposed new species *Thecotheus platyapiculatus* nom. prov.

Thecotheus platyapiculatus I. Kušan & Matočec, ***nom. prov.***
Figs. 10–13

Etymology: —lat. *platyapiculatus* = possessing a flat apiculum

Ascomata apothecial, superficial, mutually isolated, scattered to gregarious, primordia subglobular, immature conically truncate, then cylindrical (opening in prohymenial phase), turbinate to shortly stipitate when mature, hairless, margin sharply pronounced only in immature phases (Fig. 10a-d). When mature, *0.7–1.2 mm, rehydrated exsiccata †0.7–0.9 mm in diam. Whole apothecium pinkish (6A2, 7A2), both the margin and excipular flank finely pruinose, hymenium is roughened when protruding living and mature asci are present, partly semi-translucent at the base, weakly to moderately developed subiculum sometimes visible. No associated anamorph and stromatic tissues were found.

Hymenial elements: **Asci** *254–307 × 18.3–22 µm, †232–255 × 12.5–14.6 µm, *pars sporifera* *85–109 µm, 8-spored, protruding above living paraphyses up to 47 µm, uniseriate, cylindrical-strangulate, thickest in the central part, narrowing toward the apex, operculum faintly pre-delimited as a lens-shaped structure, *4–4.3 µm diam. and *0.5–0.6 µm thick, thickest at the centre, encircled by faintly thinned but rather broad circular weakened zone (of *Ascobolus* type), *0.3–0.5 µm thick, lateral wall 3-layered, *0.6–0.7 µm thick (Fig. 11a), periascal wall thickened up to *0.8 µm in strangulation belt after spore ejection (Fig. 11d); arising from compact repetitive croziers (Figs. 11c, 12d); ascal mucus present and of equal thickness throughout the whole length; in */†IKI ascal mucus diffusely weakly to moderately euamyloid but reaction is gradually strongest in lower ⅔ – ¾ of the length (Fig. 13a); in *CRB walls greenish white to light green (30A2-30A4); in *CR no differentially stained structures visible, only thin periascal wall layer dull red to greyish ruby (11C3, 12C3), †asci with prominent weakened circular zone (Fig. 13n); in †CB wall not cyanophilic, strangulation preserved in fixed living sections, acroplasm is moderately cyanophilic, lateral wall thickest at strangulation belt (Fig. 11g). **Ascospores** *(16.9)17.4–*19.1*–20.7(21) × 8–*8.7*–9.5(9.6) µm (n = 100), StD = 0.83 × 0.39, *Q =

(1.86)1.95–*2.19*–2.43(2.44); †(16.5)16.6–*18*–19.5(20.2) × (7.6)7.7–*8.4*–9.1(9.5) µm (n = 100), StD = 0.74 × 0.34, †Q = (1.91)1.97–*2.15*–2.34(2.47); narrowly ellipsoid to elongated ellipsoid or narrowly oblong to cylindric, but as a rule slightly bilaterally symmetric and/or heteropolar; hyaline, weakly refractive; laterally smooth but with bipolar flat shaped apiculi, *(0.5)1.9–2.8 µm diam., *0.2–0.7 µm high, circular from the polar view; eguttulate, 1-celled, uninucleate, nucleus centrally positioned and obscurely visible in water; proper wall 3-layered, freshly ejected ascospores embedded in a thick gelatinous sheath (Figs. 11e, 12a, 13d, g), gel condensed and visible inside *asci only on the uppermost spore as a false obtuse apiculi (with true apiculi underneath, Figs. 11a, 12b); in *IKI outer and median wall layers and apiculi unstained, inner layer greyish orange (6B3-6B5), sporoplasm greyish yellow (3B5-3B7), without glycogen accumulations, nucleus more contrasted, *3.5–3.7 µm diam., nucleolus discernable, *1.6–1.8 µm diam. (Fig. 13c, d); in *CRB spore sheath visible as a bluish violet (18A7) thin film adpressed to the spore surface while still in *asci, in ejected ascospores outer wall layer violet blue (19A8, 19B8), median layer unstained, inner layer light lilac (16A5), apiculi bluish white (23A2), sporoplasm pale blue to pastel blue (22A3-22A4), gel sheath unstained, nucleus contrasted, *3–3.3 µm diam., nucleolus invisible (Fig. 13g-i); in †CRB-KOH ascospores instantly discoloured, apiculi and gelatinous sheath totally dissolved, outer wall layer loosened especially at polar areas and sporoplasm collapsed (Fig. 13k, l), nucleus sharply visible (Fig. 13l); in *CR only inner wall layer dull red (10B4, 11B4), apiculi fading; in †CB outer wall strongly cyanophilic, not loosened, completely smooth, sporoplasm and apiculi moderately cyanophilic, without DBBs (Figs. 11f, 13o); in †KOH perispore after short exposure loosening and transversally wrinkled (Fig. 12l, m); in †MLZ outer and median wall layer loosened, especially at polar regions, lateral surface wrinkled; in †AC whole sporoplasm greyish magenta (13D5), nucleus paler and not carminophilous (Fig. 13m). **Paraphyses** hyaline, thin-walled, embedded in hyaline gel, of two types: (1) cylindrical-clavate to paddle shaped, straight, richly branched, apical cells *54.3–118 × 4.6–5.2 µm, †3–4.2 µm wide, filled with moderately refractive globular to elongated hyaline to subhyaline SVBs; (2) filiform, slender, with scattered, minute, dot-shaped weakly refractive VBs, usually arising as a branches of paraphyses type (1), apical cells *19.6–37.4 × 1.8–3 µm, †0.8–1.8 µm wide (Figs. 11b, 12c); in *IKI SVBs in greyish yellow (3B5-3B7), VBs unstained; in *CRB granular exudate contrasted, SVBs oblong greyish blue (20B6) (Fig. 13e), VBs vivid blue to turquoise (23A8, 24A8) (Fig. 13f); in †CRB-KOH exudate greyish ruby (12D7), SVBs becoming greyish ruby (12C6) (Fig. 13k), VBs instantly vanished; in †KOH SVBs instantly dissolved (Fig. 12k); in †CB both types of paraphyses with

moderately to strongly cyanophilic cytoplasm; in †AC type (1) paraphyses greyish magenta (13D5) (Fig. 13m); †MLZ. **Subhymenium** sharply differentiated, composed of hyaline thin-walled *textura angularis*, layer *40–48 µm, †30–46 µm thick, cells *2.6–8.2 µm, †4–7.8 µm wide (Fig. 10f); *IKI-; in *CR cell walls stained; in †CB walls cyanophilic; †MLZ-.

Margin: **Marginal tissue** at ½ of hymenial height *42–52 µm, †30–45 µm thick, similar to paraphyses and of two types of terminal cells: (1) globular to capitate-clavate, *8.2–13.7 µm, †5.9–10 µm wide, cytoplasm filled with subhyaline low refractive SVBs (as in paraphyses type 1) (Fig. 12h), (2) surface cylindrical to irregular cells, *2–3.7 µm, †1.8–3 µm wide, often twisted between clavate ones, cytoplasm with scattered, minute, dot-shaped weakly refractive VBs (as in paraphyses type 2); inner part composed of a mixture of swollen, globose, clavate to oblong cells, *5.2–13 µm, †4.2–9 µm wide, and intricate ±cylindrical cells, *1.9–4.2 µm, †2–2.8 µm wide, both types of cells without visible cytoplasmic inclusions (Figs. 10e, 12e, f); in *IKI SVBs greyish yellow (3B5-3B7) (Fig. 13b), other structures IKI-; in *CRB SVBs greyish blue (20B6) and in †CRB-KOH becoming greyish ruby (12C6) (Fig. 13j); in *CR cell walls stained; in †CB walls cyanophilic, strongly cyanophilic in swollen cells; in †MLZ-; in †AC surface clavate to globular cells with greyish magenta (13D5) content. ***Excipular flank:*** **Medullary excipulum** hyaline, layer *28–36 µm, †25–30 µm thick, up to *52 µm thick at the lower flank, composed of *textura porrecta-intricata*, cell rows running subparallel to the receptacle surface, cells *1.7–5 µm, †2.5–4.8 µm wide, without visible cytoplasmic inclusions (Fig. 10f); *IKI-, *CRB -; in *CR cell walls stained; in †CB walls cyanophilic; †MLZ-. **Ectal excipulum** subhyaline in outer layer, hyaline in the inner zone, *58–62 µm, †45–60 µm thick at the middle flank, predominantly composed of *textura angularis-globulosa*, cells *5.2–16.8 µm, †7–11 µm wide, thin-walled, surface globular cells filled with subhyaline low refractive SVBs (Fig. 12h), which are intermixed with narrow, undulate, thin-walled cells, *1.9–4.8 µm, †1.8–2.9 µm wide (Figs. 10f, 12e, g); in *IKI SVBs greyish yellow (3B5-3B7) (Fig. 13b), other structures IKI-; in *CRB SVBs greyish blue (20B6) and in †CRB-KOH becoming greyish ruby (12C6) (Fig 13j); in *CR cell walls stained; in †CB walls cyanophilic, strongly cyanophilic in swollen cells; †MLZ-; in †AC surface clavate to globular cells with greyish magenta (13D5) content.

Subiculum hyphae sparse, cylindrical, hyaline, †2.2–3 µm wide.

Asexual state: Not detected *in situ* nor obtained in culture (DA and DOA).

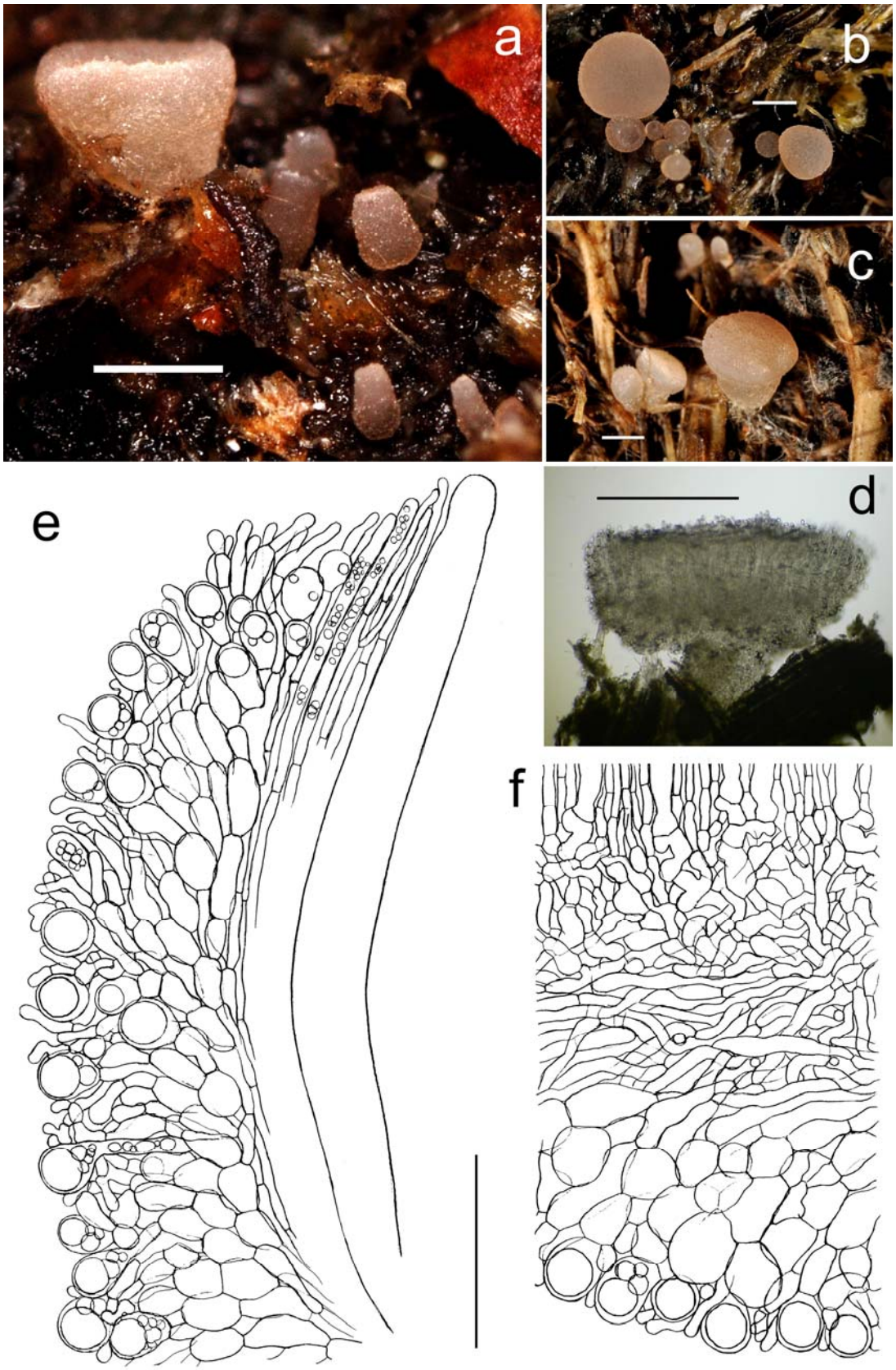
Material examined:

—CROATIA, Lika-Senj County, northern part of the Mt. Velebit, 530 m W from Vučjak peak (1644 m) (near Zavižan mountain lodge), 44.815198 N, 14.969454 E, elev. 1530 m, on *Equus caballus* dung, associated with *Podospora conica* (Fuckel) A.E. Bell & Mahoney, alpine stony grassland (pasture) with solitary *Picea abies* on limestone, 11 Jun 2011, *leg.* I. Kušan & N. Matočec (CNF 2/8950, CNF 2/9331).

