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Species Concept in the Genus *Trentepohlia* (Ulvophyceae, Chlorophyta): a Combination of Molecular and Morphological Approaches

Definice druhů u rodu *Trentepohlia* (Ulvophyceae, Chlorophyta) pomocí kombinace molekulárních a morfologických přístupů

Master's thesis

Supervisor: doc. Mgr. Pavel Škaloud, Ph.D.

Prague, 2016

Statement

I hereby state that I have completed this thesis by myself and that I have properly cited all literature and other information sources I have used. Neither this thesis nor its parts have been submitted to achieve any other academic title(s).

Prohlášení

Prohlašuji, že jsem svou závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její postatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

In Prague / V Praze, 18. 4. 2016

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Abstract

In this Master's thesis I engage in the problematics of the species concept of green subaerial alga *Trentepohlia* (Ulvophyceae, Chlorophyta). This genus has been studied intensively since the 18th century. For more than 200 years, *Trentepohlia* species have been delimited using various morphological criteria. However, recent molecular studies showed inadequacies in this approach Therefore, my goal was a precise morphological study of the European *Trentepohlia* species and the re-evaluation of their phylogenetic position using *rbc*L gene sequencing.

During the period 2013–2015, I carried out a detailed study of the oldest references of *Trentepohlia* species and compiled a delimitation key of the European species. Based on this research I concluded that it would be desirable to synonymize *T. odorata* with *T. jolithus*, as well as *T. uncinata* with *T. arborum*. I also sampled *Trentepohlia* species within Europe and carefully studied and described their morphology.

Fresh *Trentepohlia* thalli were molecularly characterized by cloning, which revealed a common mixture of *Trentepohlia* species in what on a first sight appears to be a homogenous crust. Phylogenetic analyses based on the *rbc*L confirmed the ongoing inconsistencies among morphologically and molecularly delimited species and also the existence of cryptic species. It would be benefitial to perform a revision of both species and generic concept to sort out the current chaotic situation in *Trentepohlia*.

Key words: species concept, *Trentepohlia*, subaerial algae, morphology, phylogeny

Abstrakt

Diplomová práce je zaměřena na problematiku druhového konceptu zelené aerofytické řasy *Trentepohlia* (Ulvophyceae, Chlorophyta). Tento rod byl intenzivně studován již od 18. století. Po více než 200 let byly druhy rodu *Trentepohlia* tradičně určovány na základě morfologických znaků. Nedávné molekulární studie však ukázaly, že toto tradiční pojetí má řadu nedostatků. Mým cílem proto bylo detailně morfologicky prostudovat druhy rodu *Trentepohlia* v Evropě a pomocí následné sekvenace *rbc*L genu zhodnotit jejich fylogenetickou pozici.

Během let 2013–2015 jsem detailně prostudovala nejstarší zmínky o druzích rodu *Trentepohlia* a sestavila určovací klíč pro evropské druhy. Na základě tohoto výzkumu jsem došla k závěru, že by bylo vhodné synonymizovat druh *T. odorata* s *T. jolithus*, a též *T. uncinata* s *T. arborum*. Též jsem provedla odběry vzorků druhů rodu *Trentepohlia* v rámci Evropy a jejich morfologii jsem pečlivě prostudovala a popsala.

V rámci molekulárních analýz jsem využila klonování a odhalila častou přítomnost směsi jednotlivých druhů rodu *Trentepohlia* v jinak na první pohled homogenních nárostech. Fylogenetické analýzy založené na *rbc*L potvrdily rozpory mezi molekulárně a morfologicky určenými druhy a existenci kryptických druhů. Bude vhodné provést revizi na druhové i rodové úrovni a uspořádat tak stávající chaotickou situaci v druhovém konceptu rodu *Trentepohlia*.

Klíčová slova: druhový koncept, *Trentepohlia*, aeroterestrické řasy, morfologie, fylogenetika

Content

1. Introduction

1.1 Brief Overview of the Problematics of Species Concept in *Trentepohlia*

The Trentepohliales is a group of subaerial green algae in otherwise predominantly marine class Ulvophyceae. These algae often form conspicuous and brightly coloured layers on natural as well as artificial substrates. Some of the taxa are parasites of ornamentally or agriculturally important vascular plants (Almeida, 1985), some are just epiphytes. The genus *Trentepohlia* is the richest one regarding the number of the species. Although the order Trentepohliales belongs to the Chlorophyta lineage, *Trentepohlia* possess some features typical for the lineage Streptophyta (MLS-like structures, phragmoplast present in cytokinesis; Chapman et al., 2001). *Trentepohlia* has been also known for its production of large amounts of carotenoids and polyhydroxyalcohols (Boedeker et al., 2013). This genus also often enters symbiotic relationships with great variety of fungi thus forming lichens (Nelsen, 2011).

The genus *Trentepohlia* has been intensively studied for about two hundred years now, having been already mentioned by Linnaeus (1753) for the first time. During this period, the main focus has been directed mainly towards the morphological criteria, such as the length and width of the cells, the shape of the cells, the shape of the reproductive structures, type of substrates colonized etc. (Brand, 1902; Fischer, 1922; Printz, 1964). However, in the last two decades, the first molecular data have been obtained proving large inconsistencies in molecularly and morphologically delimited species (Lopéz-Bautista et al., 2006). Many of the traditional morphological criteria used for the species delimitation rendered without phylogenetic significance and some of the species turned out to be complexes of cryptic species rather than individuals (Rindi et al., 2009a). It is now clear now that it is no longer sufficient to focus separately on morphology or phylogeny. It is more advantageous to use both approaches and study as many aspects as possible.

1.2 The Order Trentepohliales

There are two major lineages of green algae. The Streptophyta lineage, comprised of the charophytes, land plants and paraphyletic assemblage of freshwater algae; and the Chlorophyta

lineage, including the majority of green algae described so far. In these two groups, terrestrial algae can be found in many classes: Klebsormidiophyceae, Trebouxiophyceae, Chlorophyceae, Zygnemophyceae, Chlorokybophyceae and Ulvophyceae (Leliaert et al, 2012).

The class Ulvophyceae is one of the most heterogenous groups of green algae. It contains species living in marine, freshwater and terrestrial ecosystems. Apart from some subaerial genera such as *Desmochloris* (Darienko et al., 2009) or *Hazenia* (Škaloud et al., 2013b), we are aware of a whole order of green ulvophycean algae with affinity to dry land, the Trentepohliales.

There are several species within the order Trentepohliales. The most exhaustive monograph of this group so far has been written by Printz. Printz (1939) recognized five genera in the Trentepohliales: *Trentepohlia, Cephaleuros, Stomatochroon, Phycopeltis* and *Physolinum*. However, in 1992 Thompson & Wujek described the additional genus *Printzina* and transferred nine *Trentepohlia* species into this genus. The name of this genus was assigned in honour to the botanist Henrik Printz (1888–1978).

The genus *Trentepohlia* Martius 1817 contains many species. The number differs according to various scientists. Lopéz-Bautista et al. (2002) estimated the number of 36 species while Rindi et al. (2009a) about 40. According to the Algaebase (Guiry & Guiry, 2016), there are currently 48 taxonomically accepted species in this genus. Species of *Trentepohlia* grow on various natural and artificial substrates creating small tufts, cushions or crusts (fig. 1). These consist of uniseriate branched filaments usually divided into prostrate and erect parts and coloured yellow, red, orange or brown due to the presence of secondary metabolites (Fischer, 1922; Printz, 1939). *Trentepohlia* can grow epiphytically but it is not considered to be a plant parasite.

Fig. 1: *Trentepohlia jolithus* var. *yajiagengensis* growing on stone; detail of the thallus (Liu et al., 2012)

On the contrary, *Cephaleuros* Kunze ex Fries (1832) is a known parasite of many species of vascular plants in the tropics or subtropics (Almeida, 1985). It occurs on leaves, fruits and stems

of various plants of ornamental or agricultural importance such as coffee, avocado, oleander or citrus (Chapman, 1976). Infection by *Cephaleuros* is described using the terms "red rust" or "algal rust" (fig. 2). Algal filaments do not penetrate host cells but produce coloured spots on leaves. Therefore, infections of *Cephaleuros* have been often mistaken for those caused by fungi (Chapman & Henk, 1985). The thallus of *Cephaleuros* consists of prostrate and erect parts. The prostrate part is subcuticular and erect branches can erupt through the cuticle. Beneath the prostrate part, rhizoids are formed (Chapman & Good, 1983).

Fig. 2: Infection of *Cephaleuros virescens* spreading through avocado leaf in Hawaii; detail of *Cephaleuros parasiticus* thalli (Nelson, 2008)

Another plant parasite is *Stomatochroon* Palm (1934). This alga is quite rare. It lives in the stomatal chambers of vascular plants mainly in the tropics and can be found on the upper as well as lower parts of the leaf, wherever stomata are present. External parts of *Stomatochroon* can show various colours from orange to deep purple (Liu & Hu, 2013). The alga itself (fig. 3) consists of internal filaments and sometimes clustered zoosporangia located at the top of the head cells (Zhu et al., 2014).

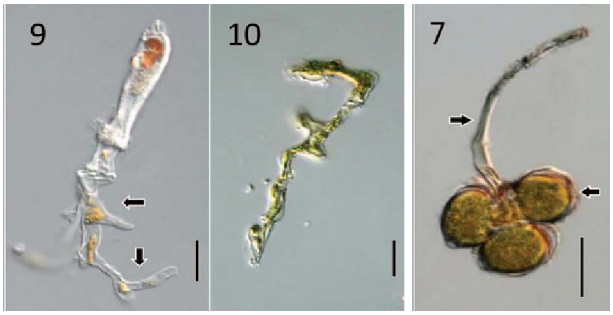


Fig 3: *Stomatochroon reniformis var. chinensis var. nov.* The internal filaments and their branches (9, 10); gametophyte (7) consisting of sterile cell: left arrow and gametangia: right arrow. (Zhu et al., 2014)

Phycopeltis Millardet 1870 is an epiphyte growing on vascular plants in tropical and temperate regions, where it occurs on the upper leaf surface. Dispersal can take place by abscission of zoosporangia, which travel by wind or rain. This procedure has been reported also in *Cephaleuros*, *Stomatochroon* and *Trentepohlia* (Good & Chapman, 1978b). The cell wall of *Phycopeltis* contains sporopollenin (Good & Chapman, 1978a). Its function is assumed to be the same as in plants, a protection from desiccation. In addition, sporopollenin may help in adhesion among filaments of the discoid thallus (fig. 4) and could be also involved in protection from fungal parasitism. Fungal hyphae probably do not penetrate algal cells (Good & Chapman, 1978a). In lichens, the alga can also maintain its own integrity and shape and it may be capable of sexual reproduction (Sanders, 2002) which is a state quite rare in algal-fungal mutualism (Law & Lewis, 1983).

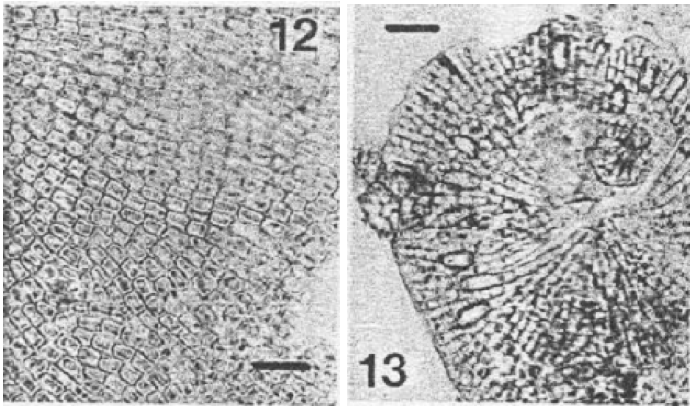


Fig. 4: *Phycopeltis aurea*. Vegetative thallus and thallus with terminal gametangia (Neustupa, 2003)

Physolinum Printz (1920) is an interesting genus and target of many doubts. It was separated from the genus *Trentepohlia* by Printz (1920) in favour of one single species, *Physolinum monile,* mainly due to its reproduction via aplanospores. In 1959, Flint reported the presence of zoospores in *Physolinum*, questioned the existence of the whole genus and suggested its reversal back to *Trentepohlia*. After a careful examination of many herbarium specimens and field collections, Cribb (1970) concluded that *Physolinum monile* could not be separated from *Trentepohlia rigidula*, a widespread tropical and subtropical species. Although Davis et al. (1989) reported aplanospores in *Physolinum* (fig. 5) and re-established the genus *Physolinum* again, the existence of this genus remains uncertain. Nowadays, many phycologists believe that some vegetative features of *Physolinum* occur in several *Trentepohlia* species and there are no morphological characters reliable enough to keep the two genera separated (Lopéz-Bautista, 2006 et al.; Rindi et al., 2009a).

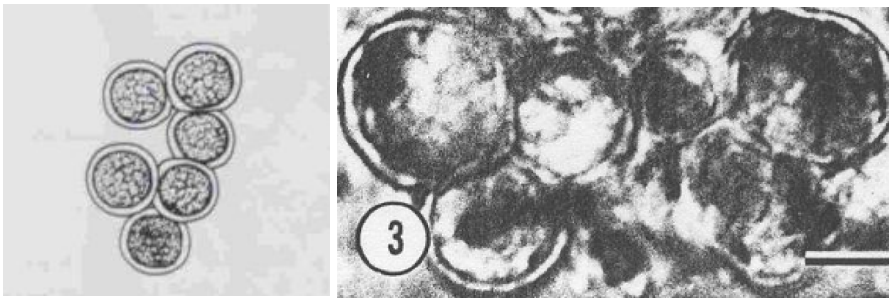


Fig. 5: Six-celled filament of *Physolinum* (Davis & Rands, 1993); group of united green aplanospores (Davis et al., 1989)

Printzina was set aside from *Trentepohlia* by Thompson & Wujek (1992). This rearrangement was based on several morphological features, including: i) globular to reniform sporangia, sessile on prostrate filaments or terminally on erect filaments, ii) a well developed prostrate system (fig. 6) while erect part scanty, iii) gametangia terminal or lateral, iv) plants of deep shade and humidity, usually green in colour. However, similar to *Physolinum*, there have been many disputes about the mentioned genus. Its validity is dubious because there are many species with morphology overlapping the circumscription of *Printzina* and *Trentepohlia*. Moreover, Rindi & Lopéz-Bautista (2007) recently stressed that the choice of the generitype *Printzina lagenifera* was unfortunate since this species does not possess a clear differentiation of prostrate and erect filaments, which even Thompson & Wujek (1992) stated: "Cell differentiation between erect and prostrate filaments often not present." In addition, Lopéz-Bautista et al. (2006) carried out the first molecular assessment of Trentepohliales and did not confirm the monophyly of either *Trentepohlia* or *Printzina*. They also claimed some of the morphological criteria commonly used for species delimitation in *Trentepohlia* and *Printzina* to be without phylogenetic significance. There is only one criterion left to distinguish *Trentepohlia* from *Printzina*: the shape of the sporangia (globular to reniform in *Printzina* and ovoid in *Trentepohlia*) which is not a strong argument to keep these genera separated.