FIGURE 10. *Thecotheus platyapiculatus* (CNF 2/8950): **a.-c.** *Apothecia. **d.** Section through the *apothecium. **e.** *Margin. **f.** *Texture of the middle excipular flank. Media: d-f H_2O. Bars: a-d = 0.5 mm; e-f = 50 µm. Phot. I. Kušan & N. Matočec. Del. N. Matočec. **(page 39)**

FIGURE 11. *Thecotheus platyapiculatus* (CNF 2/8950): **a.** Apical part of the *ascus. **b.** *Paraphyses. **c.** *Ascogenous system. **d.** †Ascus after the spore ejection. **e.** Freshly ejected *ascospores. **f.** †Ascospore. **g.** Apical part of the †ascus. Media: a-e H_2O; f-g CB. Bars = 10 µm. Del. N. Matočec. **(page 40)**

FIGURE 12. *Thecotheus platyapiculatus* (CNF 2/8950): **a.** Freshly ejected *ascospores. **b.** Apical part of the *ascus (note the uppermost spore with a false apiculi and the true apiculum underneath). **c.** *Paraphyses. **d.** Basal part of the *asci. **e.** Marginal and excipular *tissue with a part of *hymenium. **f.** *Margin (in section). **g.** *Ectal excipulum. **h.** *Margin and ectal excipulum (top view). **i.** †Asci with †mature, †submature and *mature ascospores. **j.** †Ascus with *ascospores, and †paraphysis containing SVBs. **k.** †Ascus with †ascospores, and †paraphysis without SVBs. **l.** and **m.** †Asci with †ascospores. Media: a-j H_2O; k-m KOH. Bars: a-d and f-m = 10 µm; e = 50 µm. Phot. I. Kušan & N. Matočec. **(page 41)**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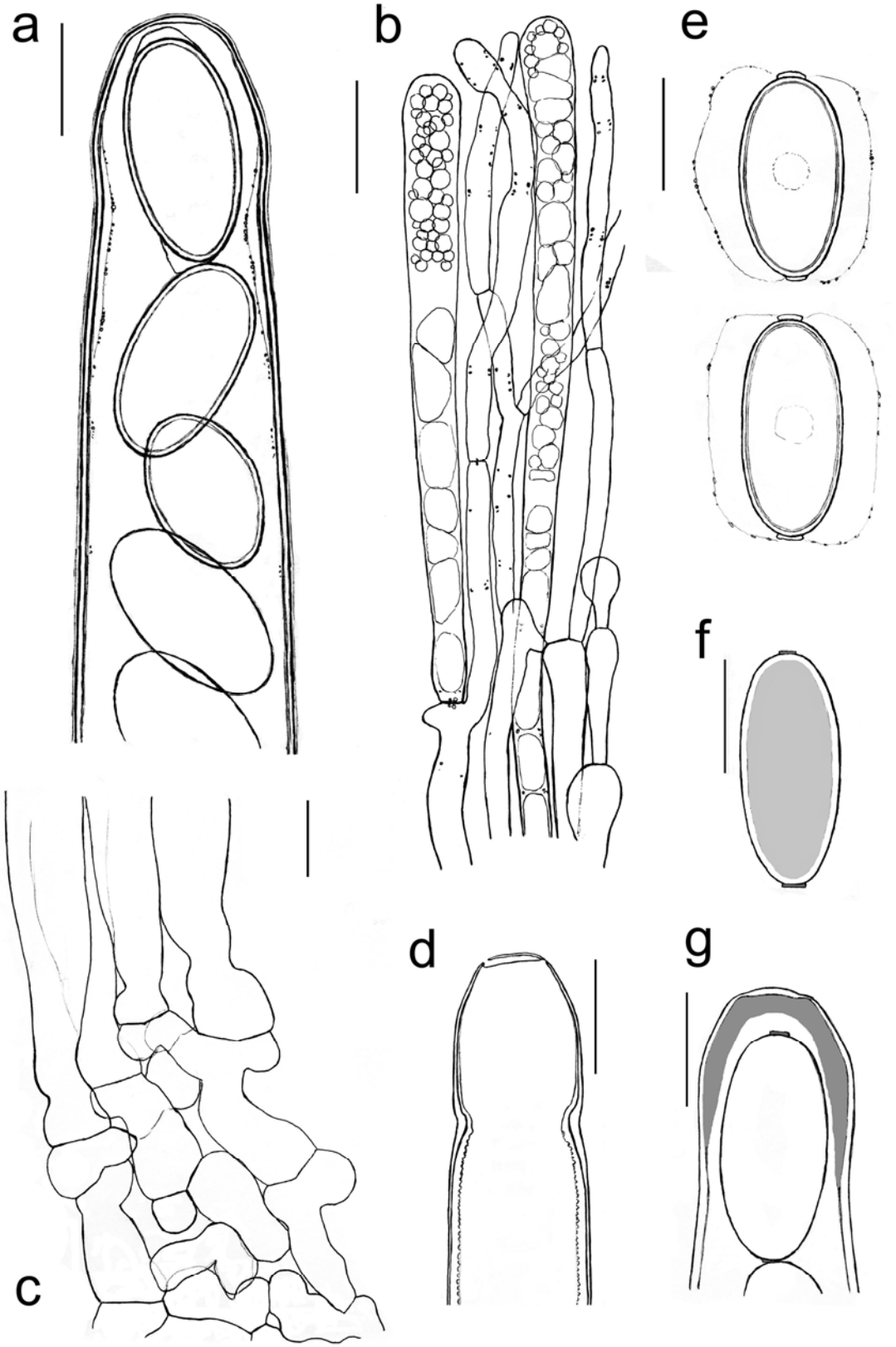
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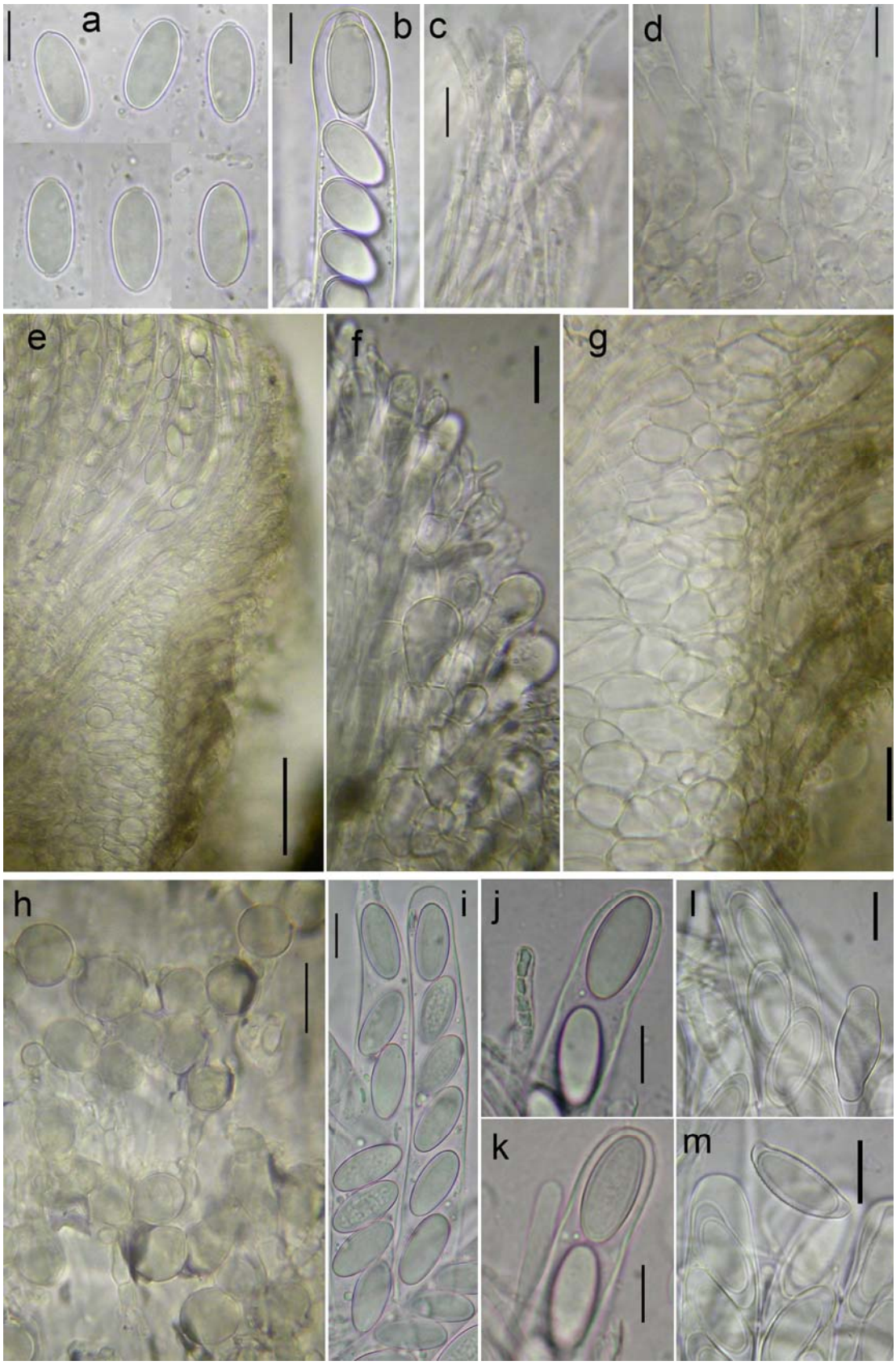
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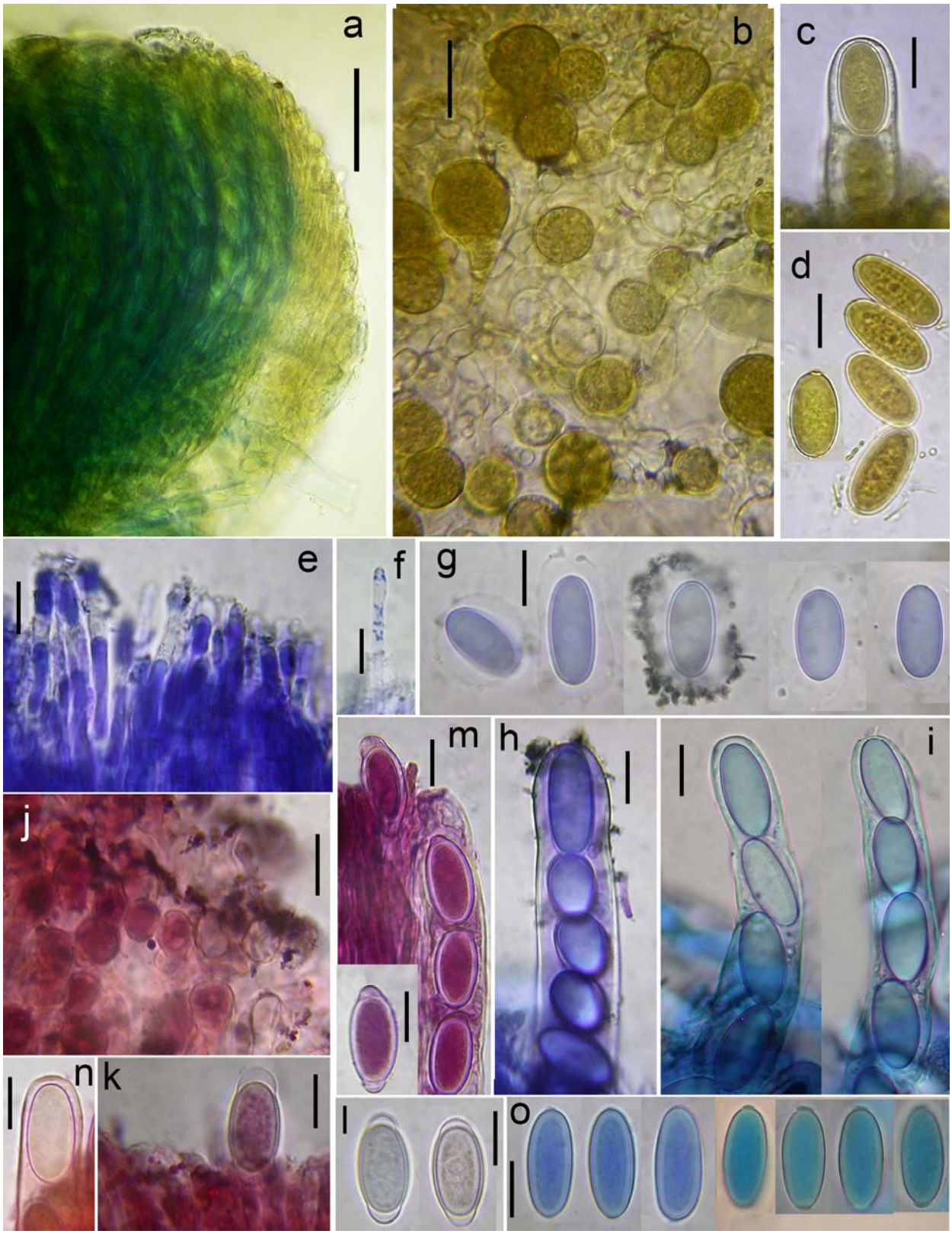
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FIGURE 13. *Thecotheus platyapiculatus*: **a.** *Section in IKI. **b.** Margin and ectal excipulum (top view) in IKI. **c.** Apical part of the *ascus in IKI. **d.** Freshly ejected *ascospores in IKI. **e.** *Paraphyses of type (1) containing SVBs in CRB. **f.** *Paraphyses of type (2) containing VBs in CRB. **g.** Freshly ejected *ascospores in CRB. **h.** Apical part of the *ascus with *ascospores in CRB. **i.** Apical part of the †asci with */†ascospores in CRB. **j.** Margin and ectal excipulum (top view) in CRB-KOH. **k.** †Ascospore and †paraphyses in CRB-KOH. **l.** †Ascospores with detaching perispore in CRB-KOH. **m.** †Ascus and †ascospores in AC. **n.** Apical part of the *ascus showing no differential staining of proper wall in CR. **o.** †Ascospores in CB. Bars: a = 50 µm; b-o = 10 µm. Phot. I. Kušan & N. Matočec. **(page 42)**

4.2.2. Worldwide identification key to the *Thecotheus* species with apiculate spores

1. Hamathecium composed only of a single type of paraphyses; always on dung 2

1'. Hamathecium composed of two types of paraphyses; on dung or plant remnants 6

2. Fully mature ascospores without lateral ornamentation (smooth-walled) 3

2'. Fully mature ascospores with various types of lateral ornamentation 4

3. Ascospores 20–22 × 10–12 µm; apiculi 4–7 µm high with collarette; walls of mature living asci without strangulation in subapical area; on elepahant dung . . . . ***T. perplexans*** (Faurel & Schotter) J.C. Krug & R.S. Khan

3'. Ascospores 24–30 × 12–14 µm; apiculi 1–4 µm high without collarette; walls of mature living asci with strangulation in subapical area; on cow dung. . . . . . ***T. neoapiculatus*** Doveri & Coué

4. Ascospores 16–19 × 7–9 µm; bilaterally symmetrical, ellipsoid with one ±flattened side; walls of mature living asci without strangulation in subapical area . . . . ***T. biocellatus*** (Petr.) Aas

4'. Ascospores longer than 20 µm and wider than 12 µm; radially symmetrical, narrowly ellipsoid to subfusiform; walls of mature living asci with strangulation in subapical area 5

5. Ascospores 25–30 × 12.5–14.5 µm; lateral ornamentation minutely verruculose to subreticulate, up to 0.4 µm wide; apiculi 2.5–6.0 µm diam., 2–3 µm high . . . . ***T. lundqvistii*** Aas

5'. Ascospores 29–38 × 14–18 µm; lateral ornamentation of isolated warts 0.3–2 µm diam.; apiculi (2)5–6.5 µm diam., 2.2–5 µm high . . . . ***T. holmskjoldii*** (E.C. Hansen) Eckblad

6. Apothecia never on dung; on rotten grasses beset with algae or on permanently soaked wood along rivulets; semiaquatic to aquatic 7

6'. Apothecia always on dung 8

7. Apothecia on permanently soaked wood; ascospores bilaterally symmetrical; 16–22.5 × 7–9 µm; apiculi single per pole . . . . . ***T. rivicola*** (Vacek) Kimbr. & Pfister

7'. Apothecia on dead grasses beset with algae; ascospores radially symmetrical; 30–37.5 × 15.5–17 µm; apiculi 1–6 per pole . . . . . ***T. phycophilus*** Pfister

8. Fully mature ascospores laterally ornamented with warts; apiculi hemisphaerical to subconical 9

8'. Fully mature ascospores without lateral ornamentation (smooth-walled); apiculi hemisphaerical or plate-shaped 12

9. Ascospores 23–28 × 12–13.5 µm; radially symmetrical; ornamentaed with rather coarse warts, 1–2 µm large; apiculi 4.5–6.5 µm diam.; on oryx dung . . . . . ***T. harasisus*** Gené, El Shafie & Guarro

9'. Ascospores shorter than 17 µm and below 10 µm diam.; bilaterally symmetrical; warts mostly less than 1 µm; apiculi less than 4 µm diam.; on other kinds of dung 10

10. Ascospores 12–16 × 7.5–9.5 µm; on elephant dung . . . . . ***T. africanus*** R.S. Khan & J.C. Krug

10'. Ascospores 17–20 × 7.5–9 µm; on cattle dung . . . . . ***T. formosanus*** Yei Z. Wang – 11

11. Ascospore lateral ornamentation verruculose, warts less than 0.5 µm diam.; apiculi without collarette . . . . . ***T. formosanus*** Yei Z. Wang f. ***formosanus***

11'. Ascospore lateral ornamentation verrucose, warts more than 0.5 µm diam.; apiculi with collarette . . . . . ***T. formosanus*** Yei Z. Wang f. ***collariatus*** Doveri & Coué

12. Apiculi constantly plate-shaped, 0.2–0.7 µm high; ascospores mostly slightly heteropolar and/or bilaterally symmetrical, walls of mature living asci with obvious strangulation in subapical area that remain in strangulated conformation even after spore discharge (as in *T. strangulatus*) as well as in fixed material . . . . . ***T. platyapiculatus*** I. Kušan & Matočec, *nom. prov*.