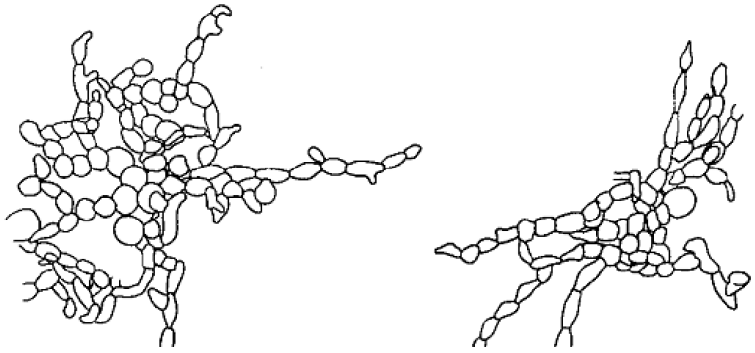


Fig. 6: *Printzina ampla*, appearance of the prostrate system (Thompson & Wujek, 1992)

There is one story left to tell. A story about a peculiar green alga named *Spongiochrysis hawaiiensis,* target of many recent queries. This alga formed golden-yellow crusts on the bark of *Casuarina* tree on the Hawaiian Islands and was collected by Dr. Rindi, who originally sampled the crust in belief that it contains species of the genus *Trentepohlia* (Rindi et al., 2006a). Microscopic observation revealed something different though. The yellow crusts comprised of globular cells without flagella, solitary or in pairs with budding-like cell division (fig. 7). Cells were 10–20 μm in diameter with red, orange or green colour. Phylogenetic analyses revealed position of *S. hawaiiensis* within the otherwise marine or freshwater order Cladophorales/Siphonocladales in the class Ulvophyceae. This unusual discovery let Rindi et al. (2006a) to the assumption that *S. hawaiiensis* may be the very first attempt of this order to colonize terrestrial habitats. So far, the order Trentepohliales has been the only subaerial group known in the Ulvophyceae.

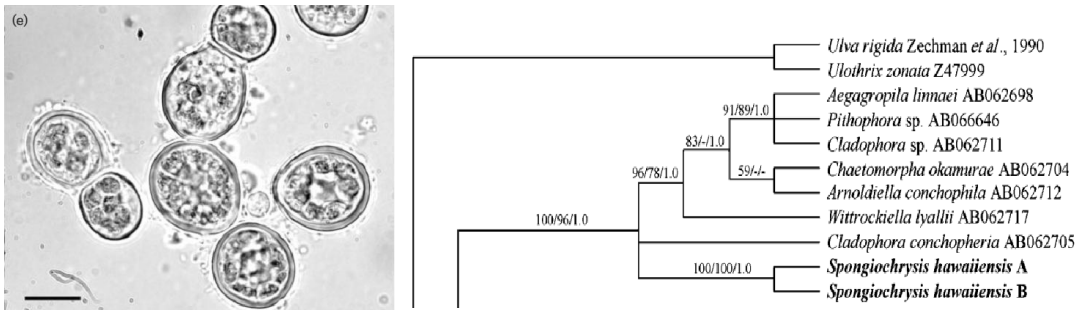


Fig. 7: Cultured specimen of *S. hawaiiensis*, segment of 18S rDNA cladogram (Rindi et al., 2006a)

However, in a few years, this alga was examined again by Boedeker et al. (2013). They re-evaluated its phylogenetic position using SSU, LSU and ITS rDNA markers. They even proceeded to low molecular weight carbohydrate (LMWC) analysis. While some features of *S. hawaiiensis*'s morphology resembled the Cladophorales (large amounts of carotenoids, budding-like cell division), the other (presence of erythritol, aeroterrestrial habitat, uninucleate cells, presence of large amounts of carotenoids covering the original colour of chlorophyll) showed striking similarity to the Trentepohliales (Boedeker et al., 2013). There were also fungal hyphae present adhering closely to the alga, which could indicate lichenization, a process quite common among the members of the Trentepohliales. More importantly, the phylogenetic position of *S. hawaiiensis* proved to be within the Trentepohliales (fig. 8).

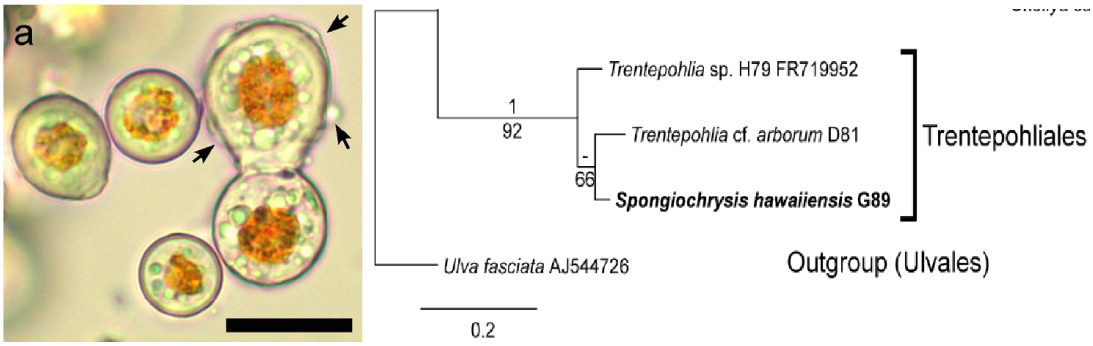


Fig. 8: Habitat of *S. hawaiiensis*: large cell in the process of so-called budding covered with fungal hyphae. Segment of the Bayesian inference phylogram based on LSU rDNA shows the position of *S. hawaiiensis* (Boedeker et al., 2013)

However, Boedeker et al. (2013) presented an ambiguous 18S rDNA dataset from the environmental sample containing both trentepohlialean and cladophoralean algal sequences. Nevertheless, *S. hawaiiensis* was the dominant organism on the tree bark forming essentially pure powder. Furthermore, the amounts of polyol erythritol in *S. hawaiiensis* were similar to the amounts found in some *Trentepohlia* samples also analysed by the same authors. Therefore, Boedeker et al. (2013) concluded that the possibility of the origin of the sequence from the contamination was improbable, thus suggesting the phylogenetic position of *S. hawaiiensis* within the order Trentepohliales. It is clear that further investigation is needed to clarify the situation. However, we cannot sufficiently reject Rindi's hypothesis until more data are collected.

1.3 Some Interesting Features of the Genus *Trentepohlia*

Subaerial algae often colonize both natural and artificial substrates and cause conspicuous colourful layers, crusts or tufts. The green algal genus *Trentepohlia* is no exception of such lifestyle. It grows on a variety of natural substrates: wood, rocks, bark, leaves and lower parts of trunks of lots of trees (Rindi & Guiry, 2002). It also often inhabits man-made concrete or metal constructions. *Trentepohlia* species are widespread mainly in the tropics and subtropics (Printz, 1939; Rindi et al., 2008). However, they are not uncommon in temperate regions either (Rindi & Guiry, 2002; Rindi et al., 2005).

The genus was firstly described by Linnaeus (1753) under the name *Byssus*. This name is currently regarded as the synonym of the name *Trentepohlia* that comes from Martius (holotype designation in 1817).

Trentepohlia possesses a combination of unique features that distinguish it from other green algae: presence of carotenoids in amounts so large, that they turn the colour of the cells from green into many shades of bright yellow, red, orange or brown (fig. 9). Unusual coloration may be visible on the tree trunks and buildings, even monuments, wherever the crusts formed by *Trentepohlia* occur. Further unique aspects are: absence of pyrenoids in the chloroplast, presence of unique flagellar apparatus in zoospores, transverse cell walls with plasmodesmata and lateral sporangia as a distinguished reproductive structure (Rindi et al., 2006b).

Fig. 9: The extended colonization of anthropogenic substrates by *Trentepohlia* species on the wall of the Palácio Nacional de Pena in the Portuguese city of Sintra (Macedo et al., 2009); building blocks at Mayan monuments of Becán in the Mexican state of Campeche (Ortega-Moráles et al., 2013)

Some of these features confused taxonomists in the past. The motile cells of the Trentepohliales showed the ultrastructure typical for the class Ulvophyceae in the lineage Chlorophyta (Hoek et al., 1995), especially the arrangement of the basal bodies and microtubular flagellar roots (anti-clockwise). On the other hand, the presence of the MLS (multi-layered structures) or MLS-like structures pointed more towards the lineage Streptophyta (Chapman & Henk, 1986). Moreover, phragmoplast, a structure present at the process of cell division in some members of Streptophyta, was found in *Cephaleuros* (Chapman & Henk, 1986) and *Trentepohlia* (Chapman et al., 2001). Lopéz-Bautista & Chapman (2003) shed light on the phylogenetic position of the Trentepohliales using phylogenetic analyses of the small subunit of rDNA (SSU rDNA). They proved the position of the Trentepohliales to be within the class Ulvophyceae in the lineage Chlorophyta. They suggested that phragmoplastin evolved perhaps independently in both Streptophyta and Chlorophyta lineages. Interrestingly, phragmoplast-mediated cytokinesis takes place in both lineages in members associated with the terrestrial habitats (Lopéz-Bautista & Chapman, 2006).

1.3.1 Adaptation to Subaerial Life

Subaerial algae have to face many kinds of stressful conditions such as a lack of water, strong UV-light irradiation, large variations in temperature and occasionally even high salinity (Gustavs et al., 2009). These algae have had to develop protective mechanisms to survive in their evolution. The Trentepohliales can manage stress using diverse ways of defence. The members of this group often produce polyhydroxyalcohols, for example erythritol or mannitol (Boedeker et al., 2013). Such chemical compounds may act as cryoprotectants, osmolytes and so-called compatible solutes causing controlled decrease of water potential. This process leads to water retention in the cell (Eggert & Karsten, 2010). The mechanism is similar in relationship to desiccation as well as to high salinity. In both cases water escapes from the cells quickly, which can lead to the cell damage. That is why the production of polyols has been known in many marine algae of a different phylogenetic origin (Karsten et al., 1999; Reed et al., 1985) or in algae living in hypersaline lakes (Zelazny et al., 1995; Chen & Jiang, 2009). Many species of the genus *Trentepohlia* also possess cellulose caps on apical cells (Brand, 1902; Fischer, 1922). These caps can play a photoprotective role thanks to the reduction of the amount of irradiation (West & Hood, 1911).

Another way how to protect algal filaments from irradiation is to produce antioxidants extinguishing reactive oxygen species (ROS) and preventing harm to the photosynthetic apparatus. Cells of *Trentepohlia* are often coloured because of accumulation of various carotenoids (Arpin & Liaaen-Jensen, 1972; Czeczuga & Maximov, 1996; Mukherjee & Borah, 2009). Carotenoids serve as a protective barrier against UV-light irradiation and can also function as additional antennas for harvesting the light spectre unobtainable by chlorophyll (Czeczuga & Maximov, 1996). Carotenoids are usually accumulated during drought or under full exposure to sunlight and are present in the cells in a form of small droplets, often covering the green colour of chlorophyll (Ho et al., 1983).

Finally, some morphological adaptations, mainly the simplification of the thallus, are worth noticing. Severe conditions of the aeroterrestrial life forced algae to obtain the most convenient shape for the best water retention and drought and irradiance resistance. Therefore, the morphology of terrestrial algae can appear almost identical (Lopéz-Bautista et al., 2007). This process is called morphological convergence (Rindi et al., 2009b) and it is the reason why we find subaerial algae mostly in three forms (fig. 10): unicellular and globular (*Bracteacoccus, Chlorella, Myrmecia,* etc.); sarcinoid, i.e. in regular packets consisting of small number of cells

(*Apatococcus, Chlorokybus, Desmococcus,* etc.) and uniseriate filaments (*Trentepohlia, Prasiola, Klebsormidium,* etc.).

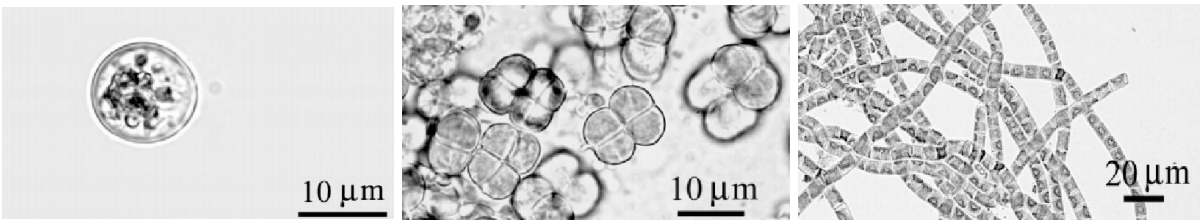


Fig. 10: *Spongiochrysis hawaiiensis* (unicellular), *Desmococcus olivaceus* (sarcinoid) and uniseriate filaments of *Klebsormidium flaccidum* (Lopéz-Bautista et al., 2007)

1.3.2 Growth Preferences

Species of the genus *Trentepohlia* prefer mainly humid tropical regions. Life conditions in the tropics and subtropics are favourable for many subaerial algae. It is also possible that the number of terrestrial algae recorded in the tropics will increase, given the under-exploration of these areas (Neustupa & Škaloud, 2008; Rindi et al., 2009b). Therefore, lumbering of rainforests and mangroves could lead to loss of diversity of many subaerial algae (Chakraborty & Mondal, 2012).

Trentepohlia species often grow on tree bark. They prefer mainly old well-developed shaded and humid forests, although they can be found also in habitats with open sky and some degree of exposure to light (Neustupa & Škaloud, 2008). Their abundance increases with a stand age. That is why they are quite scarce in young forests with clear-cuts (Hedenas et al., 2007; Neustupa & Štifterová, 2013). Another important factor is the pH of the bark surface. Neustupa & Štifterová (2013) sampled bark of various trees in the Czech Republic and some coastal areas in Italy, Slovenia and Croatia. Their results showed the affinity of *Trentepohlia* to the bark with a higher pH such as the bark of *Quercus* or *Populus* trees rather than the bark with an acidic pH (coniferous trees: *Pinus*, *Juniperus*, *Picea*) in temperate regions. Similar facts resulted from the study of Marmor & Degtjarenko (2014). They sampled the bark of *Pinus sylvestris* in northern Estonia. *Trentepohlia* species usually do not occupy its acidic bark in Europe. However, in localities close to limestone quarries, abundance of *Trentepohlia* on *Pinus sylvestris* was significantly higher. Therefore, the authors suggested *Trentepohlia* as an ecological bioindicator of alkaline dust pollution.

Another aspect important for growth of corticolous algae is the complexity of the tree bark. Neustupa & Štifterová (2013) discovered negative relationship among the abundance of *Trentepohlia* and bark roughness. On the contrary, Lüttge & Büdel (2009) brought opposite results. They found *T. umbrina* growing on the rough bark of orchard trees, while *Desmococcus* and *Trebouxia* were more associated with the smooth bark of deciduous trees (*Acer*, *Fagus*). The authors consider the ability of better water retention of the rougher tree bark as a key aspect. Rough bark holds moisture for a longer time period due to the capillary forces so the algae are less affected by desiccation. Lüttge & Büdel (2009) also proved the capability of green biofilms (*Desmococcus, Trebouxia*) to survive the longer periods of desiccation (up to 80 days) in comparison to red algal biofilms of *Trentepohlia* (30–40 days).

Looking at *Trentepohlia* biofilms, we may notice that they are usually situated on vertical substrates forming conspicuous stripes visible by naked eye (fig. 11; 12). This pattern could be connected with the way these algae reproduce. They form gametangia, which are usually globular or flask-shaped and sporangia, commonly placed on a special swollen suffultory cell, being hook-formed or funnel-like (Brand, 1902). In both cases a great impact of water and wind is expected. Gametangia are usually not separated from the maternal filament and release motile swarmers in rainy weather while being firmly attached to the filament (Printz, 1964). Sporangia also rupture when wetted and release motile swarmers through a papilla pore. This is probably the way how the stripes are created: water flows down the substrate and carries along the reproductive structures. Unlike gametangia, sporangia may be also carried by wind or insects (Rindi et al., 2006b). They can be easily separated from the suffultory cells and travel as a whole. Brand (1902) suggested that species of *Trentepohlia* with flask-shaped or globular gametangia such as *T. umbrina* inhabit only vertical substrates because of water based dispersal. On the other hand, species with funnel-like and hook-formed sporangia like *T. annulata* or *T. jolithus* are more suited to wind based dispersal, so they can also inhabit horizontal surfaces such as tree stumps and rocks.

Fig. 11: Red stripes of the *T. umbrina* biofilm on some concrete constructions in Tirrenia (left) and S. Rossore Natural Park (right) in Pisa, Italy (photo by M. Šafránková, July 2015)

Fig. 12: Orange patches of *T.* cf. *abietina* on a vertical rock near Ponte di Barano, Tuscany, Italy (photo by M. Šafránková, July 2015)

1.3.3 Reproduction of the Genus *Trentepohlia*

It is generally believed that reproduction of the genus *Trentepohlia* takes place by means of motile zoospores and gametes or fragmentation of the thalli. The asexual process of fragmentation often happens. The thallus breaks into pieces and fragments continue to grow (Hansgirg, 1886).

In the sexual part of the life cycle, an isomorphic alternation should take place, morphologically identical diploid sporophyte and haploid gametophyte alternate. Gametophyte is bearing gametangia, which undergo the process of maturation and release biflagellate gametes. Gametes fuse creating a diploid zygote. The diploid sporophyte raises from the zygote bearing zoosporangia. Zoosporangia maturate and release quadriflagellate zoospores that form haploid gametophyte (Lopéz-Bautista et al., 2002).

However, once again nothing is so simple with *Trentepohlia*. Gametangia and sporangia can obviously occur on the same filament (Rindi et al., 2005). Rindi & Guiry (2002) found no evidence of isomorphic alternation in Irish samples and cultures of *Trentepohlia*. Instead, they observed the following situation. Common life history consists of repetition of the same morphological phase, reproduction takes place via biflagellate swarmers (gametes) behaving like asexual spores, producing new cells and filaments without any act of sexual fusion. This event has been reported many times by various authors (Hansgirg, 1886; Fischer, 1922; Karsten, 1891; Printz, 1964). It seems that the biflagellate swarmers often behave like spores. I myself have seen this process at Dr. Rindi's laboratory in Ancona (in July, 2015). A sample of *T. umbrina* was placed in a small droplet of water on the underlying glass and put under a light microscope. In a few minutes, very small curved cells started to move very fast backward and forward in the specimen. On the top of these cells, two hardly visible flagella could be seen. The gametes did not interact with each other and after a while, they settled down and slowly became round in shape, just like Karsten (1891) described more than one hundred years ago.

Some phycologists solve the problem of rare sexual fusion of gametes by the theory of hetherothallic species. Gamete fusion should take place only when filaments of the opposite sex are present (Karsten, 1891; Printz, 1964; Rindi & Guiry, 2002). However, this has never been satisfactorily proven.

This raises a crucial question. Does the sexual reproduction really take place in the genus *Trentepohlia*? And if so, why does it not happen more often? Is such a process so rare here, that it has been reported only twice over two centuries by Wille (1878) and Lagerheim (1883) cit. in Rindi et al. (2005) and since then never again? Is it too bold to presume that *Trentepohlia* could be a genus with a secondary loss of the sexual reproduction? Both of the observation of gamete fusions happened a very long time ago when light microscopy was still in the process of development. Could the authors observe perhaps just two spores stuck together by an accident? Moreover, flagella of the swarmers are often very inconspicuous. In the Ulvophyceae, reproductive zoids can possess two to four flagella (Hoek et al., 1995).

Does the differentiation into zoospores with four flagella and gametes with two flagella really exist? Or are there just spores with two flagella germinating parthenogenetically, as it has been seen so many times? In his preparation for the great monograph of the Trentepohliales, Printz (1939) showed a picture of "tetrakonten Zoosporen" (fig. 13) in the sample of *T. annulata*. He stated that this picture came from Skuja. I was not able to find Skuja´s notes from 1933. However,

I assume Skuja was the first one to draw zoospores with four flagella. Printz took this drawing over into his great monograph and since then, this information has been handed down among botanists. Apart from this example, mainly pictures of biflagellate swarmers have been presented and only gametes acting like spores have been seen in the life cycle of *Trentepohlia*. Perhaps sexual reproduction is just extremely rare or it happens in some special conditions we cannot simulate. Maybe the stadium with four flagella is a planozygote that has arisen from two biflagellate gametes fusing together. This situation has been reported in ulvophycean algae (Miyamura et al., 2003) Nevertheless, sexual reproduction could be absent in *Trentepohlia* and zoospores would possess only two flagella as it has been reported by Flint (1959).

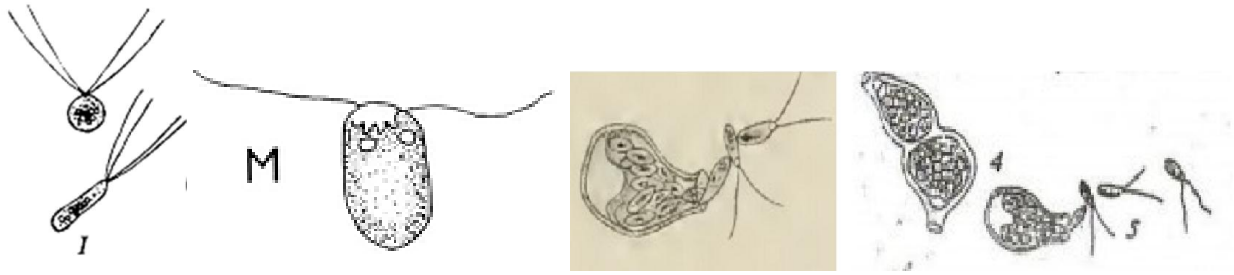


Fig. 13: Zoospores with four flagella (first from the left, Printz, 1939); Zoospores with two flagella (second, Flint, 1959); Gametangium of *T. umbrina* releasing biflagellate gametes (third, Karsten, 1891; fourth, Printz, 1964)

1.3.4 Symbiotic Relationships

Trentepohlia species can live either free or participating in symbiotic interactions. A rather curious symbiotic relationship is established with sloths in Central and South America. This is said to be a mutually beneficial association. Growth of the algae causes changes of the colour of sloth's fur thus providing efficient camouflage. Cracks in the mammal's hair are ideal substrate for the algae, because the fur can soak water like a sponge and it is able to reduce the intensity of irradiance (Suutari et al., 2010).

More importantly, the Trentepohliales tend to engage in symbiotic relationships with various fungi and form lichens (Hawksworth et al., 2011; Hedenas et al., 2007). In such state the morphology of the alga becomes different. For example, sexual reproduction of the photobiont has been only scarcely reported (Sanders, 2002; Uyenco, 1965). Secondly, trentepohlialean photobionts are often present in a compact layer in the lichen thallus, their cells have limited range

of size and restricted branching pattern (Hametner et al., 2014a). Lastly, production of carotenoids is also limited due to the protection of the alga from strong irradiation by the upper fungal layer (Czeczuga & Maximov, 1996). Such reduction suggests that the alga is controlled by the fungal hyphae (Hametner et al., 2014b). Nevertheless, the role of the lichen photobiont cannot be diminished. Almost ¼ of lichen-forming fungi associates with trentepohlialean algae (Nelsen et al., 2011). The lichens containing *Trentepohlia* as photobiont can be used as bioindicators of global warming because they respond positively to warm and rainy climates. They are adapted to moist and shaded tropical habitats and their number has been increasing in some parts of the world lately. This phenomenon could indicate the increase of the global temperature (Marini et al., 2011; Aptroot & Van Herk, 2007).

1.3.5 Biodeterioration Caused by *Trentepohlia*

Subaerial algae can inhabit various artificial substrates and cause not only some esthetical problems. Their presence can be followed by arrival of fungi and together with bacteria and blue-green algae they form microbial biofilms. These microorganisms are often responsible for local erosions and surface discolorations called patinas (Gaylarde et al., 2006; Macedo et al., 2009). Discoloration disrupts the esthetics not only of common buildings but also monuments (Gaylarde et al., 2011) and cemetery sculptures (Guiamet et al., 2012). Guiamet et al. (2012) described biodeterioration as a change in material properties due to the vital activities of the organisms. Biodeterioration is induced also by the accumulation of biological deposits on surface. Colonization by microorganisms is dependent on water availability (some materials affect water retention with their porosity and permeability), light, humidity, orientation of the surface, etc. (Ortega-Morales et al., 2013). Eradication of *Trentepohlia* biofilms is difficult and even if successful there is a possibility that the material remains stained by pigments of these algae. However, sometimes the eradication is not necessary and vast patinas can actually drag attention of tourists. An excellent example is the so-called Red-Stone-Valley in China (Liu et al., 2012).

1.3.6 Role of *Trentepohlia* in Applied Phycology

It is a deeply rooted feature of the mankind to try to gain as much as possible from the natural resources. Algae have been used in cosmetics and drug industry, they have served as food

or feeding material for cattle, they have been even useful in art (www.seaweedart.com). In the last few decades, there have been many ideas to take advantage of some abilities of *Trentepohlia* species, e.g. production of useful metabolites or intake of nutrients from wastewater (Abe et al., 2002).

A few years ago, a study about the effects of prenatal intake of nitrate residuals from drinking water lead to an unpleasant result. High amounts of nitrate in the drinking water consumed by pregnant women can cause birth defects (oral cleft defects, limb defects) in offsprings (Brender et al., 2013). Amounts of nitrogenous compounds are present in drinking water and derived from the groundwater. This can be a result of the massive use of nitrogen-based fertilizers. Residual nitrates also cause eutrophication of lakes and ponds because algae and other plants use nitrate as a source of nutrients (Abe et al., 2003). Such ability can be used in wastewater treatment. *T. aurea* is able to remove 37 % of NO_3^- in a 30 day period. In addition, biofuel can be extracted from the algal biomass afterwards (Al-Balushi et al., 2012). Some laboratories developed even small scale photobioreactors with cycling water and a biofilter with *T. aurea* (Abe et al., 2003; Abe et al., 2007). The authors are convinced that *T. aurea* possesses a great potential in wastewater treatment and production of fine chemicals such as polysaccharides, polyunsaturated fatty acids and pigments simultaneously.

Simultaneous production of β-carotene, vitamin E and C by *T. aurea* has been also proven (Abe et al., 1999). These compounds are very important as antioxidants and even cancer preventants. On the other hand, *T. aurea* is very slow-growing kind of algae. However, this can be modified by adding some other chemicals to the medium (peptone, etc.).

T. aurea was found on concrete surfaces next to highways in industrial areas by Abe et al. (1999). Therefore, the authors also suggested the role of *T. aurea* as a greenbelt over concrete surfaces near the highways thus reducing industrial CO_2 and perhaps also NO_x and SO_x.

1.4 Species Delimitation Problems and Limits of Traditional Classification

The members of the order Trentepohliales have been a subject to quite an extensive research over the past two centuries. The focus has been directed mainly towards morphological features and cultivation of these algae. It is interesting that the first molecular assessment of the order Trentepohliales was made only about ten years ago (Lopéz-Bautista & Chapman, 2003), when its

phylogenetic position within the Ulvophyceae was cleared. In 2006, Lopéz-Bautista et al. brought the results, which indicated that the traditional morphological criteria used for species delimitation do not match the phylogenetic patterns. Moreover, only *Cephaleuros* formed a monophyletic group, unlike *Trentepohlia* and *Printzina* which seemed to be polyphyletic (fig. 14). It was obvious that a major reassessment with narrowed circumscriptions of the genera will be necessary in the future. The only reliable morphological character appeared to be the position of the ostiole (escaping pore for zoospores in zoosporangia). Unfortunately, the position of the ostiole is still unknown in many species of *Trentepohlia*. Occurrence of zoosporangia in clusters located at the top at the sporangiate laterals should be a well-defined feature at least for *T. arborum*.

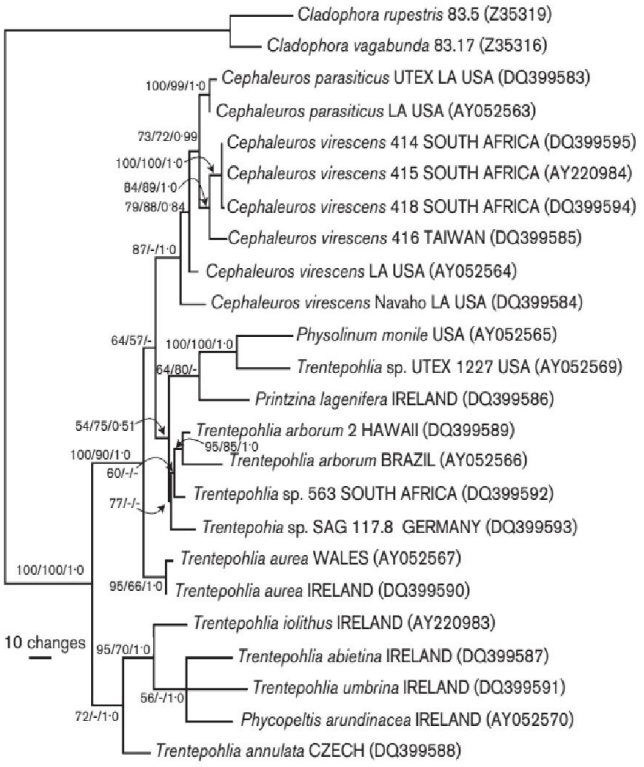


Fig. 1. Maximum-likelihood tree resulting from analysis of 18S rDNA sequences (−lnL = 5975·5283) for 22 trentepohlialean taxa (1810 characters). Support values are shown on the branches (maximum likelihood/ maximum parsimony/Bayesian).

Fig. 14: Molecular assessment of the order Trentepohliales (Lopéz-Bautista et al., 2006)

Relationships among *Trentepohlia* species are still uncertain (fig. 15). For example, circumscriptions of *T. abietina* and *T. aurea* overlap considerably according to Lopéz-Bautista et al. (2006) and the only morphological criterion to distinguish them is the width of filaments (6–10 µm in *T. abietina* and 10–20 µm in *T. aurea*) and substrate preferences (*T. aurea* prefers stone or concrete, while *T. abietina* inhabits the bark of trees).

However, several morphological aspects have turned to be phylogenetically irrelevant lately, such as the shape of the cells (cylindrical/globular/elliptical), size of the cells, habitat of the thalli (prostrate, erect) or the type of the substrate colonized (Lopéz-Bautista et al., 2006).

The classification of *Trentepohlia* species has been always based on the morphological characteristics. This could be a problem considering the polymorphism of the morphological features and also the phenomenon of the morphological convergence explained earlier. Such processes complicate the species delimitation. Although many species occur in the typical forms and make species delimitation easy, there are still a lot of specimens showing an intermediate morphology being difficult to identify with certainty (Rindi et al., 2006b).

According to Rindi et al. (2009b), some species of *Trentepohlia* should be regarded as complexes of cryptic species rather than individuals, because the morphologies of these species have evolved separately in several lineages. For example, *T. umbrina*, *T. arborum* and *P. lagenifera* form the complexes of the cryptic species affected with great morphological convergence (Rindi et al., 2009a).