12'. Apiculi normally hemisphaerical, papillate or elongated, exceeding 1.0 µm in height; ascospores either radially symmetrical and homopolar or strongly bilaterally symmetrical; walls of mature living asci without strangulation in subapical area 13

13. Ascospores 13–16 × 7–8.5 µm; strongly bilaterally symmetrical, narrowly ellipsoid with one side ±flattened; on moose dung . . . . . ***T. inaequilateralis*** Aas

13'. Ascospores 17–21 × 7.5–11 µm; radially symmetrical, oblong to cylindric; on cattle dung . . . . . ***T. keithii*** (W. Phillips) Aas

4.3. Taxonomical considerations on *Tricharina tophiseda* nom. prov. and *T. japonica*

4.3.1. Description of the proposed new species *Tricharina tophiseda* nom. prov.

Tricharina tophiseda **Matočec & I. Kušan, *nom. prov.***

Figs. 14, 15, 16 a-l

Etymology:—specific epithet *tophiseda* (lat. *tophus* = tufa, *sedere* = sitting) means "sitting" on a tuffaceous substrate.

Sexual stage

Ascomata apothecial, superficial, at first with elevated upper flanks then adpressed to the substrate until margin, broadly attached to the substrate, spatially isolated from each other, shallowly cupulate to plane, with true hairs only on the marginal area and upper flanks. When fresh 4.5–11 mm and 1.2–3.7 mm in diam. when dry. Hymenium light orange (5A4–5, 6A4–5), rarely with a pinkish white (7A2, 8A2) tinge, orange grey (6B2) in depressions, smooth, sometimes undulate, not translucent. Margin sharp, elevated above hymenium, with dense brownish red (8C8) to reddish brown (8D8–E8) protruding hairs agglutinated to form fascicles; ground orange to deep orange (5A7–8, 6A7–8). Excipular surface concolorous to the margin, sparsely covered with shorter hyaline to subhyaline hyphoid hairs mixed with subicular anchoring hyphae, uppermost area with true brownish hairs as in margin but loosely arranged in tufts. Associated visible anamorph and stromatic tissues not found.

Hymenial elements: **Asci** *226–258 × 18.4–21 µm, †174–213 × 12.5–17 µm, cylindrical-clavate, apex asymmetrically subconical-blunt, operculum slightly papillate-lensiform, *5–6 µm wide, *0.4 µm thick, *pars sporifera* *70.6–90 µm when all eight spores are fully developed and *47.2–60.3 µm when 1–3 spores are underdeveloped, uniseriate, lateral wall 3-layered, altogether *0.7–0.8 µm thick,

periascal gel very thin and visible only in *CR, base tapered, arising from small-celled perforated croziers; in */†IKI completely inamyloid; in *CR opercular wall not stained, periascal gel reddish white to reddish grey (12A2–12B2) and *0.2–0.3 µm thick; outermost layer dull red (11C3–4), median layer pastel red (10A4) and innermost layer dull but not stained. **Ascospores** *(14.1)14.3–*16.3*–18.3 × (8.6)9.2–*10.4*–11.2 µm (n = 50, in H_2O), StD = 1.01 × 0.56, *Q = (1.41)1.42–*1.58*–1.74(1.80); †(15)15.1–*16.7*–18.2(18.4) × (8.5)9–*10*–10.9(11.4) µm (n = 100, in CB), StD = 0.79 × 0.47, †Q = (1.48)1.52–*1.67*–1.83(1.85); ellipsoid-limoniform to narrowly ellipsoid-oculiform, ±radially symmetric, homopolar to occasionally faintly heteropolar, 1-celled, hyaline, smooth, sporoplasm low to moderately refractive and hyaline, uninucleate, nucleus equatorially, centrally to peritunically positioned, *3–4 µm in diam., proper wall 3-layered, hyaline, in total *0.6 µm thick, endospore hardly visible, <*0.2 µm thick, epispore *0.3–0.4 µm thick, perispore dull, *0.2 µm thick; BSG low refractive, at poles or occasionally lined along one spore side; in *IKI sporoplasm moderately refractive pale yellow (3A3), without glycogen accumulations; in *CRB perispore pale violet (18A3), not loosened nor dissolved in †CRB-KOH; in *CR nucleus sharply contrasted; in †CB without DBBs, perispore not cyanophilic, not loosening and perfectly smooth; in †AC nuclei contrasted but not carminophilic, sporoplasm purplish red (13A7) except for BSG which are unstained. **Paraphyses** cylindric-obtuse to cylindric-clavate, simple, not branching, without exudate, apical cells *41.3–90.6 × 4.8–8 µm, filled with low refractive, subhyaline, homogenous, globose or lensiform SCBs and with non-refractive, globose to oblong vacuoles, without true pigments; in *IKI and *CRB not stained; in †CRB-KOH all cytoplasmic structures instantly vanish; in †CB cytoplasm light blue (23A4–5). **Subhymenium** *54–65 µm thick, composed of greyish yellow (2B6–7) thin-walled *textura angularis-epidermoidea*, cells *3.6–8.4 µm wide, many containing highly refractive vivid yellow (3A8) carotenoid granules, wall greenish yellow (1A8), the whole layer extremely fragile and easily collapsing in living sections, pigmentation of the whole apothecia originating from the subhymenium layer.

Margin densely covered with hairs, **marginal tissue** composed of *textura globulosa-angularis*, cells *14.8–33.8 µm wide, walls thickened towards the surface, perimarginal area composed of clavate cells *20.7–59.6 × 9.4–26.8 µm, all cells with greyish yellow (4B6) walls; **marginal hairs** stiff, often also sinuous, (3)4–11(14) celled, */†(146)225–932 µm long, */†9–22 µm wide in the middle part, */†19.5–42 µm wide at the base, simple, middle cells often with internal "secondary hair", wall orange yellow (4B8), smooth, walls */†0.8–2.2 µm thick, thickest walls clearly 3-layered (>1.2 µm), apices blunt or tapered to almost pointed in longest hairs; hairs forming conical aggregations, basally organized in

fascicled groups and oriented partly in various directions and partly projecting together at the low angle, base sub-bulbous to bulbous, buried up to one cell deep, simple; in *CRB marginal hairs surrounded with gelatinous matter, wall dark blue (19E7), cytoplasm greyish violet (19C7); in †CRB-KOH durable after longer exposure to KOH (30'), cytoplasm with purple (15B7) granules, wall discoloured; in †CB marginal cell walls moderately cyanophilic; in *IKI and *CR marginal structures not stained.

***Excipular flank*: Medullary excipulum** hyaline, *110–160 µm thick at the middle flank, composed of *textura porrecta-prismatica* interspersed with narrow undulating hyphae, whole layer of homogenous *textura intricata* in dead state, cell rows running ±parallel to the receptacle surface, cells *4.2–19.7 µm wide, broadest hyphae ±completely filled with low to medium refractive subhyaline cytoplasm dotted with scattered, freely floating, medium to highly refractive globules, *0.6–1.8 µm wide, highly refractive LBs present, up to *5 µm in diam., cytoplasm in narrow hyphae non-refractive; in *IKI wide medullar hyphal cytoplasm contain reddish orange (7B8) to brownish orange (7C8) glycogene pockets along with unstained globules; in *CRB not notably stained; in †CRB-KOH durable after longer exposure to KOH (30'), cytoplasm lilac (15B5), freely floating small globules violet (17B7); in *CR walls pastel red (10A4); in †MLZ no dextrinoid reactions. **Ectal excipulum** *110–140 µm thick at the middle flank, composed of two layers, inner layer of hyaline *textura globulosa*, cells *14.1–30.6 µm wide, each contain singe large non-refractive vacuole and devoid of LBs, walls hyaline and thin, outer layer composed of yellow (2B8) *textura angularis*, cells *14–26.3 µm wide, some ±perpendicularly elongated up to *39 µm, walls thickened, highly refractive, vivid yellow (3A8), *0.4–1 µm thick; outermost cells on the upper flank giving rise to flexuous, thick-walled true **hairs**, */†70–380 µm long, */†14–18 µm wide in the middle part, */†20–38 µm wide at the base, base bulbous, wall orange yellow (4B8), */†0.7–2 µm thick; middle and lower flank with numerous subhyaline flexuous, thin-walled **anchoring hyphae**, *71.2–610 × 8–9.8 µm, base bulbous, up to *35 µm wide, wall *0.4–1.1 µm thick; in *IKI, *CRB and *CR not stained; in †CB walls moderately cyanophilic, in †MLZ no dextrinoid reactions. **Apothecial base** covered with long, rarely branching, subhyaline, hyphoid and flexuous subicular hyphae that originate from up to *30 µm wide bulbous base.

Asexual stage

Ascospores rapidly germinating on CYA, after 10 hrs over 50% swollen up to 25 × 19 µm (overmature) of which approx. 50% readily germinating with 1-2 germination points per spore. Germ

tubes *8–85 × 6.5–8.5 µm, non-septate to 1-septate, hyaline, thin-walled, not branched, ± straight and adpressed to the substrate. If secondary tube is present it is much shorter. After 18 hrs colonies macroscopically visible, reaching *3.3–11 mm diam. as yellowish white (2A2) floccose spots with irregular not differentiated rim, and without elevated outgrowths. Hyphae hyaline, smooth-walled with first lateral (shorter) branches at 60°–90° angle, not at all constricted at the junctions, with a nearest septum at the close distance from the corresponding junction (up to 11 µm). After 35 hrs colonies *19.1–25.7 mm diam., hyphae sparingly septate, hyaline, thin-walled and smooth, *2.6–3.3 µm wide with third order branches, with many anastomoses in the central area, and with short unilateral to bilateral nodules, *1.8–4.5 µm wide.

After 2 ½ days colonies covering almost the whole plate on CYA, and the same after 4 days on MEA; orange white (5A2) on CYA, yellow (3A6) on MEA; without concentric bands, reverse only slightly darker, entirely flat, central parts with watery-milky liquid film on both media; hyphae producing loops, terminal, lateral and intercalary nodules, many of them swelling to form clavate balloon-shaped outgrowths separated by septum (or septae), hyaline, smooth, formed equally on both media. Central area occupied by densely septate hyphae, many with distinctly formed smooth moniliform sporodochia-like cell aggregates, *9–23 µm wide, arranged in terminal and intramycelial (1)2-4 celled unbranched chains after 2 ½ days on CYA and 4 days on MEA. Released propagules absent. Many hyphal cells containing greenish yellow (1B8) to deep green (1C8) areas in central part of the cytoplasm but without refractive granular content on CYA, while on MEA the cytoplasm is filled with densely set highly refractive LBs of variable sizes. Some hyphal walls encrusted with ochraceous granular pattern. Hyphal walls are hyaline and thin-walled on CYA, yellowish pale yellow (2A3) and thick-walled (*0.7–1 µm thick) on MEA. Germinated ascospores remain hyaline, devoid of internal granules and 1-celled at all times. Sclerotia, stromata, pigments and secondary spores not produced. Some peripheral hyphae aerial after 5 days.

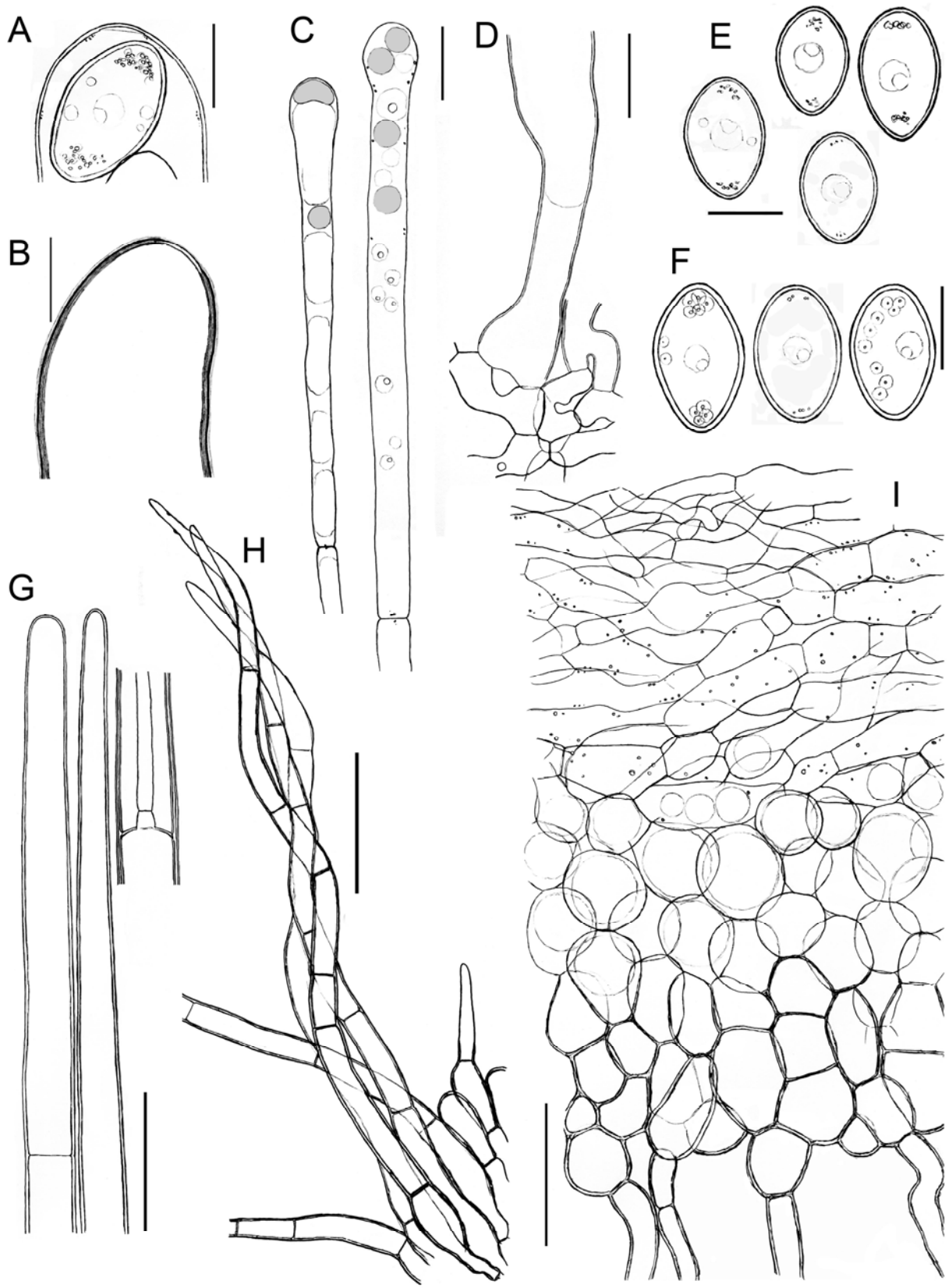
Material examined:

—CROATIA. Dalmatia: 3500 m E-SE from Skradin, right bank of Krka river in the area of Skradinski buk waterfalls, elev. 13 m, 43°48'22" N, 15°57'51" E, habitat: continuously wet tufa barrier surrounded by *Populus* sp., *Fraxinus angustifolia*, *Platanus* sp. and *Ficus carica* riparian vegetation, substrate: continuously wet tuffaceous sediment covered with mosses and scarcely beset with minute plant remnants, together with *Trichophaea woolhopeia* (Cooke & W. Phillips) Boud. s.

l., 30 November 2008, *leg.* N. Matočec & I. Kušan (CNF 2/8079); 8 May 2008, *leg.* A. Mešić, N. Matočec & I. Kušan (CNF 2/7953).