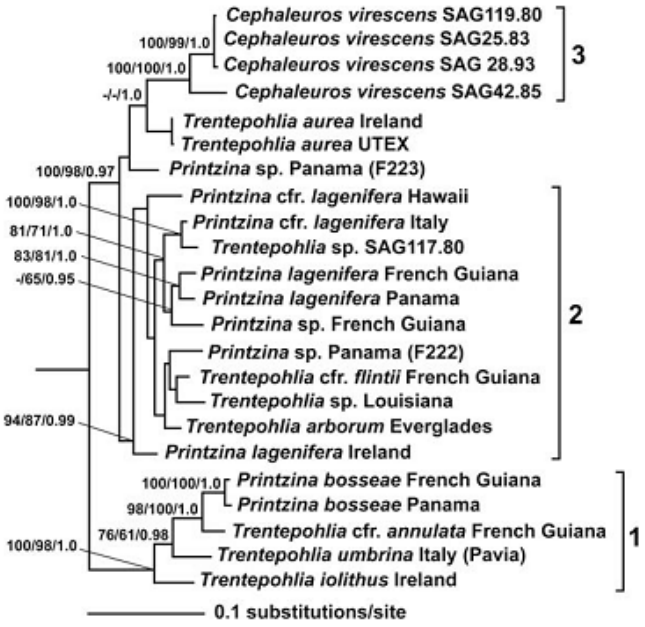


Fig. 4. Phylogeny of *Trentepohlia*, *Printzina* and related taxa inferred from Maximum Likelihood analysis of the combined dataset *rbc*L-18S rRNA. From left to right support values at nodes correspond to Maximum Parsimony BP, Maximum Likelihood BP and Bayesian PP. BP values lower than 60% and PP lower than 0.85 are not reported. The tree was rooted using outgroup sequences of Dasycladales (not shown) specified in Section 2.

Fig. 15: Phylogenetic relationships in the order Trentepohliales (Rindi et al., 2009a). Three main clades were established, yet some species are not associated with any of them. This is the most problematic in the case of *T. aurea* since it is the type species of the genus. *Printzina* and *Trentepohlia* do not form the monophyletic clades like *Cephaleuros*.

The species concept based only on the morphological data is clearly inadequate for the Trentepohliales. Additional molecular data represent the necessary piece of information required for future studies. It will also be important to find out whether supposedly conspecific strains distributed in widely separated regions belong to the same lineage or not (Hametner et al., 2014b).

1.5 Aims of This Master's Thesis

In this Master's thesis I put emphasis on both molecular data and detailed morphological observations as I believe that this is the best way how to elucidate the current situation. To do so, I carried out a complex methodological approach. First, I focused on the original descriptions of the European *Trentepohlia* species written mainly in the 18th and 19th century. Second, I studied the morphology of the fresh natural samples collected within Europe. I also maintained some cultures of these specimens, although they served more as a demonstration of a common problem of many algae, i.e. the change of the morphological and ecophysiological properties during the cultivation (Lakeman et al., 2009). For the fresh samples, I performed molecular analyses using chloroplast (*rbc*L) markers. I used cloning, insertion of trentepohlialean DNA into bacterial plasmids, in order to separate cohabitants from trentepohlialean members. Such approach helped to answer some burning and still unclarified questions. Do the crusts and tufts formed by *Trentepohlia* consist of one or more species? This aspect has never been considered by the authors collecting *Trentepohlia* into cultures, though being of great importance. Should all of the current European *Trentepohlia* species according to Brand (1902), Fischer (1922) and Printz (1939) really be considered distinct entities to date? The last, but certainly not least, goal of this thesis was to assign the most striking morphological features to the particular phylogenetic lineages. The results should help to elucidate our current knowledge of the still enigmatic relationships among the species of *Trentepohlia*.

2. Materials and Methods

2.1 Samples Collection and Examination

The samples were collected from various kinds of substrates in several parts of Europe, mainly in the Czech Republic, Portugal and Italy, during the years 2013-2015. Each sample was

removed from particular substrate using a sharp knife and placed in a paper or a plastic bag. The knife was disinfected using an antibacterial gel and clean paper handkerchieves to prevent cross-contamination among different samples. The samples were observed under the light microscope Olympus BX 51 and microphotographs were taken with the digital camera Canon EOS 1100D.

The dimensions of the cells were measured using the program ImageJ (Schneider et al., 2012) and the picture tables were constructed using the Adobe Photoshop CS3.

2.2 DNA Extraction

DNA was extracted using the modified CTAB protocol (Doyle & Doyle, 1987), as follows.

Approximately 0.5 g of algal tissue and 2-4 wolframkarbid balls were put into 2 ml Eppendorf tubes. The material was grinded in the Retsch Mixer Mill 200 for 5-6 min at 30 rev/s. The following steps were carried out in the Cruma hood. A pinch of the PVP powder (polyvinylpyrrolidone) and 700 µl CTAB solution (hexadecylytrimethyl ammonium bromide) with 2% mercaptoethanol were added to the samples in the tubes. The amount of mercaptoethanol was used according to the number of samples (calculation: the number of samples × 700 = required amount (ml) of CTAB solution, the same amount of mercaptoethanol but in µl was added to CTAB). CTAB disrupts cell membranes, PVP forms hydrogen bonds with phenolic compounds and removes them from the plant DNA extracts while mercaptoethanol removes tanins and polyphenols and helps to denature proteins.

5 µl of RNAse A (50U/mg; 10 mg/ml, Fermentas, ThermoFisher Scientific) were added to degrade the RNA. The tubes were vortexed for 10 s using Vortex Genie2 and incubated for 30 min at 60 °C with 1,400 rpm on Eppendorf Thermomixer® Comfort. In the hood, 500 µl of chloroform:isoamylalcohol (24:1) mixture was added to remove protein contamination, the tubes were turned upside down several times, left standing for 5 min and then centrifuged in the Eppendorf 5415D (max. rotational speed 13,200 rpm/max. centrifugal force 16 110 rcf) for 6 min with 13,200 rpm.

The transparent supernatant was removed carefully into the new 1.5 ml Eppendorf tubes. 500 µl of cooled isopropanol was added and let 1–3 h at −20 °C. Alcohol treatment serves for nucleic acid precipitation. Centrifugation for 5 min with 13,200 rpm was carried out to pellet the DNA. The supernatant was discarded afterwards leaving the white pellets on the bottom of the

tubes. 400 µl of cooled 96% ethanol was added to the pellets. The tubes were incubated on Thermomixer for 3 min at 37 °C and 1,200 rpm. The supernatant was discarded again. Salts and alcohol remnants were removed by adding 200 µl of cooled 70% ethanol, the tubes were left standing for 5 min. The samples were centrifuged for 3 min with 13,200 rpm. The supernatant was discarded very carefully.

The pellets were dried out on Thermomixer at 60 °C without shaking until no ethanol was left in the samples. The pellet was diluted in 30 µl of TE buffer (10mM Tris:1mM EDTA; Sigma-Aldrich) which solubilized nucleic acids while protecting it from degradation (www.sigmaaldrich.com).

The amount of the extracted DNA was measured using the NanoDrop 1000 (Thermo Scientific) spectrophotometer and stored at −20 °C.

2.3 DNA Amplification and Sequencing

DNA was amplified using polymerase chain reaction (PCR) of the *rbc*L gene with primers designated by Pavel Škaloud:

Primer name and sequence		
TrentR_F1	forward	CGTTAYAAAGGWCGWTGYTAYGA
TrentR_F2	forward	AAYGTWTTYGGTTTYAARGC
TrentR_R1	reverse	TCCAWTCTTGABWRAAGAATAC
TrentR_R2	reverse	GTWCCWGARTGTAARTGRTC

The chloroplast-encoded *rbc*L (large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase) gene was chosen according to the literature background, because it was demonstrated to give a better resolution at the genus and species level than the 18S rRNA gene. Among algae, the *rbc*L gene has been proven very useful for testing the phylogenetic hypotheses (Rindi et al., 2009a). The DNA templates were amplified in 0.2 ml thin-walled PCR tubes, in a total reaction volume of 20 µl. Each reaction consisted of: 1 µl of the DNA, 11.8 µl of rrH_2O, 4 µl of MyTaq™ Buffer (Bioline LAB MARK a. s.), a combination of the two primers, each by amount of 1.5 µl, and 0.2 µl of MyTaq™ DNA polymerase (5U/µl, Bioline LAB MARK a. s.). All the chemicals were mixed together into the Master Mix. 1 µl of DNA was added to 19 µl of

the Master Mix. The amounts of the chemicals were multiplied by the number of samples used. Amplifications were carried out in a Thermal Cycler (Eppendorff Mastercycler ep S with gradient) with an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 1 min at 94 °C, primer annealing for 1 min at 52 °C and extension for 1.5 min at 72 °C. The amplified PCR products were spread on 1% agarose gel stained with ethidium bromide (electrophoresis using Scie-Plas: HU6, SHU6, HU13, V20-CDC with voltage source Consort E132) and examined for correct length, yield and purity in the Gel Logic 100 with UV filter, SYBR Green filter and UV-light transilluminator (Herolab UVT-20M). The standard O'Gene 100 bp Plus DNA Ladder, ready-to-use (Thermo Scientific) was used as a control.

2.4 DNA purification

The amplified samples were purified using MinElute PCR Purification Kit (Qiagen) following the manufacturer's protocol. Purified PCR products were checked for the concentration with the NanoDrop 1000 (Thermo Scientific) spectrophotometer and stored afterwards at −20 °C. They were used for the subsequent cloning.

2.5 Cloning

Cloning serves two main purposes. First, it creates a large number of recombinant DNA molecules from a limited amount of starting material. Second, it serves as a purification (Brown, 2010). Field samples of *Trentepohlia* are often contaminated with other green algae living among them on the natural substrates. Since highly specific primers that amplify only *Trentepohlia* do not exist, our primers amplified the DNA of various green algae. Therefore, we decided to proceed with cloning to obtain clean sequences of *Trentepohlia*.

Cloning is a process of inserting DNA into bacterial plasmids. Bacterial colony consists of descendants of a single cell and clones in one colony possess the same molecule of DNA. Different colonies contain different molecules of DNA. For PCR, 8–10 or more colonies are desirable to amplify (and a lot of other colonies are denaturated and kept at −20 °C as a backup). Bacterial polymerase often makes mistakes. The sequences obtained have to be checked in

adequate computer program to avoid misinterpretations. It is recommended to have at least two or three sequences of the alga to check the polymeration errors.

Cloning also demands the adequate equipment. Sterility precautions must be upheld during the whole process. All the necessary tools were sterilized using ethanol and UV light and all steps were carried out in Telstar FlowBox J1001. Competent cells and aliquots from the cloning kit (Promega Corporation) were kept on ice and handled with care. First, the medium for the bacterial cells was prepared. This was the Luria-Bertani browth powder medium (LB). The LB medium with agar was prepared as follows. 12.5 g of LB was mixed with 10 g of agar and 500 ml of distilled water (Millipore Milli-Q Synthesis) and autoclaved in 120 °C for 20 min (autoclave 3150EL Tuttnauer). Firm medium served for Petri dishes. LB medium without agar contained 6.25 g of LB and 250 ml of distilled water and was also autoclaved in the same conditions. Liquid LB medium was used as the nourishment for bacterial cells.

Second, the ligation was carried out with purified DNA products after the PCR amplification. This is the process when DNA is attached to the vector (fig. 16). Ligation chemicals were used in half the amount than specified in the manufacturer's protocol: Cloning Buffer 2.5 µl, pGEM (R)-T Easy Vector System II 0.5 µl, T4 DNA ligase (5U/µl) 0.5 µl, purified DNA product 1.5 µl. Ligation mixtures were shortly vortexed and incubated for 2 h in Thermal Cycler (22 °C).

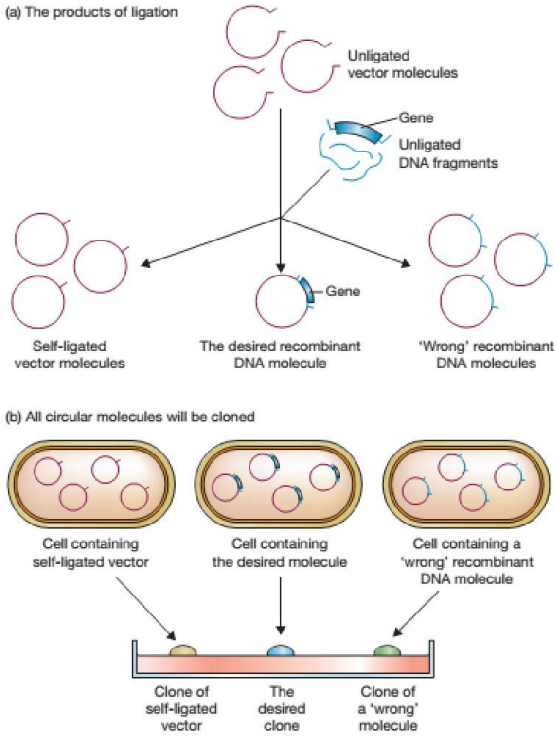


Fig. 16: Introduction of DNA into living cells (Brown, 2010)

Then, transformation was performed. This is a process which also occasionally happens in the nature when bacteria take an external molecule of DNA into their cells. In the laboratory conditions, bacteria have to undergo a particular treatment that enhances their ability to take up DNA. Such cells are called "the cloning competent cells" (Brown, 2010).

Transformation (fig. 17) took place as follows: 1 µl of ligation mixture was added to the competent cells. Their amount was calculated according to the number of the cloned samples. Calculation: 200 µl (one tube of competent cells)/number of samples. The assemblage was gently mixed by hand and kept on ice for 20 min. Then the cells were treated with heat shock being placed in 42 °C for 50 s (in Eppendorf Thermomixer® Comfort) and returned on ice for 2 min. This physical shock causes a disruption of the cell membranes and vectors with attached DNA molecules can enter the bacterial cell. After the heat shock treatment, the bacterial cells were kept in 900 µl of the liquid LB medium in the drying room (311DS Shaking Incubator, Labnet International) at 37 °C with 150 rpm for 1.5 h to adjust (Brown, 2010).

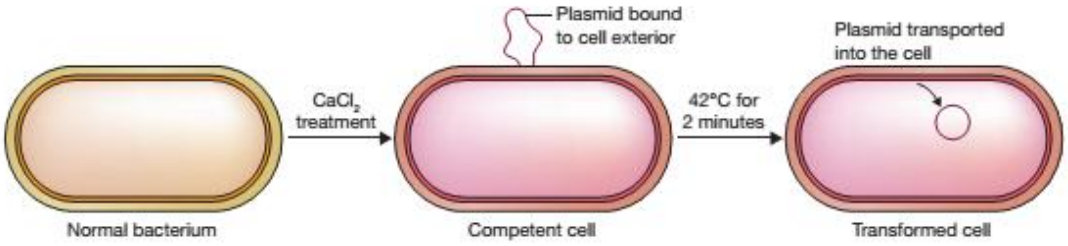


Fig. 17: The binding and uptake of DNA by a competent bacterial cell during transformation (Brown, 2010)

Most plasmid cloning vectors carry genes for antibiotic resistance. Thanks to this, we are able to distinguish the transformed cells by placing them on an agar plate containing relevant antibiotics that were added to the LB medium (ampicillin). However, such resistance genes must be firstly expressed to produce a particular enzyme which detoxifies the bacterial cell. This is why it is highly recommended to keep the bacterial cells in a liquid medium after the heat shock (Brown, 2010).