FIGURE 14. *Tricharina tophiseda*—hymenial elements and excipular structures. **a.** Ascus apex with the ascospore. **b.** Ascus apex. **c.** Paraphyses. **d.** Ascus base with a part of ascogenous system. **e-f.** Ascospores (representing all shape variations). **g.** Marginal hairs apices and a middle part. **h.** Marginal hair fascicle. **i.** Excipular texture. a–e, g, i from (CNF 2/8079); c, f, h from (CNF 2/7953). All in $^{*}H_2O$, except b which is in *CR. Scale bars: a-f = 10 µm; g, i = 50 µm; h = 100 µm. Del. N. Matočec. **(page 50)**

FIGURE 15. *Tricharina tophiseda*—apothecia, hymenial elements and excipular structures. **a-d.** Living apothecia. **e.** Section through the apothecia. **f.** Marginal and submarginal area (side view). **g.** Margin. **h.** Paraphyses and ascospores (two lower ascosp. in early stage of germination). **i.** Asci with ascospores. **j-k.** Ascospores. **l.** Asci (each in a different focal view). **m.** Middle part of the marginal hair. **n.** Ascospores (with visible BSG). **o-r.** Cells in a meddulary excipulum. a-c, e-r from (CNF 2/8079); d from (CNF 2/7953). e-j, o in $^{*}H_2O$; k in †KOH; l in *CR; m, r in *CRB-KOH; p in *IKI. Scale bars: a = 5 cm; b-c = 2 cm; e-g = 200 µm; m, o-r = 20 µm; h-l, n = 10 µm. Phot. I. Kušan & N. Matočec. **(page 51)**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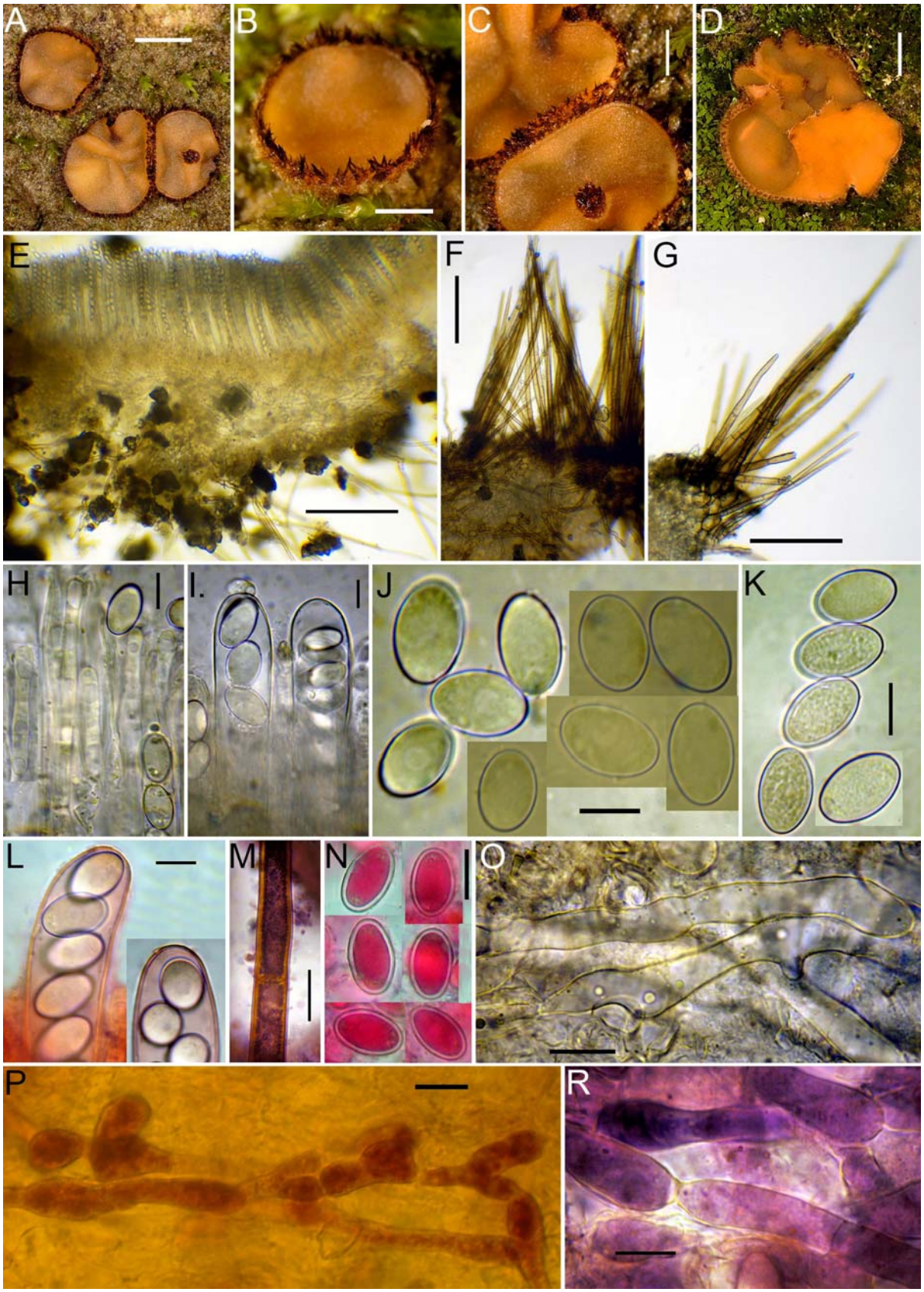
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4.3.2. Revisionary study of *Tricharina japonica*

Type material of *T. japonica* was examined for comparison along with the recent collection from Switzerland (Dougoud & De Marchi 2012). Dougoud & De Marchi (2012) provided a comprehensive description of the Swiss collection, and some important discrepancies were noticed when comparing this to the protologue. Therefore, a thorough revision of the holotype was conducted which confirmed the conspecificity between Dougoud and De Marchi's collection and the type. Because the Swiss collection contained still-living ascospores I have tried to obtain this strain in pure culture, but was unsuccessful. Here I provide the additional data that supplements the previous two descriptions, with a special emphasis on the diagnostic/differential characters (Fig. 16 m-w):

***Tricharina japonica* Chin S. Yang & Korf in Yang & Korf (1985a: 497)**

Ascomata apothecial, cupulate, †2.3–6.4 mm in diam.

***Hymenial elements*: Asci** cylindrical, regularly 8-spored, arising from crozier cells. **Ascospores** *(16.1)16.5–*18.6*–20.7(21.4) × (7.7)8.2–*8.9*–9.5 µm (n = 50, in H_2O), SD = 1.05 × 0.4, *Q = 1.91–*2.10*–2.30(2.35); †(16)16.4–*19*–21.5(23.2) × (7.4)7.7–*8.7*–9.8(10.1) µm (n = 200, in CB), SD = 1.27 × 0.52, †Q = (1.67)1.85–*2.18*–2.51(2.75), oculiform to fusiform, strongly bilaterally symmetric (trapezoidal in lateral view), ±heteropolar, hyaline, smooth, uninucleate, nucleus *3.2–3.5 µm wide, equatorially and peritunically positioned, wall highly refractive, †0.4 µm thick, cytoplasm low refractive, minute BSG weakly visible in *H_2O, partly coalesce in KOH and form larger easily visible granules, when heated in CB spore poles frequently become papillate; submature ascospores †14.4–*15.9*–17.4 × 6.2–*7.6*–8.3 µm (n = 25, in H_2O), less refractive, wall thin and not fully developed; in †CB without DBBs, perispore not cyanophilic, not loosening, and perfectly smooth, content remain hyaline; in †AC cytoplasm refractive, pink (13A5), nuclei contrasted, not carminophilic. **Paraphyses** cylindric-clavate, straight, simple, not branched, at apex †2.3–5.1 µm wide, filled with highly refractive irregular cytoplasmic remnants; in †CB cytoplasm pale blue (21A3), wall not stained. **Subhymenium** †24.5–30.6 µm thick, composed of *textura angularis-epidermoidea*, cells †4–12.2 µm wide, thin-walled, hyaline; in †CB cytoplasm pale blue (21A3), wall not stained.

***Margin*: Marginal hairs** in fascicles, ±straight, partly flexuous, subhyaline to greyish orange (6B6), densely septate, 4–9 celled, apices strongly tapered in the longest hairs, shorter hairs with rounded apex, †145–790 µm long, †5.7–13 µm thick in the middle part, †8.5–22.5(27.7) µm thick at the base, basal cells prismatic-truncate or rarely bulbous, wall 3-layered, †(1.1)1.5–2 µm thick; in †CB cytoplasm bluish white (21A2), wall not stained.

***Excipular flank*: Medullary excipulum** †50–86 µm thick, composed of *textura porrecta-intricata*, greyish orange (5B5), ±horizontally oriented, cells †3.9–12 µm wide, walls †0.5–0.6 µm thick; in †CB cytoplasm bluish white (21A2), walls not stained. **Ectal excipulum** composed of two layers: inner layer †74–111 µm thick at the middle flank, of subhyaline *textura globulosa-angularis*, largest cells elongated ±ellipsoid and oriented ±vertically to the excipular surface, cells †7.3–33 × 5.9–29 µm, many contain single small LB, wall †0.7–1 µm thick; outer layer †35–42 µm thick at the middle flank, of *textura angularis*, greyish yellow (2B6-7), wall †0.9–1.1 µm, cells organized in 1–2 rows; **excipular true hairs** covering almost whole excipular flank, flexuous, rather densely septate, subhyaline to greyish orange (6B6), †94–460 µm long, †6–17 µm wide in the middle part and †11.5–43.7 µm wide at the bulbous base, not deeply rooting, wall refractive, †1.3–2 µm thick; in †CB cytoplasm of the excipular cells and hairs bluish white (21A2) to bluish grey (21B2), walls not stained. **Apothecial base** with hyaline hyphoid subicular hyphae, richly branched and occasionally anastomosed, †5.2–8.5 µm wide, wall †0.8–1.3 µm thick. Overall texture MLZ-.

Material examined:

—JAPAN. Kyushu, Fukuoka Pref., near Fukuoka, Mt. Tachibana, on soil, 28 October 1957, *leg.* S. Imai, H. Yoshii, R. P. Korf, *et al.* (holotype CUP-K-(JA-000286))

—SWITZERLAND. Canton of Bern, Belp Municipality, Belp, river Aare, on the river bank, elev. 519 m, 29 September 2011, *leg.* R. De Marchi (R.D. 31.01.245.11).

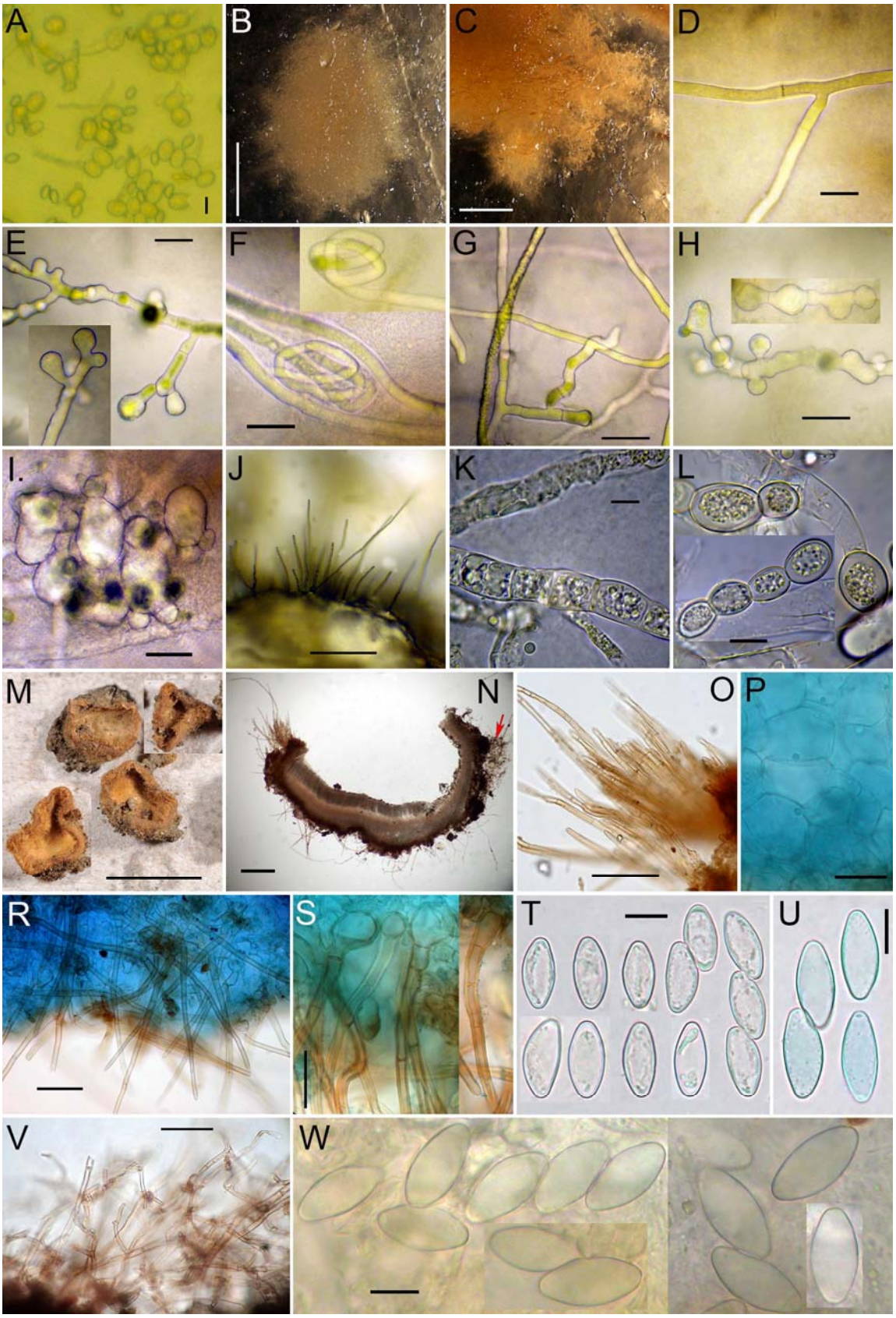
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FIGURE 16. *Tricharina tophiseda*—Colony and ascorhizoctonia-type anamorphic structures on CYA (a-j) and MEA (k-l). *T. japonica*—Apothecia, ascospores and excipular structures (m-w). **a.** Germinating ascospores. **b.** Colony after 1 day. **c.** Colony rim after 2 days. **d.** Mycelial branching with the septal positions. **e.** Terminal and lateral nodules. **f.** Mycelial loops. **g.** Smooth and rough walled hyphae. **h.** Young moniliform sporodochia-like cell aggregates. **i.** Ripe moniliform sporodochia-like cell aggregates. **j.** Aerial hyphae (after 5.5 days). **k.** Hyphae preceding to formation of sporodochia-like cell aggregates. **l.** Moniliform sporodochia-like cell aggregates. **m.** Part of the type collection. **n.** Section from the margin to the apothecial base (red arrow). **o.** Marginal hairs. **p.** LBs in the ectal excipular cells. **r.** Excipular hairs (side view). **s.** Excipular hairs with bulbous base (section). **t-u**. Ascospores. **v.** Subicular hyphae. **w.** Ascospores. a-l from (CNF 2/8079); m-o, r, t-v from (CUP-K-(JA-000286), holotype); p, s, w from (R.D. 31.01.245.11). d-k, w in $^{*}H_2O$; n-o, t, v in †KOH; p-s, u in †CB. Scale bars: b-c, m = 5 mm; n = 200 µm; j, o, r = 100 µm; s, v = 50 µm; a, h-i, p = 20 µm; d-g, k-l, t-u, w = 10 µm. Phot. I. Kušan & N. Matočec. **(page 54)**

4.3.3. Proposition of the emendation of the genus *Ascorhizoctonia*

Ascorhizoctonia **Chin S. Yang & Korf**, Mycotaxon 23: 468 (1985) ***emend. prov.*** **Matočec & I. Kušan**

Apothecia sunken in the substratum up to halfway up the apothecial flanks; ascospores ellipsoid with conspicuously tapered ends, walls thick (0.7–1 µm), perispore cyanophilic, not loosening in CB, smooth or finely verrucose, RBI 1–2, containing only BSG; asci arising from compact croziers; hairs distributed only on marginal area, hair base narrow; colonies concentrically banded on MEA, of moderate growth (reaching 5–6 cm diam. on MEA at fourth day); ascorhizoctonia-type anamorph normally formed on MEA; mycelia forming loops on water agar and CMA; saprotrophic; so far including species with yellowish to ochre hymenia in fresh apothecia, occurring only on burnt substrates (see Table 7).