Selective medium helps us to get rid of non-tranformants. Now we have to determine which of the transformed colonies contain the recombinant DNA molecules and which are just bearers of the self-ligated vector molecules (see fig. 16). Modern plasmid vectors carry the ampicillin resistance gene and another gene, which will cause the cell recombinants' inability to synthesize β-galactosidase. Screening for β-galactosidase presence or absence is easy. This enzyme usually

helps the breakdown of lactose to glucose and galactose. However, it can also catalyse a reaction with a lactose analog called X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranose, Bioline LAB MARK a. s.). The enzyme helps the breakdown of X-gal, which is followed along with the production of deep blue colour. If X-gal and its inducer IPTG (isopropylthiogalactoside, Bioline LAB MARK a. s.) are added to the agar plates along with ampicillin, the non-recombinant colonies, which synthesize β-galactosidase, will be coloured blue. The recombinants unable to produce β-galactosidase stay white. Both ampicillin resistance and presence/absence of β-galactosidase screenings (fig. 18) are conveniently carried out on a single agar plate (Brown, 2010).

Petri dishes with the firm LB medium were placed outside the refrigerator to gain the room temperature. Afterwards, 50 µl of IPTG and 50 µl of X-gal were applied on their surface. The incubated transformed cells were centrifuged in Sigma 4–16K (max. rotational speed 15,000 rpm/max. centrifugal force 25,155 rcf) for 3 min with 3,500 rpm, the LB medium was carefully removed and 100 µl of the liquid LB medium was added for thickening of the mixture. The transformed cells were placed on the Petri dishes using sterilized laboratory glass and stored upside down in the drying room at 37 °C for at least 16 hours.

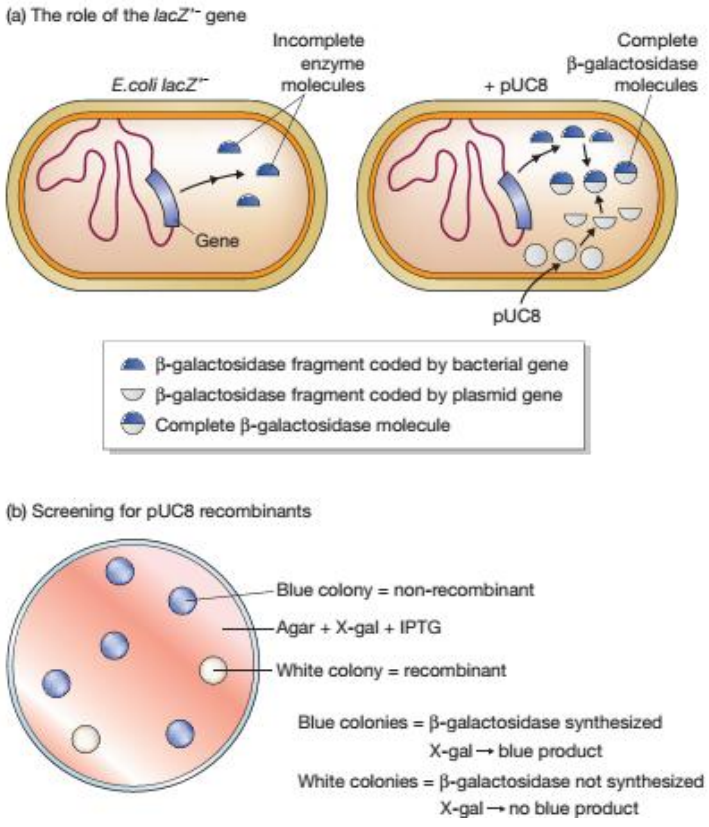


Fig. 18: Ampicillin and β-galactosidase presence/absence screening (Brown, 2010)

For following PCR amplification only white colonies were used. The DNA was either taken directly from the colonies on the Petri dishes using sterilised toothpicks or 1 µl from the backup denaturated colonies was used (the colonies were placed into 20 µl of rrH_2O and incubated in the Thermal Cycler at 95 °C for 10 min. This way, the material can be stored for a long time period at −20 °C).

2.5.1 DNA Amplification after Cloning

After cloning, the DNA was amplified using bacterial primers:

Primer name and sequence		
M13F	forward	GTAAAACGACGGCCAGT
M13R	reverse	GCGGATAACAATTTCACACAGG

The Master Mix contained the following chemicals (the amounts were multiplied by the number of the samples): 14 µl of rrH_2O, 4 µl of MyTaq™ Buffer, a combination of the two primers, each in the amount of 0.4 µl and 0.2 µl of Mytaq™ DNA polymerase, respectively. 19 µl of the Mix was placed to the 0.2 ml thin-walled PCR tubes with 1 µl of the DNA in a total reaction volume of 20 µl. The amplifications were carried out in the Thermal Cycler with an initial denaturation step of 95 °C for 1 min, followed by 35 cycles of 20 s at 95 °C, primer annealing for 30 s at 60 °C and extension for 1.5 min at 72 °C. The total amplified products were visualized after the electrophoresis under UV light on a 1% agarose gel stained with ethidium bromide and examined for correct length, yield and purity. The standard O'Gene 100 bp Plus DNA Ladder, ready-to-use was used as a control.

2.5.2 DNA Purification and Sequencing

The samples were purified again using the MinElute PCR Purification Kit following the manufacturer's protocol. The amount of the purified DNA amplification products were quantified using the NanoDrop 1000 spectrophotometer and sent via FEDEX to the Macrogen sequencing service (primers M13F and R were used for sequencing).

2.6 Culturing

Several samples from Italy were cultivated using the modified Bold's Basal Medium with vitamins. The algae were placed on approximately 30 ml of the agarized medium in the plastic Petri dishes and placed into the cultivation chamber at Università Politecnica delle Marche (Ancona, Italy, July–August, 2015) and stored at 21 °C in 16: 8 h light: dark cycle with 50–200 µmol photons m^{-2} s^{-1}. The algae grew very slowly. They were examined carefully under the stereoscope afterwards. The microphotographs of field-collected material were taken by the digital camera (Canon EOS 1100D). The DNA of the cultured and field specimens of the samples from Italy (TA2, TA5, TA9, TA10, TA11 and TA17) was extracted using the DNeasy Blood and Tissue kit (Quiagen) following the manufacturer's protocol and then sent to Prague. The PCR amplification of the cultured specimens from Italy was carried out in Prague using the protocol described above. Cloning of the field specimens from Italy was also carried out in Prague using the protocol described earlier.

2.7 Sequence Alignment

Two datasets of *rbc*L sequences were analysed. The first one contained sequences of *Trentepohlia* species obtained from the cloning (35 sequences) placed among 48 GenBank (http://www.ncbi.nlm.nih.gov/) sequences of *Cephaleuros, Trentepohlia* and *Printzina* (*rbc*L sequences of *Stomatochroon* and *Phycopeltis* are still missing in GenBank). *Acetabularia acetabulum*, *Halimeda tuna* and *Ulva lactuca* were selected as the outgroup. The second dataset was made of the sequences of the green algal cohabitants present in the samples. These were mostly the members of Trebouxiophyceae (45 sequences) and they were placed among 71 GenBank sequences of Trebouxiophyceae. Additionally, three sequences of *Trentepohlia* (*Trentepohlia abietina* TA28, *Trentepohlia jolithus* TA17 and *Trentepohlia umbrina* TA2) from previous dataset were selected as the outgroup. Apart from Trebouxiophyceae, only few members of Cyanobacteria, Chlorophyceae and Ulvophyceae were present among the cohabitants and they were not included in the following analyses.

The sequences were adjusted manually using the SeqAssem software (Hepperle, 2004). With respect to the error rate of bacterial polymerase, a minimum of three sequences of the same species was required in the trentepohlialean dataset. Whenever it was possible, three and more

sequences were mutually corrected in SeqAssem and assembled into one consensus sequence. In the rest of the sequences where no comparison was available, no consensus sequence was assembled (only sequence ends were trimmed). In the dataset of cohabitants, sequence ends were trimmed, no consensus sequences were created.

The sequences were aligned manually using MEGA5 (Tamura et al., 2011). Both datasets were checked for presence of putative chimaeras using the programmes RDP4 (Martin et al., 2015) and Bellerophon (Huber et al., 2004). In the Greek mythology, Chimaera was said to be a savage beast made out of three different creatures and was slain by the hero named Bellerophon. In molecular biology, the term chimaera may refer to a sequence made out of more than one species. Such situation is one of the cloning artifacts which occur when two or more unrelated DNA fragments are present at once in the same vector (www.nucleics.com). Chimaeras obviously cannot be regarded homologous and they could bias further analyses. All putative chimaeras identified were also checked manually and whenever proven to be true, excluded from the alignment. They were present in the Trebouxiophyceae dataset where only some sequences were mutually compared and larger portion of sequences was present only in one copy. Here, seven chimaeras were found and confirmed. However, in the *Trentepohlia* dataset, no evidence of the recombination has been found.

The alignment was minimized to the mask for the first, second and third codon position using BioEdit (Hall, 1999). The protein-coding sequences such as *rbc*L tend to show unequal substitution rate variation across sites. The third codon position sites usually evolve much faster than the first and second codon sites because most of the mutations located here are silent. However, mutations in the second codon position sites always result in the change of the amino acid and are therefore eliminated by natural selection (Ronquist et al., 2011). On that account, the first, second and third codon positions were separated and examined independently by the Jmodeltest (Darriba et al., 2012; Guindon & Gascuel, 2003) to search for the best nucleotide substitution model.

In case of *Trentepohlia* dataset, following models were assigned: SYM+Γ (the first codon position), K80+I+Γ (the second codon position) TVM+I+Γ (the third codon position). In case of Trebouxiophyceae dataset following models were assigned: TIM1ef+Γ (the first codon position), TVMef+I+Γ (the second codon position) TVM+I+Γ (the third codon position). The models mentioned above were used in the following phylogenetic analyses.

2.8 Phylogenetic Analyses

The analyses were executed in MrBayes 3.2 (Ronquist et al., 2011). This programme is based on the Metropolis Coupling Monte Carlo Markov Chain sampling. It simultaneously runs two completely independent analyses, each starting from different random trees. By default, there are four chains, three cold and one heated. They examine the "landscape" of the possible topologies and exchange the information (the cold chain swaps states with the heated chains). As the analysis proceeds, a state of equilibrium should be reached where the two runs converge (the two tree samples are now similar) and the chains move around in the area of the best tree topologies. These topologies are used for the construction of the consensus tree. Before the state of equilibrium is reached, the values of log-likelihood increase rapidly. This is called the "burn-in" phase and all the trees sampled in this part of the analysis should be discarded.

After some initial trials, the number of generations was set to 8,000,000 for both datasets to ensure a good sample from posterior probabilities (convergence of runs). The number of chains was set to four and the diagnostic frequencies were sampled at every 100th generation. At the end of both analyses, the average standard deviation of split frequencies was checked (0.03 for Trebouxiophyceae, 0.001 in case of the Trentepohliales) as well as the PSRF statistics (reasonably close to 1.0). The burn-in was specified according to the plots pictured at the end of the analyses and by checking the values to log-likelihood ratio at the value of 1,000 for the Trebouxiophyceae as well as the Trentepohliales. The final phylogenetic tree was processed via FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

In the case of Trebouxiophyceae, when using both Bayesian and Maximum likelihood analyses with partitions into the three codon positions, the result was unsatisfactory (see more in discussion). Therefore, maximum likelihood analysis without the partitioning and with the GTR+Γ+I model was executed in Garli (Zwickl, 2006) and is shown in the results.

In contrast to Maximum Likelihood (ML), Maximum Parsimony (MP) and Distance methods, in MrBayes the branch lengths can never reach zero even in the case of very similar sequences. This is said to be caused by the usage of priors in the Bayesian statistics. For this reason, the identical sequences were merged in the alignment so the dataset would be free of additional false variability.

Statistical support for grouping of the taxa into clades was performed using the Bootstrap method via Maximum Parsimony performed in PAUP4.0 (Swofford, 2003) and Maximum

Likelihood performed in Garli. For the posterior probabilities, only values higher than 0.97 are shown. In case of bootstrap (MP, ML), only values higher than 50% are displayed. Final trees were graphically rearranged using the Adobe Illustrator CS3 to become more reader friendly.

3. Results

3.1 Morphological Observations

Several species of the *Trentepohliales* were sampled in Europe (see the map, fig. 19) and also scarcely apart from it (samples T10 and T12). Europe should host 9 species of the order *Trentepohliales*: *Trentepohlia umbrina*, *Trentepohlia annulata*, *Trentepohlia arborum*, *Trentepohlia abietina*, *Trentepohlia aurea*, *Trentepohlia jolithus*, *Trentepohlia uncinata*, *Trentepohlia odorata* and *Printzina lagenifera.*

However, after the careful examination of various literature sources I felt growing doubts regarding possible synonymy of some species mentioned above. Therefore, I carried out a detailed analysis of the oldest literature references I was able to find. As a result I have compiled the complex delimitation key of the European *Trentepohlia* species (see the appendix). The very original descriptions are typed in bold and citations are placed in the reference part. After my detailed comparison of the species circumscriptions, I have concluded that not all species can probably be considered distinct entities up to date. This applies to two species: *Trentepohlia odorata* and *Trentepohlia uncinata*. I am afraid that various authors have named the same species differently and *T. odorata* is in fact *T. jolithus* as well as *T. uncinata* would be *T. arborum*.

T. jolithus was for the first time described by Linnaeus (1753) while *T. odorata* was described by Wiggers (1780). The circumscriptions of both species made by the original authors, as well as by their followers, are almost identical (appearance of the thallus in crusts or tufts with developed prostrate system, violet-like smell, cell dimensions, hook-formed cell supporting sporangia, fondness of temperate zones, etc. See the appendix for more information). Cribb (1989) also mentioned the resemblance of *T. odorata* and *T. jolithus.* His drawings and pictures of other authors are in agreement with this similarity. Therefore, I would suggest *T. odorata* to be the synonym of *T. jolithus*.

The same case represents *T. arborum*. *T. arborum* was described by Agardh (1824) whereas *T. uncinata* was decribed only 3 years later by Gobi. Both descriptions clearly overlap (appearance of the thallus in tufts, cell dimensions, sporangia in groups attached to a swollen suffultory cell, etc.) Hariot (1889) mentioned that the occurance of sporangia in the group on the suffultory cell in *T. arborum* resembled *T. uncinata*. Gobi's notes are a little disorganized. However, his drawings show the similarity of *T. uncinata* to *T. arborum* very clearly. Therefore, I would suggest *T. uncinata* to be the synonym of *T. arborum*.

During the years 2013–2015, the following species were collected and studied from the morphological as well as molecular point of view. With respect and honour to the work of the great scientists mentioned above, morphological observations were written down in the same fashion including hand drawn pictures of observed features.