Type: *Ascorhizoctonia praecox* Chin S. Yang & Korf, Mycotaxon 23: 475 (1985)
= *Peziza praecox* P. Karst., Notiser ur Sällskapets pro Fauna et Flora Fennica Förhandlingar 10: 124 (1869)

= *Lachnea praecox* (P. Karst.) Sacc., Sylloge Fungorum 8: 183 (1889)

= *Scutellinia praecox* (P. Karst.) Kuntze, Revisio Generum Plantarum 2: 869 (1891)

= *Tricharia praecox* (P. Karst.) Boud., Icones Mycologicae, Tome 2: pl. 349 (1905–1910)

= *Tricharina praecox* (P. Karst.) Dennis var. *praecox*, Kew Bulletin 25(2): 338 (1971)

Other species:

Ascorhizoctonia intermedia Egger, Chin S. Yang & Korf, Mycotaxon 23: 474 (1985)

= *Tricharina praecox* (P. Karst.) Dennis var. *intermedia* Egger, Chin S. Yang & Korf, Mycotaxon 24: 507 (1985)

5. DISCUSSION

5.1. Biogeography and re-description of the taxonomically imperfectly known species *Hamatocanthoscypha rotundispora* (*Helotiales*)

The first impression of *Hamatocanthoscypha rotundispora* hymenial elements under oil immersion is that living asci contain mostly ± roundish ascospores. In such asci ascospores are apically densely packed under turgor with their long axes ± perpendicularly oriented to the ascus axis and aligned in a polar view. This appearance might have leaded the species authors to name the taxon after a polar view of the spores (Galán & Raitviir 1994). The protologue gives spores as "broadly ellipsoid to subglobose" which means that the Q value should be 1.05–1.30 according to Bas (1969). Re-examination of the type and treatment of additional collections showed that the Q value range is 1.39–2.09 in living spores and 1.29–2.50 in dead spores. Regarding the basic discrepancies between the species name (epithet "*rotundispora*"), the protologue, and observed spore shape in this study, I have proposed revised and supplemented mathematical delimitation of straight, prolate, radially and bilaterally symmetric spore shapes (Tables 2, 3) in the Materials and methods chapter. Thus, *Hamatocanthoscypha rotundispora* living spores are subciborioid to ciborioid or rarely amygdaliform in lateral view and limoniform to oculiform in dorsiventral view.

A new type of cell inclusion was discovered in this research and described here as refractive cytoplasmic globules (RCGs) (Fig. 6e, Fig. 8b, d, e, h, j). I assume that this type of microscopic element is cytochemically most similar to vacuolar bodies (cf. Baral 1992). The main differences between those two elements are: (a) CRB stains RCGs greyish violet versus turquoise blue in VBs; (b) in IKI RCGs are not stained whereas VBs are frequently stained in a shade of rusty ochre to orange yellow; (c) RCGs are here found exclusively in surface cells of marginal tissue, basal hair cells and ectal excipular flank cells but not in the paraphyses and apical hair cells where VBs typically occur. Metachromatic corpuscles (MCs) differ from RCGs by being invisible in tap water and IKI in living cells. Furthermore, MCs appear during the flocculation process inside non-refractive globose vacuoles when CRB is added and after a certain period of exposure whilst RCGs are readily visible and stable prior and after application of all these staining procedures.

The applied ascospore morphometrical analysis produced the following conclusions: **(a)** A significant intraspecific variability was found within *Hamatocanthoscypha rotundispora* collections in both living and dead material (see Table 4). This could be a consequence of different ecological conditions (e.g. different substrates and bioclimates) and/or of intraspecific evolutionary divergence (i.e. existence of geographical races). The four collections that were analysed statistically differed significantly from each other, perhaps because they originate from substrates of three different host species and three countries: *Juniperus thurifera* from Spain (AH6696), *J. phoenicea* from Croatia (CNF 2/8847) and *Cupressus macrocarpa* from France (CNF 2/8889 and CNF 2/9110). Additional cause of significant differences could originate from methodological issues when studying ascospores in the dead state (living ascospores fixed with KOH vs. dead ascospores obtained from exsiccata mounted in KOH). Testing of these hypotheses will be possible after higher number of analysed statistical samples. Therefore, I do not propose to establish any taxonomic implications at the moment until more material will be analysed using the above standardised procedure. **(b)** The use of living material for ascospore morphometrical analysis is more reliable than the traditional approach based on dead material (which could suffer from non-homogeneous ascospore shrinkage) (see Table 5). Baral (1992) wrote that L and W are reduced for 10–20% and V for 30–50% in dead spores (treated with MLZ) when compared to living state (in tap water). Spore size mean values from HR(CNF 2/8847) and FR(CNF 2/9110) demonstrate a shrinking effect in L of 15–35%, in W of 15–32% and in V of 38–70%. The observed higher values of shrinkage in our samples can be the result of using KOH instead of MLZ as was used by Baral (1992) and particularly differences in cell wall elasticity between the taxa. **(c)** The use of non-parametric statistics is a more reliable approach to describe morphometrical variability of ascospores, because distributions of their dimensions could significantly deviate from normality (see Table 4).

Seven species of the genus *Hamatocanthoscypha* are confined to or usually found on conifers and five of them [*H. rotundispora*, *H. laricionis* (Velen.) Svrček, *H. minutissima* (Velen.) Raitv., *H. phaeotricha* (K. Holm & L. Holm) Raitv. and *H. uncinata* (W. Phillips) Huhtinen] are exclusively foliicolous. Apparently, a discovery of *H. rotundispora* on decaying leaves of *Juniperus thurifera* by Galán & Raitviir (1994) was accidental since coniferous substrate is the ecological domain for *H. laricionis* var. *laricionis*. According to Huhtinen (1990) *H. laricionis* var. *laricionis* is confined to various litter elements of *Pinaceae* and acicular-leaved *Cupressaceae*, i.e. *Juniperus nana* (Müller

1968), and accidentally it can invade also *Lycopodium* and *Rhododendron*. Apparently, *H. rotundispora* is confined to scale-leaved *Cupressaceae* (*Cupressus* and *Juniperus* subgen. *Sabina*) limited to the warm Atlantic – Circum Mediterranean area (cf. Vidaković 1982). Its known distribution is limited to three European countries: France (Hairaud 2006, this study), Spain (Galán & Raitviir 1994, E. Rubio *pers. comm.*) and Croatia (this study). All known localities of this species are roughly aligned to the January isotherm of +5 °C mean air temperature (Fig. 17).

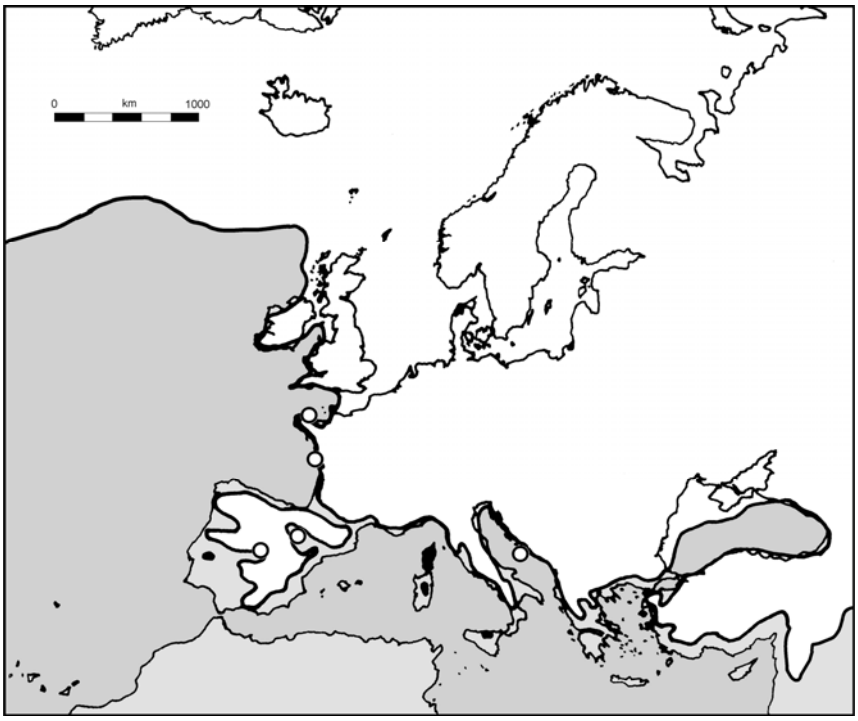


FIGURE 17. Known distribution of *Hamatocanthoscypha rotundispora* with superimposed January isotherm of +5 °C mean air temperature after Stanners & Bourdeau (1995).

5.2. Taxonomic studies on *Thecotheus* species with apiculate spores (*Pezizales*)

Differential characters of *Thecotheus platyapiculatus* nom. prov. and *T. keithii*

Thecotheus platyapiculatus is very similar to the common and widely distributed *T. keithii* sharing the same type of substrate (horse dung), a hamathecium that contain two kinds of paraphyses, ellipsoid ascospores without lateral ornamentation and similar ascospore dimensions: (16)17–21(22) × (7)7.5–10(11) µm in *T. keithii* (Aas 1992) and *(16.9)17.4–20.7(21) × 8–9.5(9.6)

µm / †(16.5)16.6–19.5(20.2) × (7.6)7.7–9.1(9.5) µm in *T. platyapiculatus* (this study). Even though ascospore dimensions are so similar, other qualitative and quantitative characters separate these two species. These are: (1) constantly flat and plate-shaped apiculi of low height (0.2–0.7 µm) vs. much longer, variable apiculi in *T. keithii* that can be long and narrow or hemispherical to pulvinate, papillate to acute or pad-like and are 1.4–5.6 µm high (Aas 1992) in the ascospores with undisturbed surface structures and (2) *T. keithii* has radially symmetric and homopolar ascospores vs. *T. platyapiculatus* with predominantly slightly heteropolar and/or bilaterally symmetric ascospores. Strongly inequilateral spores are found in *T. inequilateralis* but this species has smaller spores (13–16 × 7–8.5 µm) with hemispherical apiculi (1–2 µm high; 2–3 µm diam.). Additionally, *T. platyapiculatus* has true strangulate asci, as in *T. strangulatus* (Velen.) Aas & Lundqvist and *T. uncinatus* Aas, both of which have non-apiculate spores. *Thecotheus platyapiculatus* and *T. keithii* can be further differentiated by details in excipular tissue. The medullary excipulum is comparatively thinner and lacks globose to subglobose and wide elongated cells in *T. platyapiculatus* when compared to *T. keithii*. Also, cytoplasmic pigments and/or encrustations in terminal cells on excipular surface as well as in paraphysis apical cells are lacking in *T. platyapiculatus*. In this study I did not re-examine the type material of *T. keithii* but rely on detailed description and numerous microphotographs given by Aas (1992) who analysed and verified 14 collections from Europe and North America. I consider the differences in *T. keithii* vs. *T. platyapiculatus* (represented with two collections) as well established. Even though Aas (1992) considered *Ascophanus appendiculatus* Alf. Schmidt as a most probable synonym of *T. keithii*, it cannot be verified since no voucher specimen is known to exist. This species surely belongs to the genus *Thecotheus* and according to Schmidt (1912) has ascospores with cap-like apiculi, 1-2 µm high, and therefore it cannot be conspecific with *T. platyapiculatus*.

There is almost no molecular phylogenetic research on the genus *Thecotheus*. National Center for Biotechnology Information (NCBI) database holds one DNA sequence data of a single *Thecotheus* species, *T. holmskjoldii* (Landvik *et al*. 1998a).

Living cell inclusions

According to Aas (1992), Wang & Kimbrough (1993), Wang (1994) and Doveri & Coué (2008), some species in the genus *Thecotheus* have two types of paraphyses: (1) cylindrical-obtuse to

cylindrical-clavate and enlarged in the apical part and (2) filiform and slender. In *T. platyapiculatus* paraphyses of type (1) possess subhyaline semi-resistant vacuolar bodies (SVBs) and type (2) contains scattered minute true vacuolar bodies (VBs). These semi-resistant vacuolar bodies (SVBs) are here described as new microscopic elements that are cytochemically most similar to vacuolar bodies (cf. Baral 1992). The main differences between these two elements are: (a) CRB stains SVBs greyish blue (Fig. 13e) vs. vivid blue to turquoise blue in VBs; (b) in IKI SVBs are stained greyish yellow (Fig. 13b) whereas here VBs remain unstained; (c) by adding KOH to CRB mount SVBs are "semi-resistant", turning greyish ruby and are only partially and very slowly dissolved (Fig. 13j), while VBs are instantly and completely dissolved without giving any colouration, and (d) in AC test paraphyses cytoplasm of type (1) turn greyish magenta (Fig. 13m) whereas VBs instantly vanish. Mutual features for both of the cell inclusions are their position in the apical paraphyses cells and terminal cells of excipular surface and they are both instantly dissolved after KOH is added to the water mount (Fig. 12k).

Apiculi and the lateral ornamentation in the genus *Thecotheus*

Taxonomic significance of the ascospore apiculi together with the spore lateral ornamentation in the genus *Thecotheus* is unquestionable. Roughly half of the species never produce apiculi. Apiculate-spored species are sufficiently differentiated solely by apiculi and lateral ornamentation in most cases (see key, page 42). The apiculi within the genus *Thecotheus* are distinctly morpho-chemically vulnerable and can easily vanish without any trace or could severely get altered depending on the applied microscopic method and markedly depend on ontogeny (Bronckers 2011). Therefore, guidelines on how to study the ascospores in the genus *Thecotheus* are proposed here. In the course of my vital taxonomical study I have observed and documented in several vital mounting media (H_2O, CRB, CR and IKI) a vast number of fully mature and freshly ejected ascospores in a number of collections of apiculate-spored species within the genus (e.g. *T. holmskjoldii*, *T. lundqvistii*, *T. platyapiculatus* and *T. rivicola*). The apiculi always showed its stable shape, structure and low variable dimensions. Only through the standardised procedure all species could be thus efficiently characterized, identified and mutually compared. Brief guidelines are here recommended as follow:

(1) *Ontogeny*. The apiculi in immature spores are not yet developed or just have started to develop and are not representative for a given species. Spores in the first phase of germination (Baral 1992: 375) (i.e. overmature spores) can easily alter or loose their original apiculi and also should be disregarded in the study on apiculi. Only mature and freshly ejected ascospores (from living mature asci) are reliable and possess the original apicular structures. However, cases can exist where some living mature asci contained spore sets with one or few underdeveloped spores among normally developed ones (Pfister 1981, Bronckers 2011). Therefore, we can expect the occurrence of spores without apiculi or with underdeveloped apiculi to be present at low numbers among the accumulations of freshly ejected spores in the microscopic mount. Such spores should be disregarded from the study of apiculi.