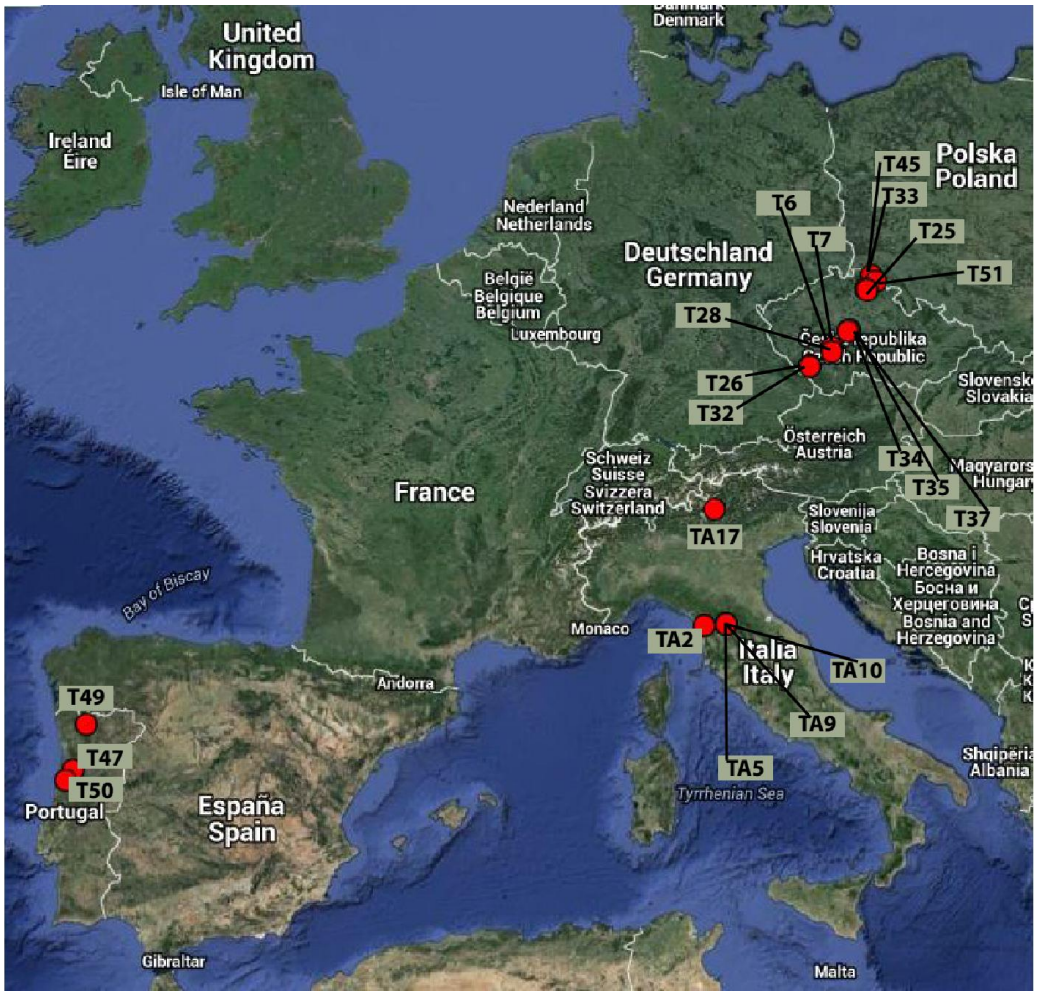


Fig. 19: The map of the sampling sites within Europe

Trentepohlia annulata F. Brand

Sample ID	Locality	Country	Substrate	Habitat
T33	The Krkonoše mountains, Bratrouchov, 750-800 h. a. s. l.	CZ	Old tree	Shaded

This peculiar sample was found in a shady and moist forest on the stump of a tree so old and rotten that it was not possible for me to determine its kind. Vertical as well as horizontal side of the stump was inhabited with a bright golden dense moss-like texture which turned out to be *Trentepohlia annulata.* The bright coloured tufts were up to 1.5 mm high and divided into prostrate and erect filaments. The cells were straight and rectangular, sometimes a little bit swollen in the middle, with the dimensions 13.8 µm × 32.5 µm and a rather thin cell wall. There were many sporangia present at the tips of filaments. The terminal sporangia were oval to elliptical with the dimensions 22 µm × 40 µm. There were also a few transparent funnel-like ring structures visible at the bottom of the sporangia where they attached to the filaments (fig. 20.3). Remembering the traditional terminology of the German researchers, I would call such reproductive organs the "Trichtersporangia" (fig. 20.4, 6, 7). Some terminal cells were slightly tapered and then enlarged at the top (fig. 20.1). From what I saw, it could be a remnant after sporangia detachment (I believe the detachment takes place between the cellulose funnel-like rings). Some filaments could continue growing after the sporangium department. Whenever this happened, the filament was bounded on sides with some small parallel pieces of cellulose (fig. 20.2). Some filaments seemed to be sterile and ended with a transparent cellulose cap (fig. 20.5). See also fig. 21 for more features.

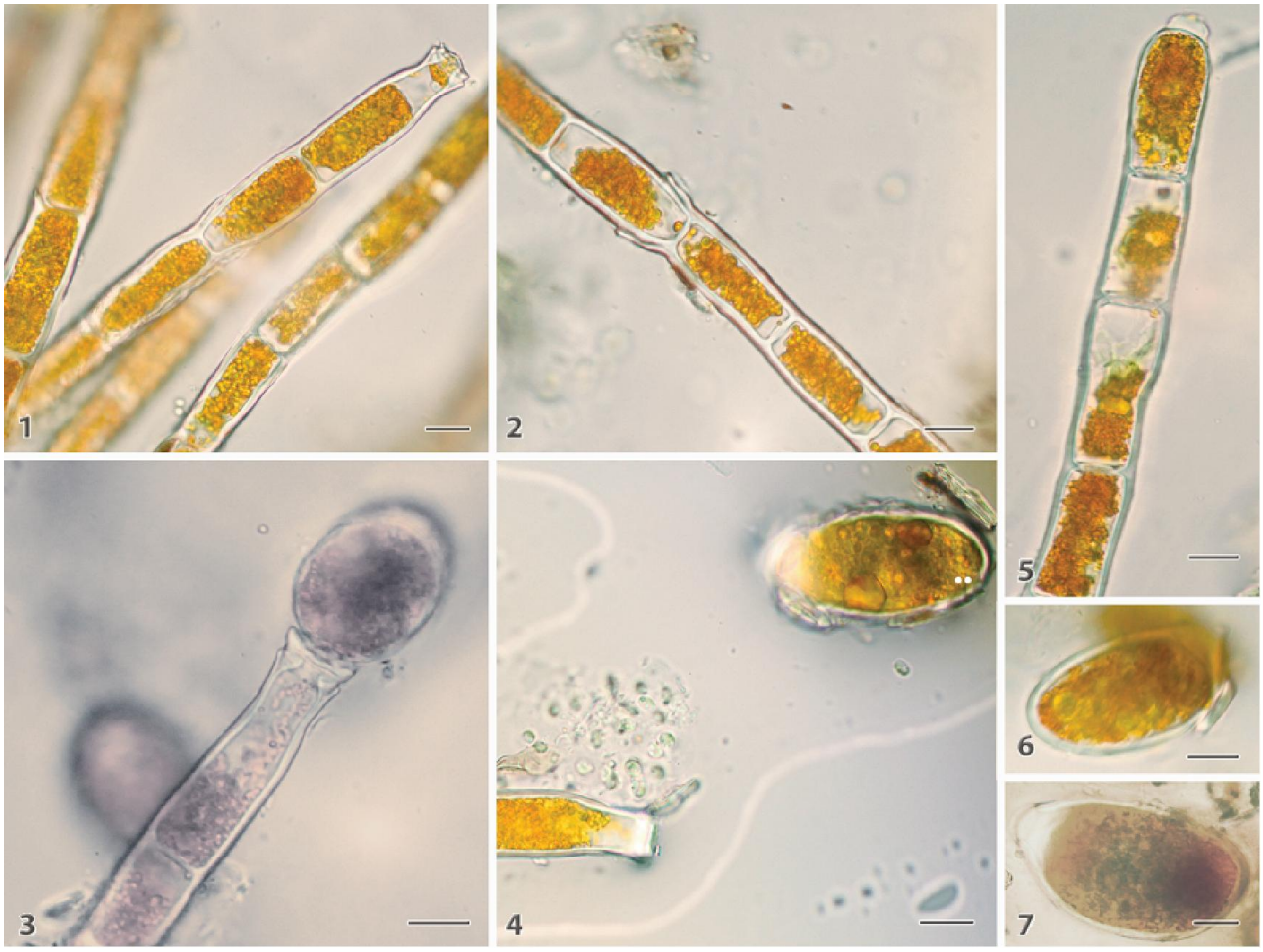


Fig. 20: *Trentepohlia annulata*. Scale bar represents 10 µm. 1–7: sample T33

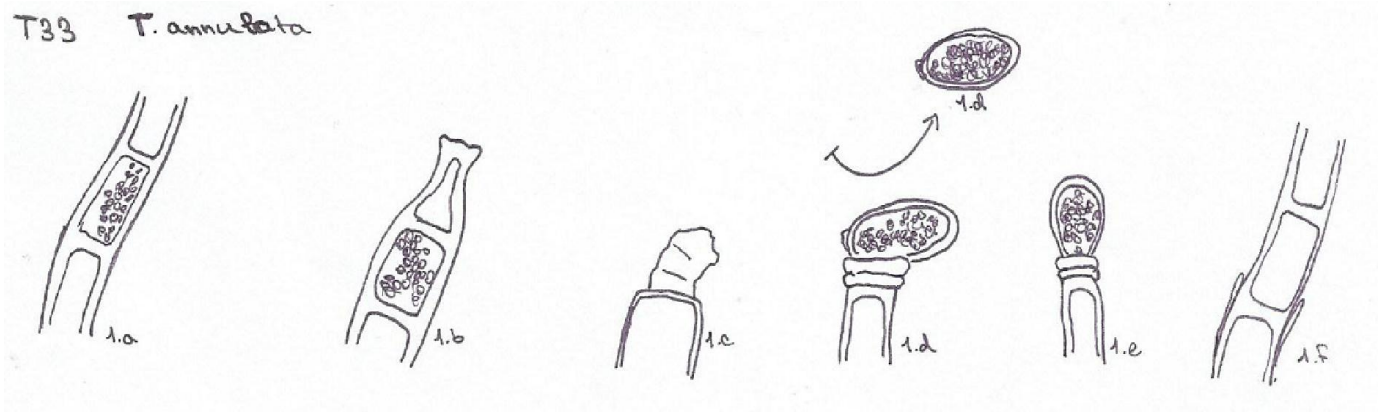


Fig. 21: Hand drawn pictures of *Trentepohlia annulata*. T33 1.a: appearance of a filament; 1.b: shape of the filament tip after presumed sporangia detachment; 1.c: cellulose cap at the tip of a filament; 1.d: a sideview of a trichtersporangium attached to funnel-like structures, detachment of the trichtersporangium; 1.e: rear view of the trichtersporangium; 1.f: cellulose remnants on sides of a fertile filament with continuous growth after sporangia detachment.

Trentepohlia umbrina (Kützing) Bornet

Sample ID	Locality	Country	Substrate	Habitat
T6	Varvažovská Paseka	CZ	Plum tree	Half-shaded
T7	Varvažovská Paseka	CZ	Pine tree	Half-shaded
T25	Český Ráj,Vyskeř	CZ	Concrete pillar	Shaded
T34	Konopiště	CZ	Granite wall	Shaded
T35	Tvoršovice u Benešova	CZ	Apple tree	Half-shaded
T37	Konopiště	CZ	Linden tree	Exposed
T49	Near Albufeira da Barragem de Travassos lake	PT	Concrete	Half-shaded
T51	Pelešany u Turnova	CZ	Concrete	Shaded
TA2	Tirrenia, Tuscany	I	Concrete pillar	Exposed

All samples of *T. umbrina* showed a similar morphological pattern (fig. 22, 23). The filaments showed no differentiation into prostrate and erect part. They appeared in the form of bright reddish or rusty dust on the substrate. The filaments easily broke into clusters of globular to elliptical amber coloured cells with a thin cell wall (fig 22.3). Some cells even occurred solitarily. There were usually not many gametangia present on the thalli. However, a problem with recognition of the gametangia of *T. umbrina* is that they strongly resemble the vegetative cells and even a trained eye might fail to notice them. The only difference may be in the size. The gametangia tend to be a little bit bigger than the vegetative cells and have a thicker cell wall (fig. 22.6, the upper cell). When present, they were positioned terminally, laterally and intercalarly. Dimensions of vegetative cells were not consistent in all species: 3–3.9 µm × 3.5 µm (T6 and T7; fig. 22.1, 2), 7.8 µm × 8.6 µm (TA2), 12–13 µm × 12–15 µm (T25, T34, T37, T51; fig. 22.4, 6, 8), 14.5 µm × 18 µm (T49; fig. 22.7).

In case of some samples of *T. umbrina* from Italy, whose gametangia were more mature, an interesting process could be seen. When the sample was wetted and one was patient enough to

wait five to ten minutes carefully observing the cells under the light microscope, some gametangia erupted due to suction of the water and released motile cells. These were so-called biflagellate swarmers, quite small and curved cells with two barely visible flagella (fig. 23.1.d). One could see them move vividly through the water and then settle down after a while, loose the flagella and slowly become round in shape. I have never seen these cells to fuse. Sometimes two of them met and got stuck together, but a few seconds later they separated and continued to follow their own paths. Conclusively, the motile cells did act in no way like gametes, their behaviour was more spore-like. In other samples (T34, T35; fig. 22.1), transparent globular structures could be spotted sitting among other cells in the filaments. I presume these were empty ghosts of once mature gametangia which had released their precious cargo.

In my humble experience, *Trentepohlia umbina* was the most widespread species in Europe. I could only speculate why. When sampling this species by scraping the crust of the substrate, the light dusty particles float through the air with grace and settle down everywhere in the surrounding area. I would assume the spreading success of this species lies in the easy fragmentation of the thallus (it is indeed a much easier process than in case of the other European *Trentepohlia* species). In constrast to the filamentous species like *T. annulata* or *T. abietina* which form moss-like tissue sticking together, *T. umbrina* scarcely creates the filaments. Besides the thalli fragmentation, *T. umbrina* perhaps creates quite a lot of gametangia, although one can determine their precise amount only with difficulties.