(2) *Living material*. The importance and the methods of studying the living material (contains living cells and tissues) was elaborated in detail by Baral (1992). Only fresh material containing living mature asci of a given *Thecotheus* species will produce the spores with unaltered original surface structures. To reduce the appearance of immature spores with underdeveloped or not developed apiculi make the apothecial vertical median sections by hand using a razor blade and do not apply any pressure to coverslip. After a short time freshly ejected ascospores should accumulate above the hymenium if the specimen contains living asci (markedly protruding above paraphyses level). Freshly ejected spores can be also caught in a hanging drop of tap water placed above the ripe apothecia. Living spores can be observed also in fresh material if trapped inside dead asci but only the spores displaying apiculi should be taken into account. If a given specimen still contains living mature spores inside dead asci they will often display the apiculi if treated directly with CB. When working with CB the precaution should be taken not to overheat the sections. The specimen of *T. platyapiculatus* dried at the room temperature still contained ca. 50% of living mature spores 3.5 years after its collection (Figs. 12i, 13i).

(3) *Aggressive mounting media*. Aggressive media such as KOH, MLZ and AC can quickly alter the original surface structures including apiculi. Apiculi can be either dissolved or irreversibly loosened / swollen (Fig. 12l). Therefore these media should not be used for observing size and shape of the apiculi. The species with smooth lateral spore walls may easily become wrinkled in such media (Fig. 12m). Cotton blue is also classified as aggressive media because the procedure includes heating and clarifying the sections with 50% (wt/wt) chloral hydrate, but this media if applied properly is of great value and should be used because it provides opportunity for a number of important

observations: cyanophility, clear visibility of ornament including its fine details and separability of the perispore.

(4) *Recommended mounting media*. In order to observe the ascospores with fully developed and unaltered original surface structures (apiculi and lateral ornamentation) it is important to use "vital" mounting media (e.g. H_2O, CRB, CR and IKI). Because of their isotonic feature and non-aggressive chemical properties, the apiculi of fully mature ascospores should always retain in its native configuration and dimensions (cf. Baral 1992).

(5) *Documentation*. In most cases, the original, unaltered apicular structures cannot be obtained on dead spores. It is therefore essential to make sufficient documentation (i.e. microphotographs and notes) to record the original, native state of these important taxonomic characters for future use along with the rest of the voucher material (dried specimen, permanent slides, etc.). The importance of reliable and sufficient collection documentation is also explained in Huhtinen (1990) and Baral (1992). It is equally essential that the worker clearly quote the observation technique in which the given character is documented / measured.

Thecotheus culturing

There are several references about obtaining *Thecotheus* in pure culture. Dodge (1912) successfully cultured primary mycelium of *T. pelletieri* (P. Crouan & H. Crouan) Boud. and Conway (1975) obtained primordial development of *T. pelletieri* and ascomata of *T. cinereus* (P. Crouan & H. Crouan) Chenant as well as imperfect state of both species but conidial production only in *T. pelletieri*. Pfister (1981) recorded only spore germination of *T. phycophilus*. The entire life-cycle (from ascospore to ascospore) was reconstructed in culture by Ranalli & Mercuri (1998) who obtained mature apothecia of *T. cinereus*, *T. pelletieri* and *T. crustaceus* and anamorph with abundant conidial production similar to *Sporothrix* Hektoen & C.F. Perkins in the latter two species.

Culture studies performed in the present study did not yield spore germination on two tested media (DA and DOA). The reason that ascospore germination was not successful in this procedure might be because living ascospores were applied to the agar surface by placing the rehydrated apothecial fragments instead of shooting spores from the living asci and / or keeping incubated material in a constant dark (cf. Ranalli & Mercuri 1998).

Biogeography and ecology

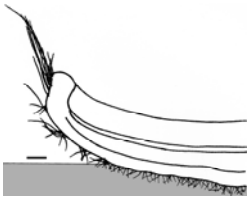
The most similar species to *T. platyapiculatus*, *T. keithii*, is reported by Kimbrough (1969) from Canada and USA under the name *T. apiculatus* Kimbr. Aas (1992) verified its existence in Finland, Sweden, United Kingdom, Canada and USA, but records from Japan, Poland and former USSR remained unverified by him. Prokhorov (2004) reports it from Tajikistan and Turkmenistan as *T. apiculatus*. Bronckers (2011) recorded this species in the Netherlands. In addition, several internet resources quote this species also for Belgium, Germany and Norway. *Thecotheus platyapiculatus* is so far known only from western Croatia, the area above 1500 m a.s.l. situated among the highest peaks of northern part of coastal Adriatic Mt. Velebit. The habitat is the alpine grassland (pasture) developed on a very thick limestone beds (hyperkarst) where horses have been traditionally bred and kept outdoors for the most of the year. The area represent the sharp climatic barrier and it is subjected to very harsh ecological conditions, exposed to extreme and frequent bora wind strikes, occurring as abrupt irruptions of very dry and cold air masses. Winters are very cold, with long persistent snow cover. Summers are characterised by strong droughts, enhanced by highly permeable limestone bedrock often interrupted by strong cyclonic activities. Frosts are possible in night and in the morning even during the summer months.

5.3. Taxonomic studies in the genus *Tricharina* (*Pezizales*)

Comparison of *Tricharina tophiseda* nom. prov. and *T. japonica*

Microscopic analysis of living apothecia of *Tricharina tophiseda* showed that this species is similar to *T. japonica* based on the very long marginal hairs (the longest in the genus) and size of the ascospores (cf. Yang & Korf 1985a). However, the ascospores of *T. japonica* were described as trapezoidal, a shape that was not observed in *T. tophiseda*. Therefore, it was necessary to examine the type material along with the European collection (Dougoud & De Marchi 2012), which confirmed this character. My examinations further showed that *T. japonica* differs from *T. tophiseda* in several important microscopic and macroscopic characters, summarised below (Table 6).

TABLE 6. Differential characters of *Tricharina tophiseda* and *T. japonica*. Scale bar in the diagrammatic sections = 0.2 mm.

	Tricharina tophiseda	***Tricharina japonica***
*Hymenium colour	light orange	whitish or dirty grey to pale grey
Ascospores:		
Shape / †Q	ellipsoid-limoniform to narrowly ellipsoid-oculiform / 1.52–1.83	oculiform to fusiform / 1.85–2.51
†Dimensions	15.1–18.2 × 9–10.9 µm	16.4–21.5 × 7.7–9.8 µm
Symmetry	radially symmetric	bilaterally symmetric (laterally trapezoidal)
Poles in CB	slightly tapered	tapered to papillate
Marginal hairs: middle pt. diam. / basal pt. diam.	9–22 µm / 19.5–42 µm	5.7–13 µm / 8.5–22.5 µm
Excipular true hairs	only upper flank	whole excipular flank
Distribution of excipular LBs	only in medullar wide cells	in medullar wide cells (Dougoud & DeMarchi 2012) and cells of ectal excipular inner layer
Contact with the substratum	sunken in the basal half 	centrally attached apothecia 

Most notably, the ascospore length/width ratio in *T. tophiseda* is much lower than that seen in *T. japonica* (cf. Table 6). During the revision of *Tricharina japonica*, 100 ascospores were measured from each dried collection to obtain statistically reliable sample sizes in order to discriminate between *T. japonica* and *T. tophiseda* using ascospore length and width. The analysis showed that the spore length in *T. japonica* is slightly different than stated in the protologue, i.e. 16.4–21.5 µm rather than the initially reported 15.8–19 µm. Living ascospores from the recent collection of *T. japonica* (R.D. 31.01.245.11) were also measured for comparison with *T. tophiseda*. These measurements indicated that young and immature spores in *T. japonica* do not display larger spore dimensions, contrary to the observations in the protologue (Yang & Korf 1985a). Instead, they are considerably smaller (14.4–17.4 × 6.2–8.3 µm), less refractive than mature spores, and possess an under-developed and thin spore wall.

The marginal hairs of *T. tophiseda* and *T. japonica* are similar, being straight, partly stiff and thick-walled, with strongly tapered apex in the longest hairs, but also with rounded apices in shorter hairs (Figs. 14g-h, 15f-g, 16o). However, the shape of the basal cell differentiate these two species, being sub-bulbous to bulbous in *T. tophiseda* vs. prismatic-truncate or rarely bulbous in *T. japonica*. In

T. japonica, true excipular hairs were observed to extend down the whole excipular flank, which is an important but hitherto unreported feature (Fig. 16n), while those of *T. tophiseda* are confined only on the upper excipular flank. The apothecial bases of both *Tricharina* species are covered with hyphoid subicular hyphae with bulbous bases, but in *T. japonica* they are richly branched and occasionally anastomosed (Fig. 16v), while in *T. tophiseda* they are rarely branching and not anastomosing at all.

Both species are probably saprotrophs, living on different substrates in humid habitats. *Tricharina tophiseda* was found twice on a continuously wet tufa barrier (outside of an area with tufa-forming mosses), surrounded with scarce minute plant remnants, and shaded by *Populus* sp., *Fraxinus angustifolia*, *Platanus* sp. and *Ficus carica* riparian vegetation. In contrast, the Swiss collection of *T. japonica* was found on bare sandy soil in a riparian forest with *Quercus* sp., *Fraxinus* sp., *Alnus* sp. and *Corylus* sp. (Dougoud & De Marchi 2012). More precise ecological data for the type collection were not provided (Yang & Korf 1985a). Cultural studies of *T. tophiseda* and *T. japonica* were unsuccessful from rehydrated apothecia on the same media that Yang & Korf (1985b) used in their ascorhizoctonia-type anamorph studies. The acidity of agar plates was pH 4 (more acidic than CYA pH 6.3 and MEA pH 5.3 used for inoculation from fresh apothecia of *T. tophiseda*) which may have inhibited ascospore germination, especially for *T. tophiseda* which inhabits an alkaline tuffaceous substratum.

Re-evaluation of some taxonomic features in the genus *Tricharina* and allied taxa

Small granules of low refractivity (here abbreviated as BSG, cf. Table 7) are generally found in polar areas of the *Tricharina* ascospores (Yang & Korf 1985a). These granules do not stain in IKI, CRB, CB and AC, and partly coalesce after adding KOH and in dead spores (present study). All these features correspond to the lipid bodies (Baral 1992) but Yang & Korf (1985a) ascertain that the granules are not stained with Sudan IV in ethyl alcohol and therefore could not consist of lipids. Their assumption that these granules might be glycogen accumulations (in IKI staining red brown) in the scale of the whole genus is not correct. The presence of glycogen in spore polar areas, beside BSG, is confirmed only in *Tricharina ascophanoides* (Boudier) Chin S. Yang & Korf (personal unpublished data). The same feature is also depicted in Lindemann (2013, p. 45). Seemingly the same structures in the spore polar areas demonstrated in *Paratricharina poiraultii* (Boud.) Van Vooren, U. Lindemann, M. Vega, Ribes, Illescas & Matočec (Table 7) are, however, true lipid bodies as ascertained in Van

Vooren *et al*. (2015). They readily coalesce to form larger guttules in freshly ejected living spores in a short period of time and when rehydrated from the dried material, spores are biguttulate. Unlike the polar lipid bodies, BSG partly coalesce only after thorough cellular desiccation or in aggressive media such as CB and KOH, and retain original conformation in AC (Fig. 15n).

Attempts to study *Tricharina tophiseda* in axenic culture were successful, and mycelium producing an ascorhizoctonia-type anamorph was obtained on CYA (Fig. 16a-j) and MEA (Fig. 16k–l). Cultures developed rapidly, covering the whole 9 cm Petri dish in 2.5 days on CYA and in 4 days on MEA. This is the most rapid growth in the whole genus followed by *T. ochroleuca* (Bresadola) Eckblad which reaches 6.9–8 cm and *T. cretea* (Cooke) K. S. Thind & Waraitch 7–7.6 cm on MEA in 4 days. The complete absence of concentric bands on colonies was hitherto known only in *T. cretea*, and is now also noted in *T. tophiseda*. In *T. gilva*, which is a type species of the genus *Tricharina*, concentric bands become distinguishable only in aged plates and mycelial growth reach 6.8–7.4 cm on MEA on the fourth day. Unlike *T. tophiseda*, *T. gilva* does not produce sporodochia on MEA.

The true excipular hairs in *T. japonica*, extending down the whole excipular flank, resemble those found in the genus *Wilcoxina* (Yang & Korf 1985a). However, I did not find any other evidence that *T. japonica* should be placed in this genus. Ascospores from the recent collection from Switzerland failed to germinate following the procedure of Yang & Korf (1985b), and so no anamorph currently known to help assign this species to a more appropriate genus. The genus *Wilcoxina* is considered a mycorrhizal genus (E-strain fungi, Table 7) with all members forming mycorrhiza with *Pinaceae*, but data about the presence of conifers on *T. japonica* collection sites has not been discussed in the literature (Yang & Korf 1985a, Dougoud & De Marchi 2012).

In this study *T. japonica* and *T. tophiseda* are accommodated in the genus *Tricharina* on the account of the following characters: thin-walled ascospores with RBI ~0.5 (Table 7), presence of BSG, absence of cyanophilic perispore, asci arising from perforated croziers (Fig. 14d), rapidly growing colonies (regularly reaching above 7 cm diam. on MEA on the fourth day), colonies not banded (MEA) and the ascorhizoctonia-type anamorph produced by *T. tophiseda* (Table 7). However, since a number of phylogenetic studies have demonstrated polyphyly in this genus (Egger 1996, Perry *et al*. 2007, Hansen *et al*. 2013, Stielow *et al*. 2013, Van Vooren *et al*. 2015), a phylogenetic analysis of the former two species is planned as part of my ongoing research in pyronemataceae fungi.

TABLE 7. Character diversity in selected *Tricharina* species groups and similar allied genera in *Geopora-Tricharina* clade (cf. Tedersoo *et al.* 2006).

Genus / ecology	RBI[a]	Spore sheath / ornamentation & perispore		Paraphysis pigment	True hair vestiture	Apothecial contact with substrate	Germination; mycelial growth / anamorph	True hairs
Wilcoxina ECM, ECEM[b] (E-strain fungi)	~0.5 BSG	none /smooth CB+ perispore		none or scanty	marginal & lateral	basal	slow; complexipes-type	single type: arising from thick-walled isodiametric cell rows
Tricharina gilva (type species) saprotroph	~0.5 BSG	delicate & temporal, only in CRB-KOH /without perispore, CB- wall		none, restricted to subhymenial cells	marginal only	⅔ sunken	rapid; ascorhizoctonia-type (6.8–7.4 cm on MEA)	single type: gradually widened towards the base
Tricharina japonica & *T. tophiseda* saprotroph?	1 BSG	none /smooth CB- perispore		none, restricted to subhymenial cells	marginal & lateral	basal	rapid; ascorhizoctonia-type (8.5 cm on MEA)	two types: marginal longer than lateral, fascicled; arising from bulbous cell
"*Tricharina*" *ochroleuca* group ECM	nearly 0 BSG	none /without perispore, CB- wall		none to scanty	marginal only	½ sunken	rapid; ascorhizoctonia-type (various growth rates)	single type: basally gradually broader
"*Tricharina*" (*T. praecox* & *T. intermedia*) saprotroph, on burnt substrate	1-2 BSG	none /minute CB+ verrucose or smooth CB+ perispore		yellow, granular, scattered & free from vacuoles	marginal only	½ sunken	moderate; ascorhizoctonia-type (4.9–6.2 cm on MEA)	single type: basally narrow
Paratricharina poiraultii ECM?	2 LBs	persistently encapsulated in all media /CB- verrucae & CB+ perispore		scanty, granular in vacuoles	marginal & lateral	basal	unknown	two types: marginal shorter than lateral, fascicled
Hoffmannoscypha ECM?	4-5 LBs	none / finely verrucose, perispore presence and cyanophility unknown		abundant, orange, granular, scattered & free from vacuoles	marginal & lateral	basal	unknown	single type: basally narrow
Geopora s.l. ECM	4-5 LBs	none /smooth, perispore absent in fully mature spores		none	marginal & lateral[c]	⅔ sunken to hypogeous	unknown	two types: marginal ±reduced to pustulate texture, shorter than lateral

[a]RBI: 0 = no content; 1 = 1–5%; 2 = 6–20%; 3 = 21–40%; 4 = 41–70%; 5 = 71–90% of total sporoplasm volume; [b]ECEM = ectendomycorrhiza; [c]In epigeous spp.

Yang & Korf (1985b) established the genus *Ascorhizoctonia* to accommodate anamorphs of some *Tricharina* species. Three species [*A. cretea* Chin S. Yang & Korf, *A. intermedia* and *A. praecox* Chin S. Yang & Korf] were soon reduced to varieties of *T. praecox* (P. Karst.) Dennis, based on apothecial morphology, despite some morphological variation and distinct *Ascorhizoctonia* anamorphs. A monograph of the genus *Tricharina* (Yang & Korf 1985a) treated these taxa as *T. praecox* var. *praecox*, *T. praecox* (P. Karst.) Dennis var. *cretea* (Cooke) Chin S. Yang & Korf and *T. praecox* (P. Karst.) Dennis var. *intermedia* Egger, Chin S. Yang & Korf. *Tricharina praecox* is consistently positioned at a distance from the core *Tricharina* clade comprising *T. gilva* (the type species of *Tricharina*) and other *Tricharina* species in all phylogenetic studies involving *Tricharina* (e.g. Egger 1996, Wei *et al*. 2010, Hansen *et al*. 2013, Stielow *et al*. 2013, Van Vooren *et al*. 2015) (Figs. 18, 19). *Tricharina gilva* differs from *T. praecox* by a number of important characters (viz. non-cyanophilous perispore, blunt ascospore ends, perforated ascogenous cells, inability to form an ascorhizoctonia-type anamorph on MEA, and different ecological features, as detailed in Table 7). Because of these divergent characters, it is proposed here to accommodate *T. praecox* var. *praecox*, and its apparent closest relative *T. praecox* var. *intermedia*, in a genus of their own, separate from the genus *Tricharina*. *Tricharina cretea* [in Yang & Korf (1985a) as *T. praecox* var. *cretea*] is here considered to be more distant from the previous two taxa due to the basally attached apothecia covered with hairs over almost the whole excipular flank, white hymenium of fresh apothecia, elongated fusiform spores, rapid colony growth, colonies not banded on MEA and delimiting septum at 5–7 μm from the corresponding hyphal junction in mycelia.

Since the anamorph of the *Tricharina praecox* is described by Yang & Korf (1985b) under the name *Ascorhizoctonia praecox* and is designated as the type species of the anamorphic genus *Ascorhizoctonia*, here I propose to use the generic name *Ascorhizoctonia* in a restricted sense for *T. praecox* var. *praecox* and the closely related *T. praecox* var. *intermedia*. Although these taxa are currently considered varieties of the same species, morphological differences in their anamorphic stages and ascospore characters suggest these should be considered as distinct species.

Other *Ascorhizoctonia* species [viz. *A. cretea*, *A. gilva* Chin S. Yang & Korf, *A. groenlandica* Chin S. Yang & Korf, *A. hiemalis* Chin S. Yang & Korf, *A. ochroleuca* Chin S. Yang & Korf and *A. ascophanoides* Chin S. Yang & R. Kristiansen], remain synonymous under the corresponding *Tricharina* species until further taxonomic evidence is established. The ascorhizoctonia-type

anamorph for *T. striispora* Rifai, Chin S. Yang & Korf is not named but is described in Barrera & Romero (2001).

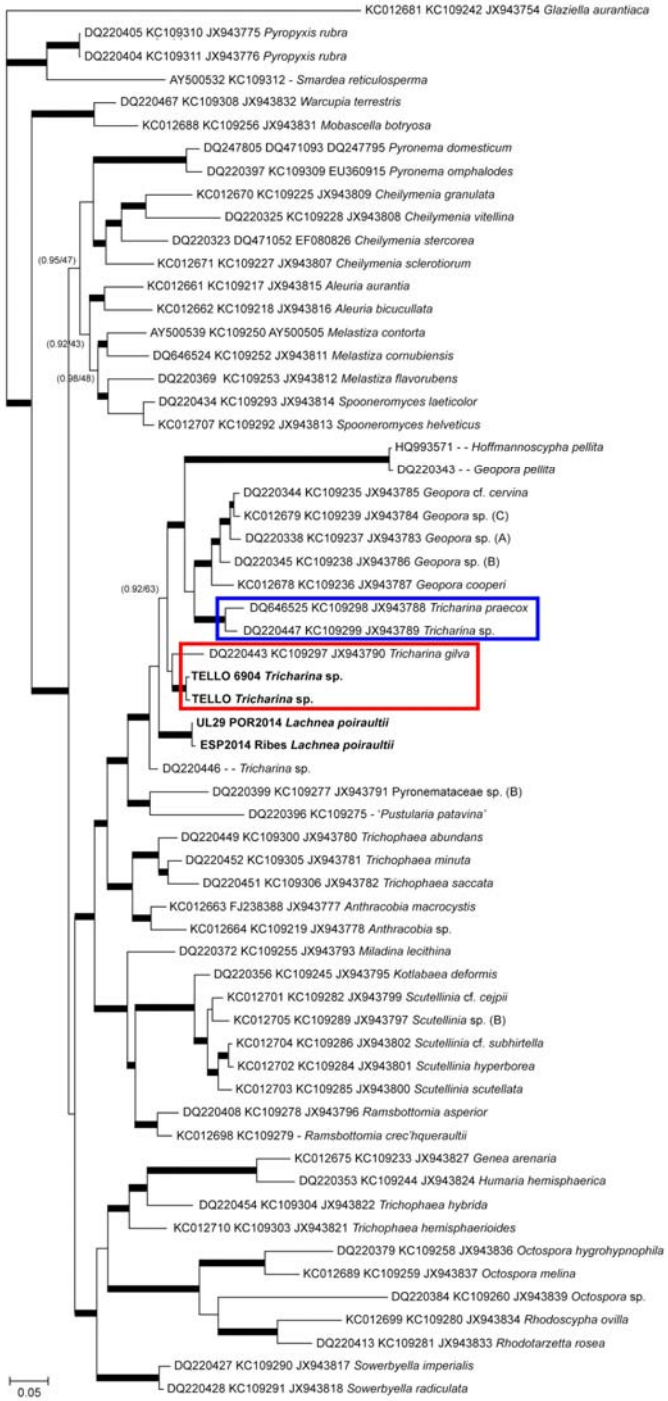


FIGURE 18. Consensus 28S-tef 1α-rpb2 phylogram of the family *Pyronemataceae* (Van Vooren *et al*. 2015). Red rectangle is showing the clade of *Tricharina* s. str. and blue one the position of *Tricharina praecox*.

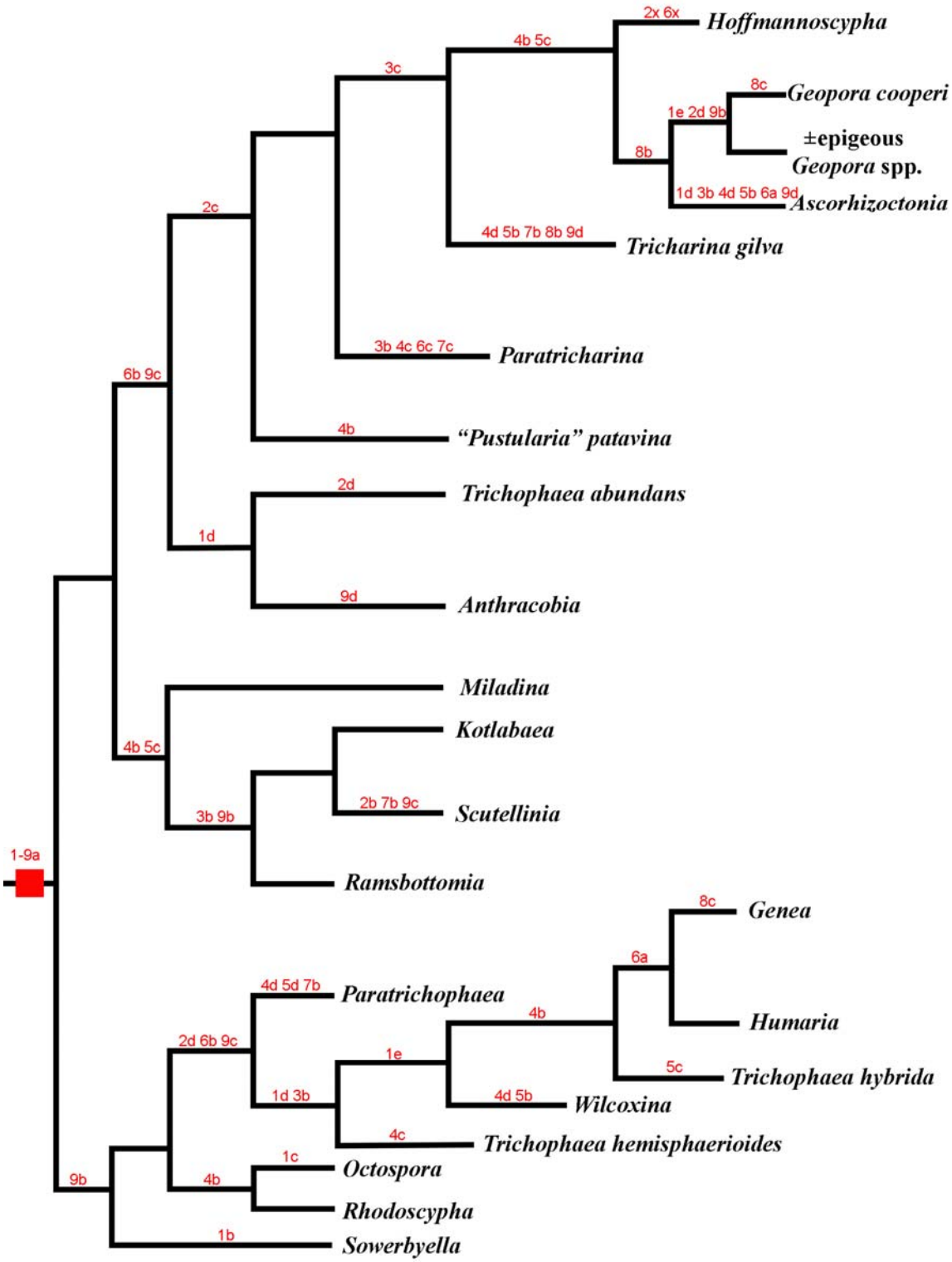


FIGURE 19. A simplified phylogenetic tree taken from Van Vooren *et al*. (2015) modified to display position of the genus *Wilcoxina* with superimposed non-molecular proposed phylogenetic markers (character states). See explanation of character states in the Table 8.

TABLE 8. Explanation of charaters and character states superimposed in Fig. 19.

	Character	Character states
1	Trophic status	1a – terricolous saprotroph; 1b – humus saprotroph; 1c – bryophilous biotrophy; 1d – anthracophilous; 1e – ECM biotrophy
2	Carotenoid pigmentation	2a – abundant, reacting green with IKI; 2b – crystallised, reacting purple with IKI; 2c – reduced (mostly to subhymenial layer); 2d – absent; 2x – abundant, reaction in IKI unknown
3	Perispore	3a – non-cyanophilic; 3b – cyanophilic; 3c – absent
4	Spore RBI content	4a – RBI=3; 4b – RBI=4-5; 4c – RBI=2; 4d – RBI=0-1
5	Spore guttulation	5a – LBs polar; 5b – BSG polar; 5c – guttules dispersed; 5d spores devoid of guttules
6	Spore ornamentation	6a – cyanophilic; 6b – absent; 6c – non-cyanophilic; 6x present but of unknown nature
7	Spore sheath	7a – absent; 7b – delicate or tough and cyanophilic; 7c – permanent non-cyanophilic encapsulation
8	Apothecia vs. substrate	8a – epigeous, basally attached; 8b – epigeous, sunken into the substrate; 8c – hypogeous
9	Apothecial hairs	9a – absent; 9b – hyphoid; 9c – setose; 9d – reduced to margin

Since a number of recent phylogenetic studies demonstrated polyphyly of the genus *Tricharina*, further splitting into several monophyletic small genera would apparently be necessary. Simplified phylogenetic tree (Fig. 19) with superimposed non-molecular characters depicts high overlapping of characters obtained by "vital taxonomy" methods (Table 8) with phylogenetic structure in some lineages of the family *Pyronemataceae*. This shows applicability of these methods in taxonomic considerations based on phylogenies.

5.4. General discussion

In this section, the synthesis of the "vital taxonomy" methods contribution to the general taxonomy of *Ascomycota* is elaborated, focusing mainly on the advantages and implications to effective and clear taxa recognition.

"Vital taxonomy" methods were applied in all aspects to the material collected and treated within this study according to Baral (1992). In my treatment of genera *Thechotheus* and *Tricharina* (*Pezizales*) these methods were expanded with the propagation of living material to axenic culture. The problem of inadequately described taxa is clearly elaborated in the case of *Hamatocantoscypha rotundispora*. The species is thoroughly analysed, both using fresh and dried samples with addition of type material re-examination. Galán & Raitviir (1994) who treated dead material provided its short description without recognizing the true variability of some important quantitative characters (e.g. ascospores dimensions). As well, very important qualitative character – spore shape was

completely erroneously given as “broadly ellipsoid to subglobose”. My analysis of type material and own collections proved that cells and tissues in this tiny ascomycetous fungus irreversibly change in the drying process and tissues are shrinked to the level that separate layers could not be observed any more. On the other hand, the analysis of living structures enabled us to detect higher number of features that could be used in the future taxonomic analyses of other *Hamatocanthoscypha*, *Thecotheus* and *Tricharina* species, i.e. vacuolar bodies in the paraphyses, metachromatic corpuscles in spores, refractive cytoplasmic globules (different from VBs in paraphyses) in hairs, marginal tissues and excipular flank cells. These cell inclusions are easily visible by applying “vital” stains (viz. Lugol solution, Brilliant cresyl blue) to the water mounted sections.

While *Hamatocanthoscypha rotundispora* is a member of the order *Helotiales* which is one of the main focus of Baral (1992), *Thecotheus platyapiculatus* and *Tricharina tophiseda* are members of the order *Pezizales*. Species within the order *Pezizales* are still highly underexplored by means of “vital taxonomy” methods when existing literature data are taken into account. Fruitbodies of the pezizalean fungi are composed of average larger cells and cell inclusions, i.e. vacuoles, glycogene accumulations, lipid bodies, bipolar spore granules and nuclei that are easily visible in living cells. If visible, none of these inclusions retain its original constellation in dead cells.