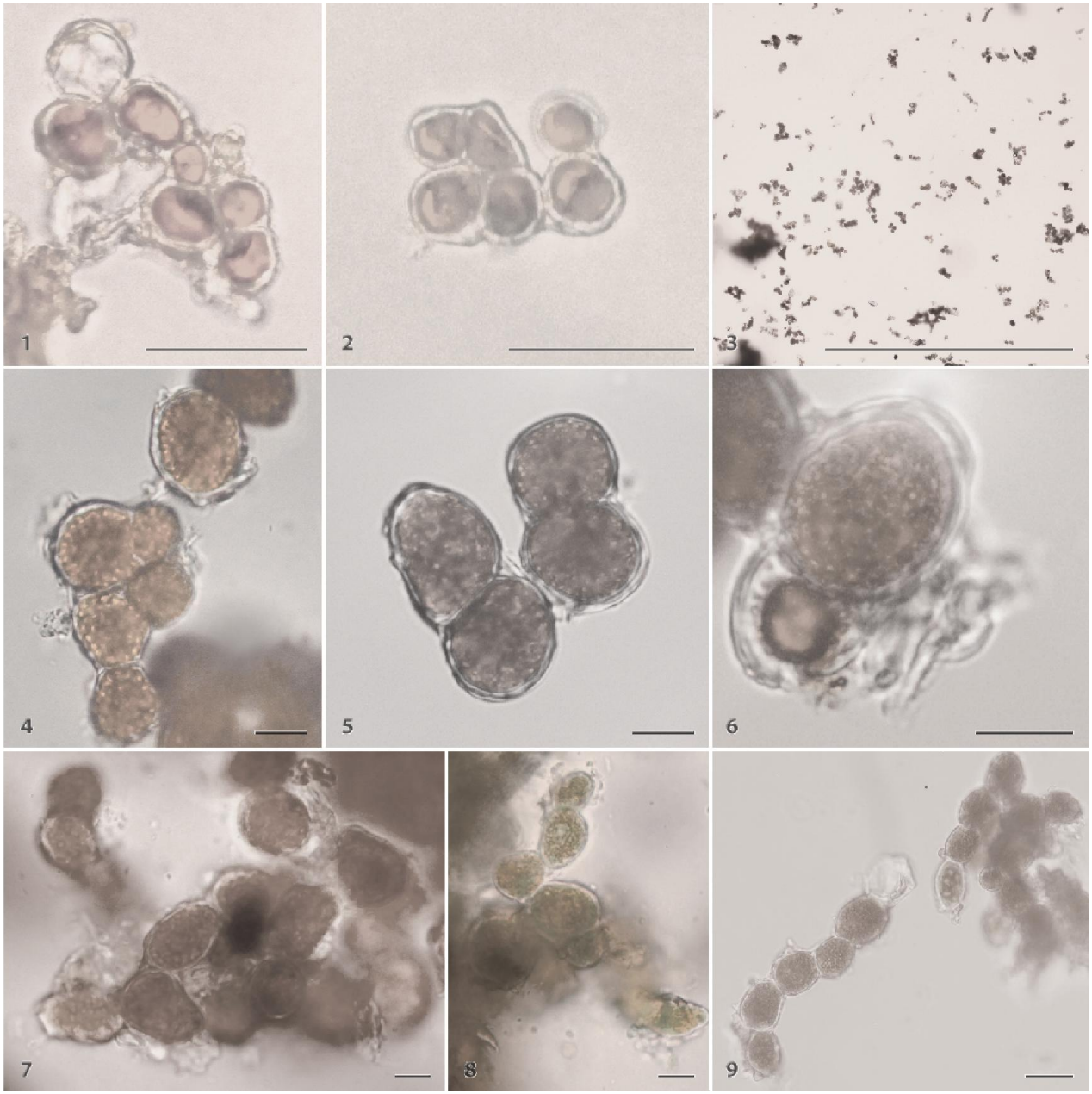


Fig. 22: ***Trentepohlia umbrina*****. Scale bar represents 10 µm. 1: sample T6; 2: sample T7; 3: sample T25; 4: sample T34; 5: sample T35; 6: sample T37; 7: sample 49; 8: sample T51; 9: sample TA2**

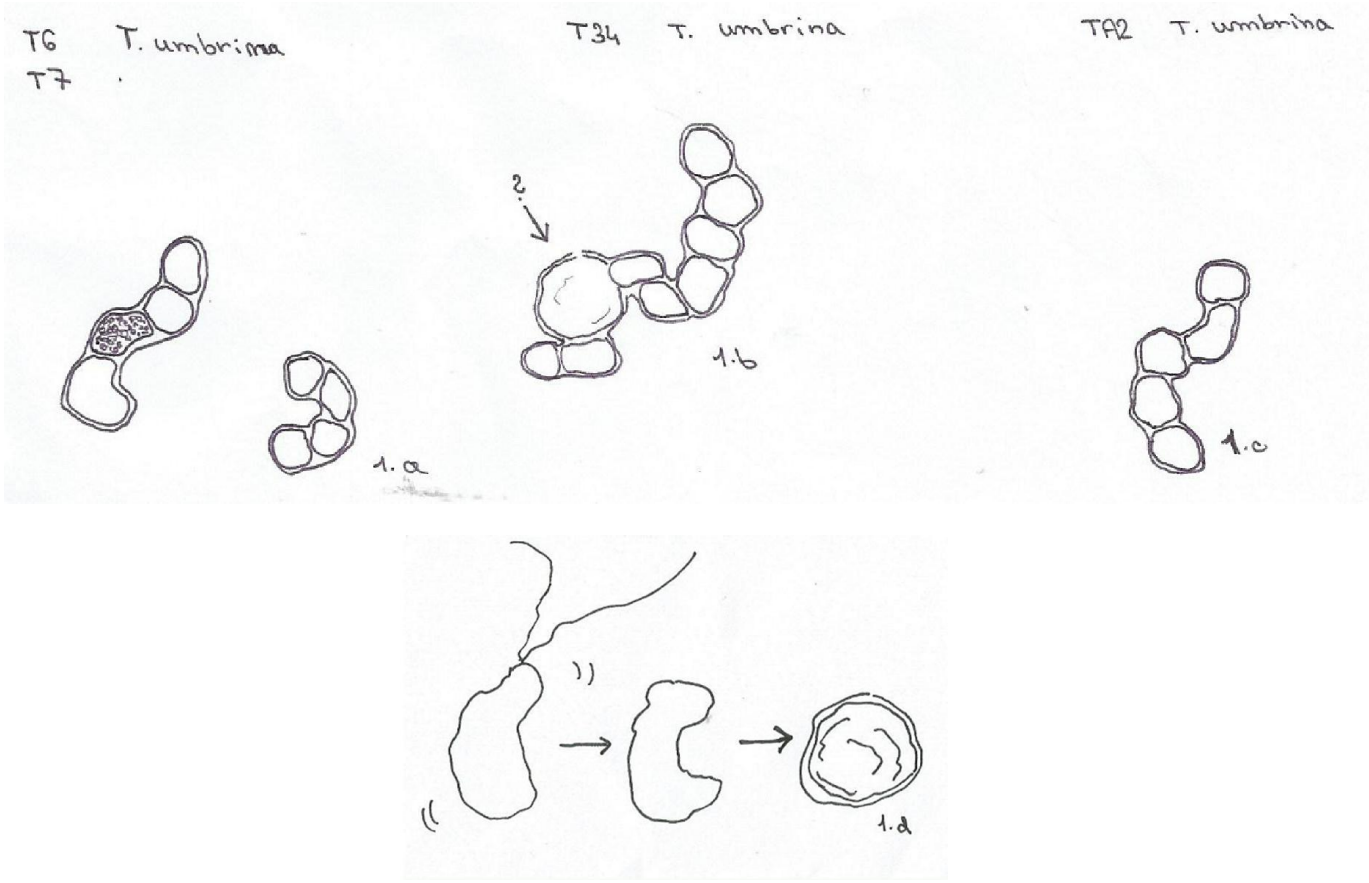


Fig. 23: Hand drawn pictures of *Trentepohlia umbrina*. T6, T7 1.a: arrangement of filaments; T34 1.b presumed empty gametangium; TA2 1.c sterile filament. Picture 1.d. represents biflagellate swarmers present in some samples from Italy.

Trentepohlia arborum (C. Agardh) Hariot				
Sample ID	**Locality**	**Country**	**Substrate**	**Habitat**
T10	Vieng Xai	LAO	Unknown tree	xxx
T12	Luong Namtha	LAO	Unknown tree	xxx

I am grateful to my friend and colleague Helena Bestová for having obtained the only two outside-European samples T10 and T12 (fig. 24; 25). They formed thick moss-like cushions which consisted of very wide filaments with specific angle of branching always 90° (fig. 24.1, 3). The cells were straight in shape with conspicuous carotenoid droplets coloured orange to brownish (fig. 24.4). The cell dimensions were 17–20 µm × 37–40 µm. The terminal cells were never tapered and did not possess any cellulose caps (fig. 24.2). The cell wall was smooth, transparent and quite thin. The reproductive organs were not present.

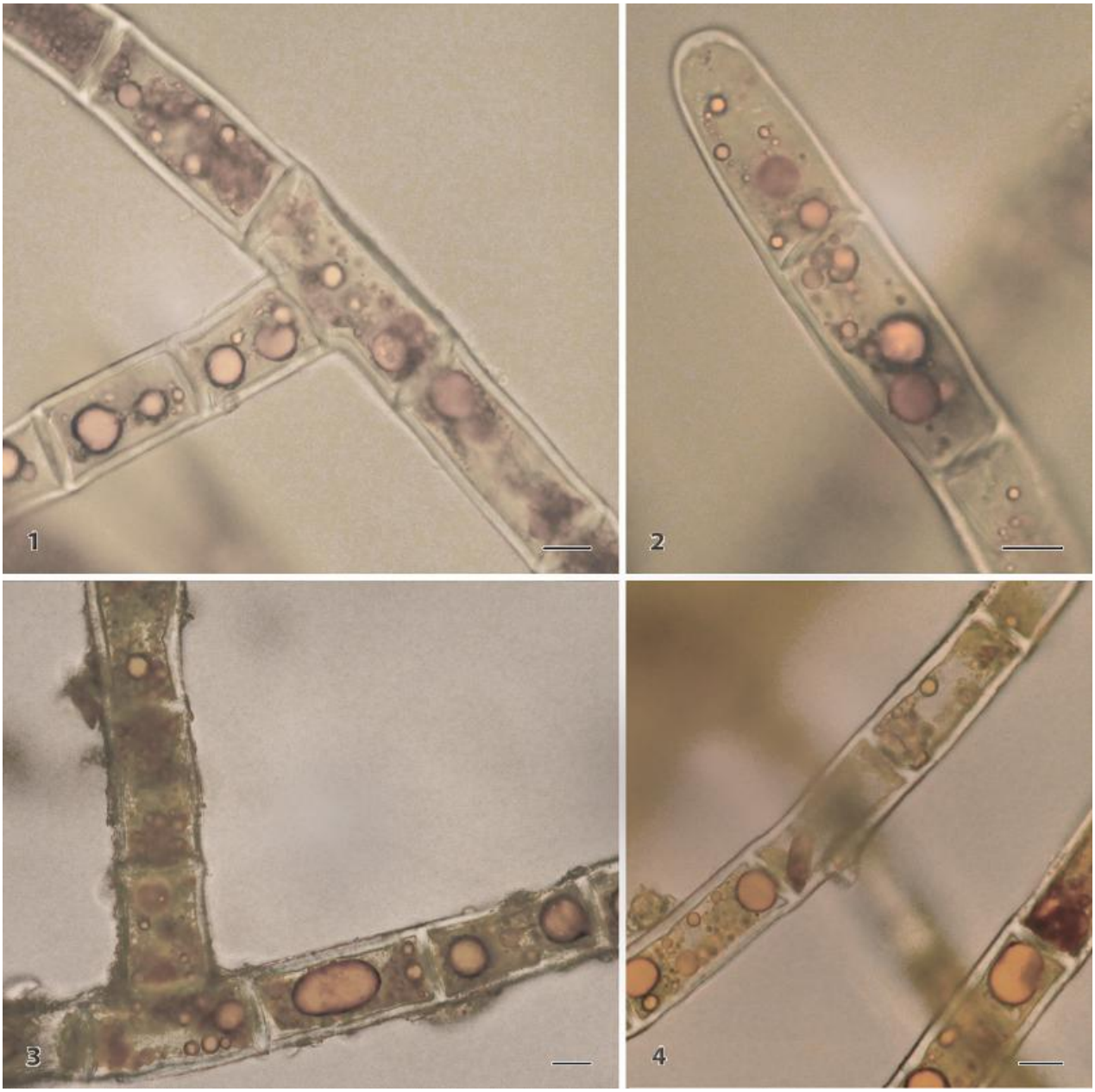


Fig. 24: *Trentepohlia arborum*. Scale bar represents 10 µm. 1–2: sample T10; 3–4: sample T12

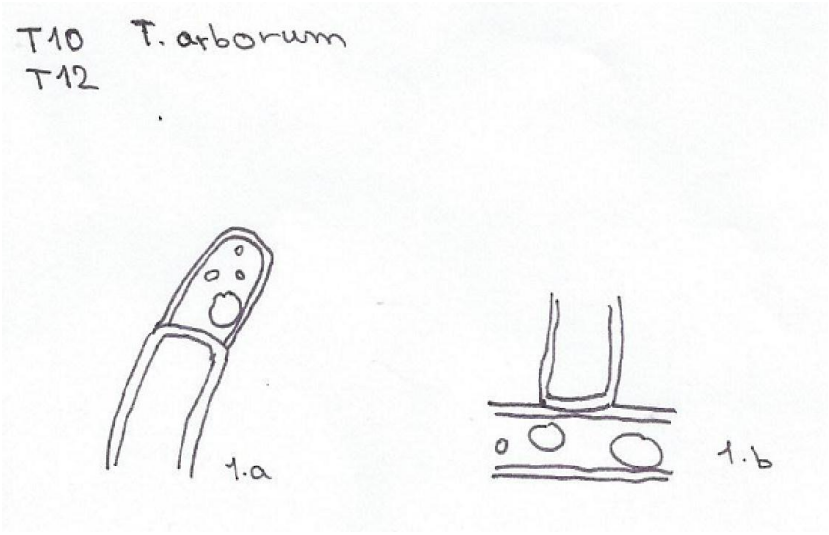


Fig. 25: Hand drawn pictures of *Trentepohlia arborum*. T10, T12 1.a: tip of a filament; 1.b: branching pattern (always in the angle of 90°).

Trentepohlia abietina (Flotow ex Kützing) Hansgirg				
Sample ID	**Locality**	**Country**	**Substrate**	**Habitat**
T28	Topělec u Písku	CZ	Birch tree	Shaded
T47	Near Pateira de Fermentelos lake	PT	Willow tree	Shaded
T50	Shore of Lagoa Salgueira lake	PT	Oak tree	xxx
TA5	Ponte di Barano Tuscany	I	Elm tree	Shaded
TA9	Ponte di Barano, Tuscany	I	Stone on a river bank	Shaded

Apart from the T28 thallus which formed dark red patches, the cushions made out of the other four specimens were usually bright orange up to 0.5 mm high and very conspicuous. The filaments were richly branched, quite thin and formed by rectangular short cells with a rough cell wall. The terminal cells were often swollen (a few cells in a row) and crowned with a small rectangular cellulose cap (fig. 26.3, 4). There were a lot of globular gametangia present (fig. 26.5). They occurred terminally or on the bases of the filaments but mostly laterally (fig. 26.6). In TA5 and TA9, the gametangia were often empty (fig 26.7). This was also the case of T28 although the

gametangia were scarce in general (fig. 26.2). When empty, the gametangia often appeared to be sort of deformed (see also the hand drawn pictures, fig. 27). The cell dimensions: 7–8 µm × 17 µm (T47, T50; not measured for T28), 3.7–3.9 µm × 9.1–10.3 µm (TA5, TA9). The dimensions of the gametangia: 9–10 µm × 9–11 µm (T47, T50), 7.7 µm × 7.8 µm (TA5, TA9).