The use of more advanced statistical analysis in a mycological taxonomic research is rather poor and its usefulness is often neglected. Only in a minority of cases, authors apply descriptive statistics (e.g. Harmaja 1977, Kullman 1982, Huhtinen 1990, Matočec 1998), in a way presented in cases of *Thecotheus platyapiculatus*, *Tricharina tophiseda* and *T. japonica* for ascospore measurements. In the species descriptions length, width and length/width ratio ("Q" value) are given as: (min.) stat. min. – arith. mean – stat. max. (max.) where “min.” = minimum (lowest measured value), “stat. min.” = statistical minimum (arithmetic mean minus two times standard deviation), arith. mean = arithmetic mean, “stat. max.” = statistical maximum (arithmetic mean plus two times standard deviation), “max.” = maximum (highest measured value). Standard deviations of spore length and width are also calculated. Calculations of ascospore measurements are performed either on 50 (in *Hamatocanthoscypha rotundispora*) or 100 ascospores (in *Thecotheus platyapiculatus*, *Tricharina tophiseda* and *T. japonica*). Number of measurements ≥ 50 per slide ensures much clearer limits of the very important quantitative taxonomical characters such as length, width and “Q”value of spores, whereas number of measurements normally performed in the majority of mycological papers

is about 20–25 or even less (e.g. Schumacher 1990, Aas 1992, Palmer *et al*. 1994). Some authors do not provide data on number of measured spores (e.g. Carpenter 1981, Yang & Korf 1985a, Van Brummelen 1995) at all, or do not specify the number of measured spores per collection (Huhtinen 1990). The "Q" value clearly delimits proposed new species *Tricharina tophiseda* from *T. japonica* even though individual measurements of length and width of spores are partially overlapping. More advanced statistical treatment was applied in the case of *Hamatocanthoscypha rotundispora* where beside descriptive statistics, a test for normality (Shapiro & Wilk 1965) was also performed for length, width, Q ratio and volume of spores. This was performed in order to analyse the variability of spore morphometrical variables. Mann-Whitney test (Lehmann 2006) was used for pairwise comparisons of: (1) statistical samples on living and dead spores of the same collection (in order to estimate influence of shrinkage to spore morphometry), (2) statistical samples on living spores of different collections (in order to estimate intraspecific ascospore morphometrical variability using "vital taxonomy") and (3) statistical samples obtained on dead spores of different collections (in order to estimate intraspecific variability based on fixed material). The influence of shrinkage to spore morphometry was once more confirmed here and was similar to those presented in Baral (1992). Because of non-homogenous spore shrinkage, statistical analysis of the variables obtained from living spores was proven to be more reliable than those from dried samples with dead spores. This fact is strongly supporting use of "vital taxonomy" methods vs. traditional ones. Certain intraspecific variability among Croatian, French and Spanish samples of *Hamatocanthoscypha rotundispora* were dicussed in order to be connected with different substrates/plant host species. Additional analyses (e.g. a larger number of spore measurements) should be applied to evaluate possible taxonomic implications.

Ascospores are produced endogenously by compartmentalization of the ascus cytoplasm, in a process termed "free cell formation" by Harper (1897). After meiosis, some processes occur that are typical for the given species or higher taxa: additional mitosis (one or more) and specific pattern of developing the spore wall around nuclei (including the wall ornamentation). These processes are genetically controlled (Turian & Hohl 1981) and are of high importance at the species, genus and family level (cf. Berthet 1964, Bellemère 1994). Qualitative (e.g. shape), quantitative (e.g. measurements) and meristic (e.g. number of nuclei) ascospore features are among the most important taxonomic characters in the phylum *Ascomycota*. Despite of their unquestionable

taxonomic importance, these characters were too often erroneously reported or interpreted in ascomycete species descriptions. The three examples treated in this thesis are not exceptions. After re-examination of type material and analysis of both fresh and dried samples I found both spore shape and size to be erroneously defined in the protologue of *Hamatocanthoscypha rotundispora* (Galán & Raitviir 1994). The importance of fungal spore geometry was previously pointed out by Domínguez de Toledo (1994) and spore shape naming by Bas (1969). Because of inconsistencies and lack of clear criteria in spore shape naming, a standardised tool for determining the ascospore shape regarding the spore elongation, spore symmetry and polarity for prolate straight symmetric spores using geometric criteria is proposed here. Similarly, the protologue of *Tricharina japonica* also gives ascospores variation ranges different (with somewhat lower values) than it is ascertained here, while ascospore apiculi in *Thecotheus* although essential in species recognition, are quite often inadequately analysed and interpreted in some papers (Wang 1994, Doveri & Coué 2008, Bronckers 2011).

The importance of living cell inclusions (e.g. nuclei and nucleoli, vaculoar bodies, lipid bodies, metachromatic corpuscles, KOH-soluble cytoplasmic bodies etc.) in taxonomy of ascomycetous fungi is ascertained and elaborated by Baral (1992). Cell inclusions disappear or irreversibly change in dead cells and its presence is easily detectable by use of "vital" stains/reagents, i.e. Lugol's solution, Brilliant cresyl blue and Congo red. It is shown in this research that some cell inclusions are still unreported and/or are undescribed to science. In the case of *Hamatocanthoscypha rotundispora*, refractive cytoplasmatic globules (RCGs) are recognized and newly described. Beside differences in vital staining, these inclusions are found in *H. rotundispora* exclusively in surface cells of marginal tissue, basal hair cells and ectal excipular flank cells but not in the paraphyses and apical hair cells where vacuolar bodies usually also occur. In the case of *Thecotheus platyapiculatus*, a new microscopic element that is cytochemically most similar to vacuolar bodies is described and named as semi-resistant vacuolar bodies (SVBs). These cell inclusions are situated in one of the two types of paraphyses, usually present in some species in the genus *Thecotheus* (Aas 1992), which are cylindrical-obtuse to cylindrical-clavate and enlarged in the apical part. The other type of paraphyses is filiform and slender and devoid of such cell inclusions but contain very similar scattered minute true VBs. Semi-resistant vacuolar bodies are noted also in some other *Thecotheus* species (pers. data). Mutual features for both of the cell inclusions are their position in the apical

paraphyses cells and terminal cells of excipular surface and they are both instantly dissolved after KOH is added to the water mount. Cell inclusions in spores of *Tricharina* spp., present both in living and dead state, are defined here for the first time as bipolar spore granules (BSG). These small granules of low refractivity are normally found in polar areas of the *Tricharina* spp. ascospores (Yang & Korf 1985a). Even though its features correspond to the lipid bodies (Baral 1992), Yang & Korf (1985a) ascertain that the granules do not consist of lipids. Their assumption that these granules might be glycogen accumulations in the scale of the whole genus is not correct. For now, the presence of glycogen in spore polar area, is confirmed only in *Tricharina ascophanoides* (pers. unpublished data) where it is clearly separated from bipolar spore granules that do not stain in Lugol's solution.

Every adequately designed area in biological and spatial sense that is under law protection (such as national parks or nature parks) is of a paramount importance to the taxonomy in general. If such protected area includes ecologically valuable, rare or endangered and vulnerable habitat types, then it provides lasting protection of security and survival for the vast number of fungal species. This is very important for highly specialized species confined to a certain habitat types (such as tuffa barriers, traditionally managed grassland, endemic species as hosts for specific fungal species etc.). The areas of such exceptional biological value, ascertained in this paper are Sjeverni Velebit National Park and Krka National Park in which two proposed new species are found: *Thecotheus platyapiculatus* and *Tricharina tophiseda*. However, I cannot conclude that these two are endemic species until more research in this respect is done. While horse dung on which *T. platyapiculatus* is found represents a world-wide substrate type, tuffa barriers are highly specific, small and highly isolated habitat type which could theoretically house endemic species.

When the low global mycobiodiversity exploration level is taken into consideration (Mueller & Schmit 2007, Lumbsch *et al*. 2011), it is clear that there are still a vast number of fungal taxa waiting to be discovered and described. Many of previously described species are inadequately documented and poorly known. Thus, taxonomists should be encouraged to produce high quality re-descriptions for such taxa, especially of those described from the sole type collection; to lodge cultures where possible and to produce sequence data (Korf 2005). Because the current mycological practices have shown the existence of many cryptic (sibling) species (Frisvad & Samson 2004, Jaklitsch 2009, 2011, Liu *et al*. 2012, Muggia *et al*. 2014, Perrone *et al*. 2011, etc.) it is advisable to include

physiological, chemotaxonomic, ontogenetic and molecular phylogenetic methods because only the polyphasic (holistic) approach could define differences between closely related taxa (Kuhnert *et al.* 2014, Maharachchikumbura *et al*. 2012, Pažoutová *et al*. 2013, Udayanga *et al*. 2011, 2012). This is particular important for plant pathogenic species (Sharma *et al*. 2013, Udayanga *et al*. 2013, Yang *et al*. 2009).

6. CONCLUSIONS

"Vital taxonomy" methods provide an insight into the larger number of characters and character states in cells and fungal structures in general. Qualitative and meristic taxonomical features are more numerous, less variable and taxonomically more informative than those from dried material. A relationship between taxonomic characters obtained on living material and those from type material is possible to establish after thorough re-examination of the same collection in dried state providing us data comparable to the type material (exsiccata). Application of "vital taxonomy" methods and evaluating its results in taxonomical procedures ensure more confident and better re-classification at the generic level. Although comparatively time demanding and rather complex than traditional methods, "vital taxonomy" is proved to be very useful in successful delimitation of cryptic taxa within *Ascomycota*.

Poorly known and inadequately documented fungal species, especially those described from the sole type collection, should be treated using both "vital taxonomy" methods and traditional procedure based on dried material. Whenever possible, type material should be re-examined in detail and subsequently compared with the data obtained from dried material. In the end, detailed synthesized taxon re-description should be provided including all gathered data to avoid any further misapplication. Taxon with well-defined variation limits will reduce subsequent misidentification and enable reliable use in applied sciences.

Specific microhabitats in any given geographic region that might have been neglected in previous field research (such as dung of wild or domestic animals) or were apparently never explored before in a greater geographic scale (e.g. tufa barriers) can house a certain number of unique species yet unknown to science. In this work two species are recognized on the account of presented taxonomic evidence and are here described as *nomina provisoria* until effective publication is issued: *Thecotheus platyapiculatus* nom. prov. and *Tricharina tophiseda* nom. prov. These species are clearly delimited from the most similar ones by use of "vital taxonomy" methods. Both species are found in the protected areas (national parks), which are in general highly important to the taxonomy because they are designed to maintain both species and habitat diversity thus offering

subsistence to a great number of rare and/or still undescribed species. In this respect, protected areas' *loci classici* could provide taxonomically valuable biological material for a long time.
Molecular phylogeny methods should be used to ascertain position of *Tricharina japonica* and *T. tophiseda* within *Tricharina* s. str. group of species. More systematic work in this scope that will combine taxonomically informative microscopical data with molecular phylogeny and culture studies is still to be done in the genus *Thecotheus* and at the species level in *Hamatocanthoscypha rotundispora* to ascertain whether statistically significant ascospore variation reflects in taxonomical domain.

Morphometrical analysis of *Hamatocanthoscypha rotundispora* ascospores showed the existence of a significant intraspecific variability within collections from different countries (Croatia, France and Spain) and different substrates (*Juniperus phoenicea*, *J. thurifera* and *Cupressus macrocarpa*) in both living and dead material. Four collections that were analysed statistically differed significantly from each other. Because of a non-homogenous ascospore shrinkage, the use of living material for ascospore morphometrical analysis is more reliable than the traditional approach based on dead (freshly fixed and/or dried) material which was proven by a Mann-Whitney statistical test. The use of non-parametric statistics is a more reliable approach to describe morphometrical variability of ascospores, because distributions of their dimensions could significantly deviate from normality. For a good delimitation of ascospore variation range (spore length and width), at least 50 to recommended 100 spores should be measured and included in the taxon description. In the living material those spores should be randomly selected among mature spores freshly ejected from the living asci.

When describing a new species, it is essential to give a thorough description of the holotype and not to delimit only on *a priori* defined and widely used characters, because one cannot predict which characters will turn out to be essential in delimiting such species from the large number of species yet to be discovered and described.

Qualitative (e.g. shape, type of ornamentation), meristic, and quantitative (measurements) ascospore features are among the most important taxonomic characters in the phylum *Ascomycota*. The need of standardised procedures in their naming, describing and treatments is therefore

inevitable. Use of standardised spore shape naming in the cases of *Hamatocanthoscypha rotundispora* and *Tricharina tophiseda* vs. *T. japonica* ensured that species could be efficiently characterized, identified and mutually differentiated. This postulate has universal value in ascomycete taxonomy.

In the case of *Thecotheus* species with apiculate spores the following conclusions regarding ascospore apiculi should be adopted:
In most cases, the original, unaltered apicular structures cannot be obtained on dead spores. Only mature and freshly ejected ascospores are reliable source of taxonomically important data and possess the original apicular structures. Overmature and immature spores should be disregarded in the study on apiculi. To reduce the appearance of immature spores with underdeveloped or not developed apiculi, apothecial vertical median sections should be made by hand using a razor blade without applying any pressure to coverslip. If a given specimen still contains living mature spores inside dead asci they will often display the apiculi if treated directly with CB. Aggressive mounting media such as KOH, MLZ and AC can quickly alter the original surface structures including apiculi. Apiculi can be dissolved or irreversibly loosened / swollen. Therefore these media should not be used for observing size and shape of the apiculi. In order to observe the ascospores with fully developed and unaltered original surface structures (apiculi and lateral ornamentation) it is important to use "vital" mounting media (H_2O, CRB, CR and IKI). A fragile nature of *Thecotheus* spore apiculi is recorded in some other pezizalean species with apiculate spores (pers. unpublished data), so proposed procedure on how to study ascospore apiculi might have wider taxonomical application.

In most cases *Thecotheus* species having apiculate spores are sufficiently differentiated solely by apiculi and lateral ornamentation. This fact is applied in compiling the worldwide key for identification of apiculate-spored *Thecotheus* species given in this work.

Living cell inclusions similar to vacuolar bodies are described for the first time in this work. These are refractive cytoplasmatic globules (RCGs) in *Hamatocanthoscypha rotundispora* and semi-resistant vacuolar bodies (SVBs) in *Thecotheus platyapiculatus*. The existence of these inclusions should be tested in other species in the future taxonomical work.

Lodging cultures in *Tricharina tophiseda* and gaining ascorhizoctonia-type anamorph is of a great value in taxonomy because ecophysiological data and information on species' life history are highly taxonomicaly informative in general and cannot be obtained outside highly controlled conditions that exist in axenic cultures. This is generally one of the research aspects with highest value in polyphasic taxonomy in phylum *Ascomycota*.

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8. CURRICULUM VITAE

Ivana Kušan was born on the April 4th 1980 in Sisak, Croatia. She finished primary school education in Sunja and high school education in Zagreb. In 2006 she graduated at the Department of Biology, Faculty of Science, University of Zagreb, as a master of biology (mag. biol.) and master of educational biology (mag. educ. biol.). As an excellent student, she was granted a scholarship from the Ministry of Science, Education and Sports. During the study of biology she got interested in mycology and graduated with a thesis: Taxonomical, ecological, and biogeographical features of the family *Vibrisseaceae* (*Ascomycota*) in the regions of Croatia, Slovenia and Austria. Since then, her main scientific focus is in mycology, especially taxonomy, biogeography, biodiversity and ecology of the ascomycetous fungi.

From 2010 she works as a research assistant in the Division for Marine and Environmental Research, Ruđer Bošković Institute, Zagreb, and enrolled to a Postgraduate Interdisciplinary Doctoral Study in Protection of Nature and Environment in 2011. During mycological specialization she attended several international mycological workshops and two training schools. Also, she participated in more than 10 professional and research projects of various scopes, mostly inventories of *Ascomycota* in the protected areas of Croatia.

Ivana Kušan published eight original scientific papers with additional one accepted for publication, co-authored two books and participated in one national and two international conferences. She is a peer-reviewer of the Mycotaxon, the international journal of fungal taxonomy and nomenclature. She is a member of Croatian Mycological Society and ADIPA – Croatian Natural History Research and Conservation Society where she has appointed as a secretary.

Papers published in peer-reviewed journals cited by Current Contents (3):

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