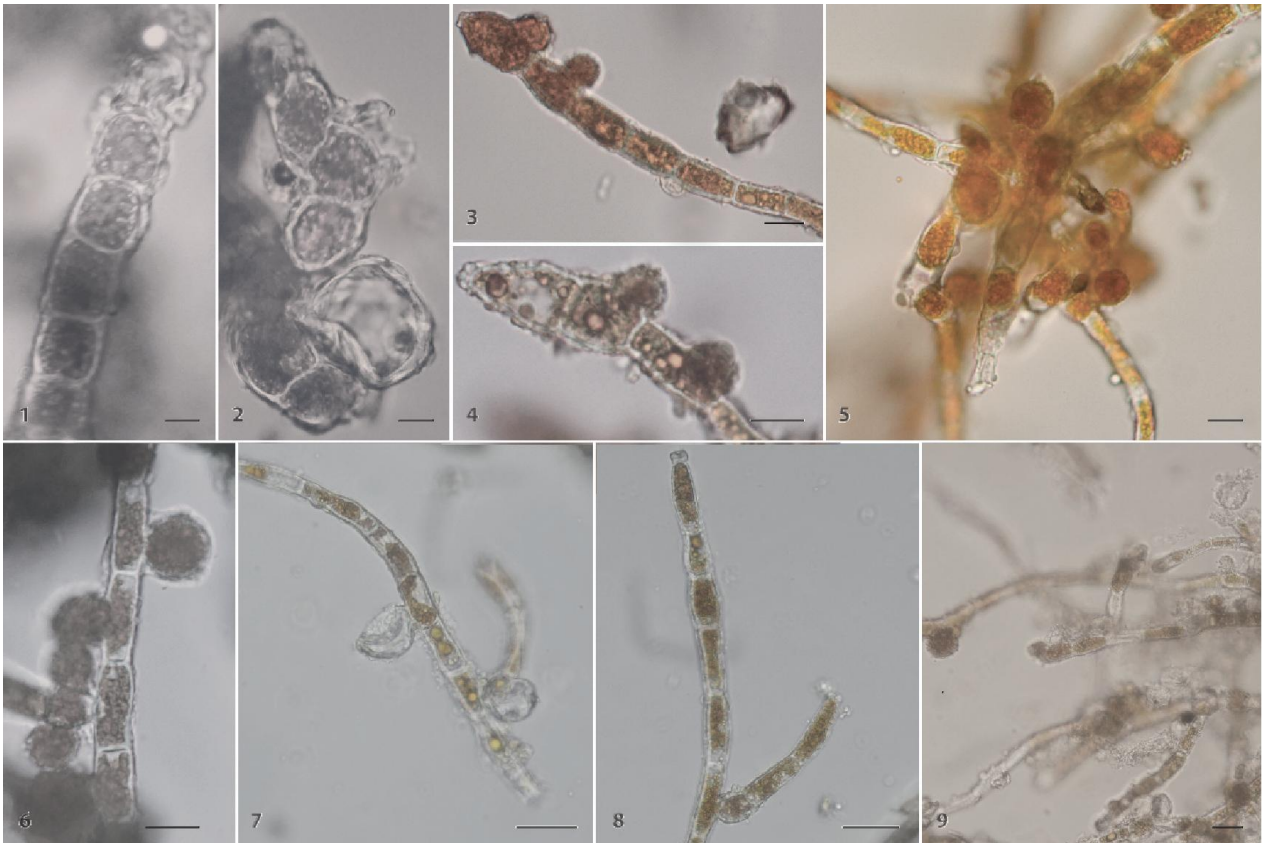
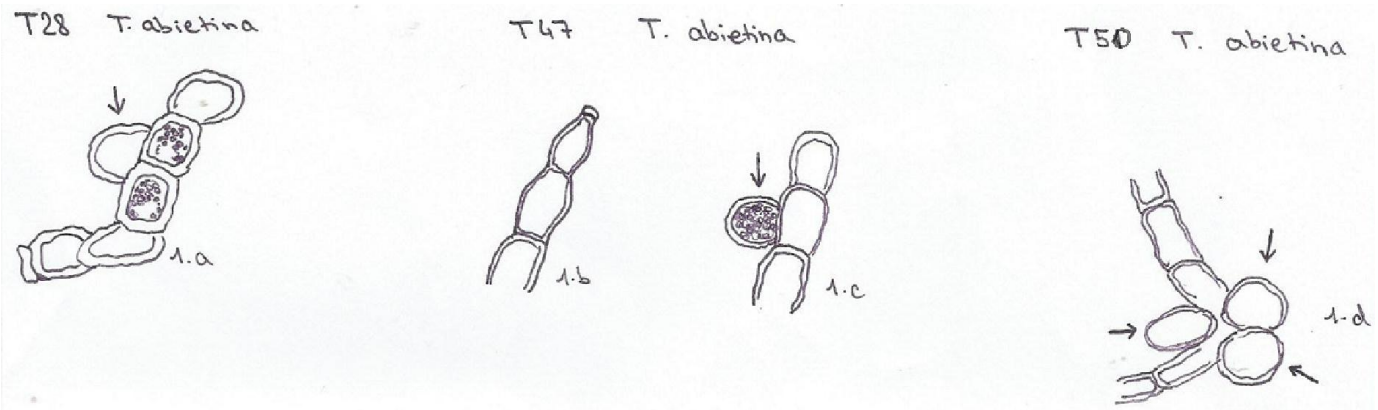


Fig. 26: *Trentepohlia abietina*. Scale bar represents 10 µm. 1–2: sample T28; 3–4: sample T47; 5–6: sample T50; 7–8: sample TA5; 9: sample TA9



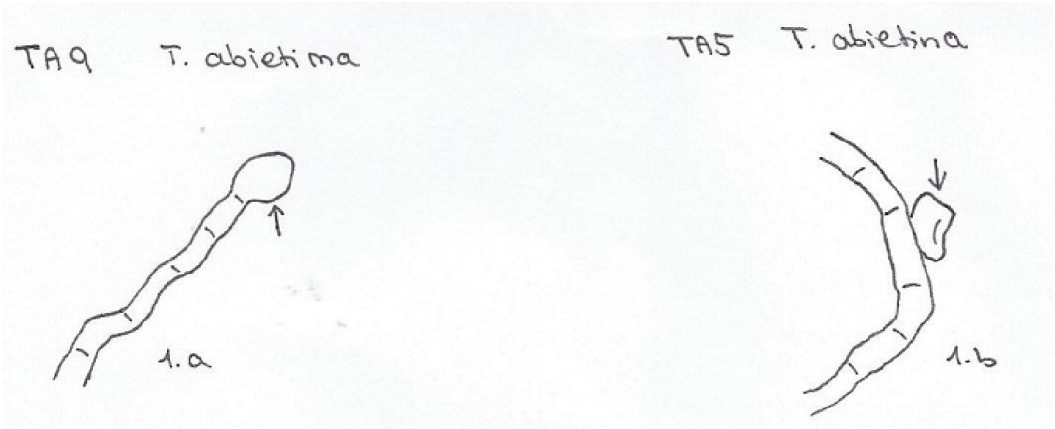


Fig. 27: Hand drawn pictures of *Trentepohlia abietina*. T28 1.a: a filament with an empty lateral gametangium; T47 1.b: a filament with small terminal cellulose cap; 1.c: lateral gametangium; T50 1.d: intercalary cluster of globular gametangia on the prostrate part; TA9 1.a: a sketch of a filament with terminal gametangium; TA5 1.b: a sketch of a filament with an empty lateral gametangium.

Trentepohlia jolithus (Linnaeus) Wallroth				
Sample ID	**Locality**	**Country**	**Substrate**	**Habitat**
T32	Šumava	CZ	Stone	xxx
T45	Bukovec, Jizerka	CZ	Rock	xxx
TA17	Sarca Val Nambrone, north Italy	I	Granite stone on a river bank	xxx

The filaments of these algae were very wide with a thick rough cell wall that appeared a bit shaggy (fig. 28.1–4; 29). The cells were orange to amber in both prostrate and erect filaments (fig. 28.3, 4). The prostrate cells were smaller, usually stuck together, more globular and little bit *umbrina*-like (fig. 28.3). However, unlike *T. umbrina* cells, these sticked together tight and did not fall apart. The dimensions of the cells of erect axes were 18–21 µm × 25–30 µm (T32, T45) and 9.8 µm × 12.3 µm (TA17). The terminal cells of the erect filaments were narrower and could have been prolonged, curved and tapered occasionally (fig. 28.4, 6, 7). When I scraped the patches of the substrate I detected a smell similar to violets and remembered some researchers mentioning this peculiarity (as far back as Linnaeus, 1753). I guess the problem with the morphological delimitation of *T. jolithus* lays in its similarity with *T. umbrina*. Sometimes the prostrate parts of both species could resemble each other very intensively.

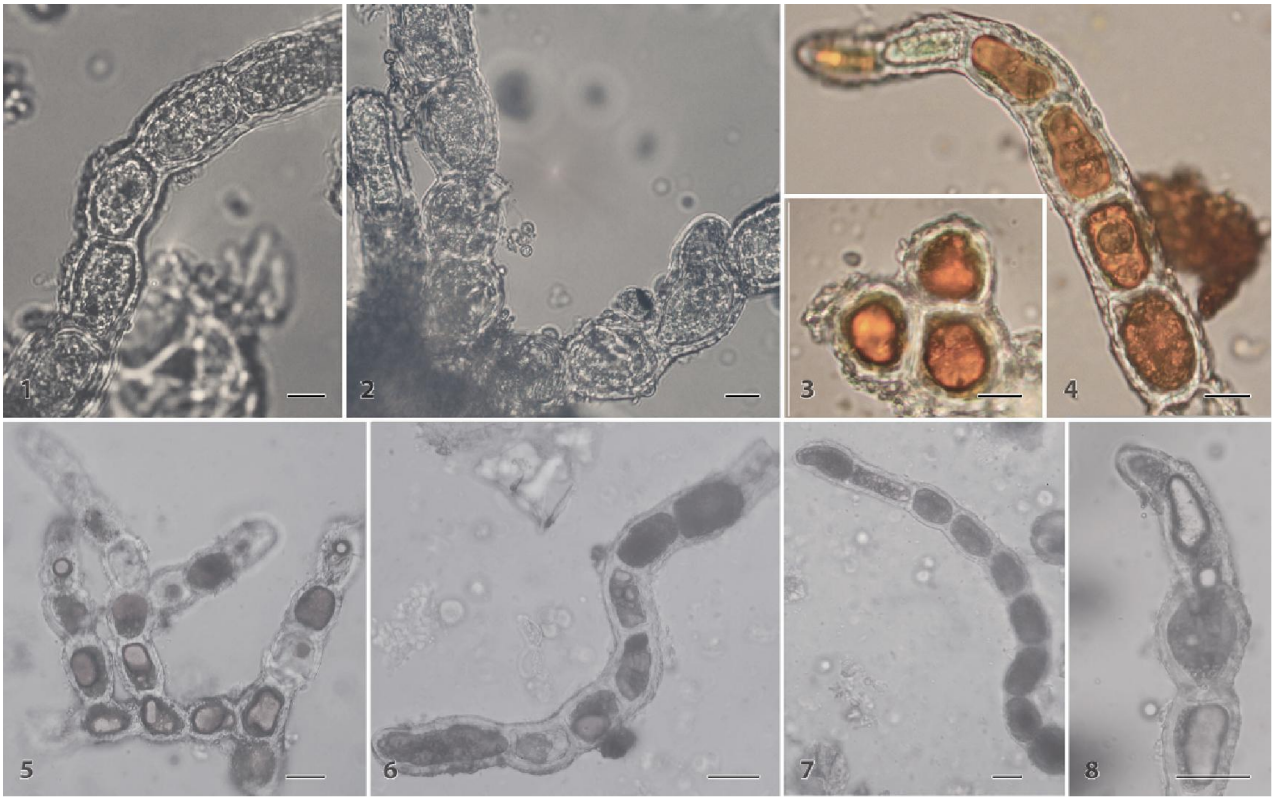


Fig. 28: *Trentepohlia jolithus*. Scale bar represents 10 µm. 1–2: sample T32; 3–4: sample T45; 5–8: sample TA17

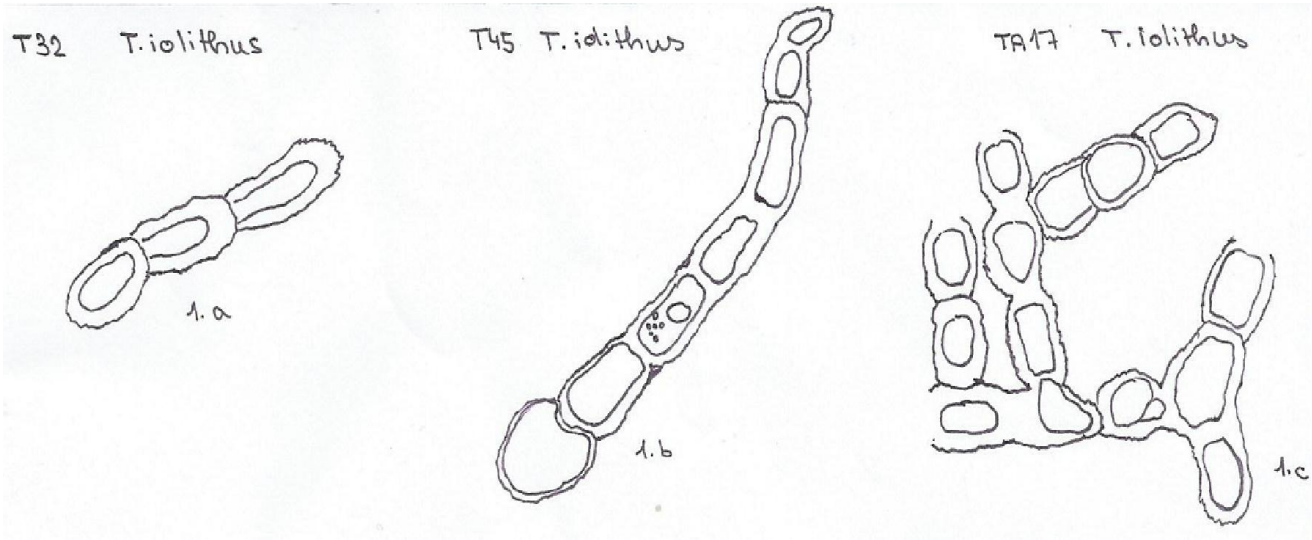


Fig. 29: Hand drawn pictures of *Trentepohlia jolithus.* T32 1.a: an arrangement of a filament; T45 1.b: a filament with curved tip and rough cell wall; TA17 1.c: branching pattern.

Trentepohlia aurea (Linnaeus) C. F. P. Martius

Sample ID	Locality	Country	Substrate	Habitat
T26	Šumava, Srní	CZ	Stone bridge above a river	Shaded
TA10	Ponte di Barano, Tuscany	I	Vertical rock in the splash zone of a small waterfall	Shaded

The filaments of these algae (fig. 30; 31) created the orange red (T26) and bright orange (TA10) cushions up to 1 mm high with the prostrate filaments thickly entwined and the erect filaments formed more loosely and growing upwards. The filaments were richly branched. The branches were quite thin and the terminal cells usually tapered to the tip (fig. 30.3, 4). The cells were rectangular with a smooth cell wall and with the dimensions 10.8 µm × 35.2 µm (T26), 6.8 µm × 15.8 µm (TA10). The inconspicuous transparent cellulose caps sat on the tips of a small amount of the terminal cells (fig 30.4). The gametangia were bottle-shaped, placed on the prostrate part but present only scarcely in T26 (fig 30.1). On the contrary, a lot of globular to elliptical gametangia with the diameter of 12 µm grew laterally, terminally in clusters and solitarily or grouped on the prostrate part in TA10 (fig. 30.5, 6). When cultivated, the sample TA10 became sterile, the filaments changed colour to bright yellow or golden and dimensions and shape of the filaments changed as well (the cells became noticeably bigger: 8.6 µm × 23.6 µm and seemed to be barrel shaped, the tips of filaments were no more tapered but some of them stayed prolonged).

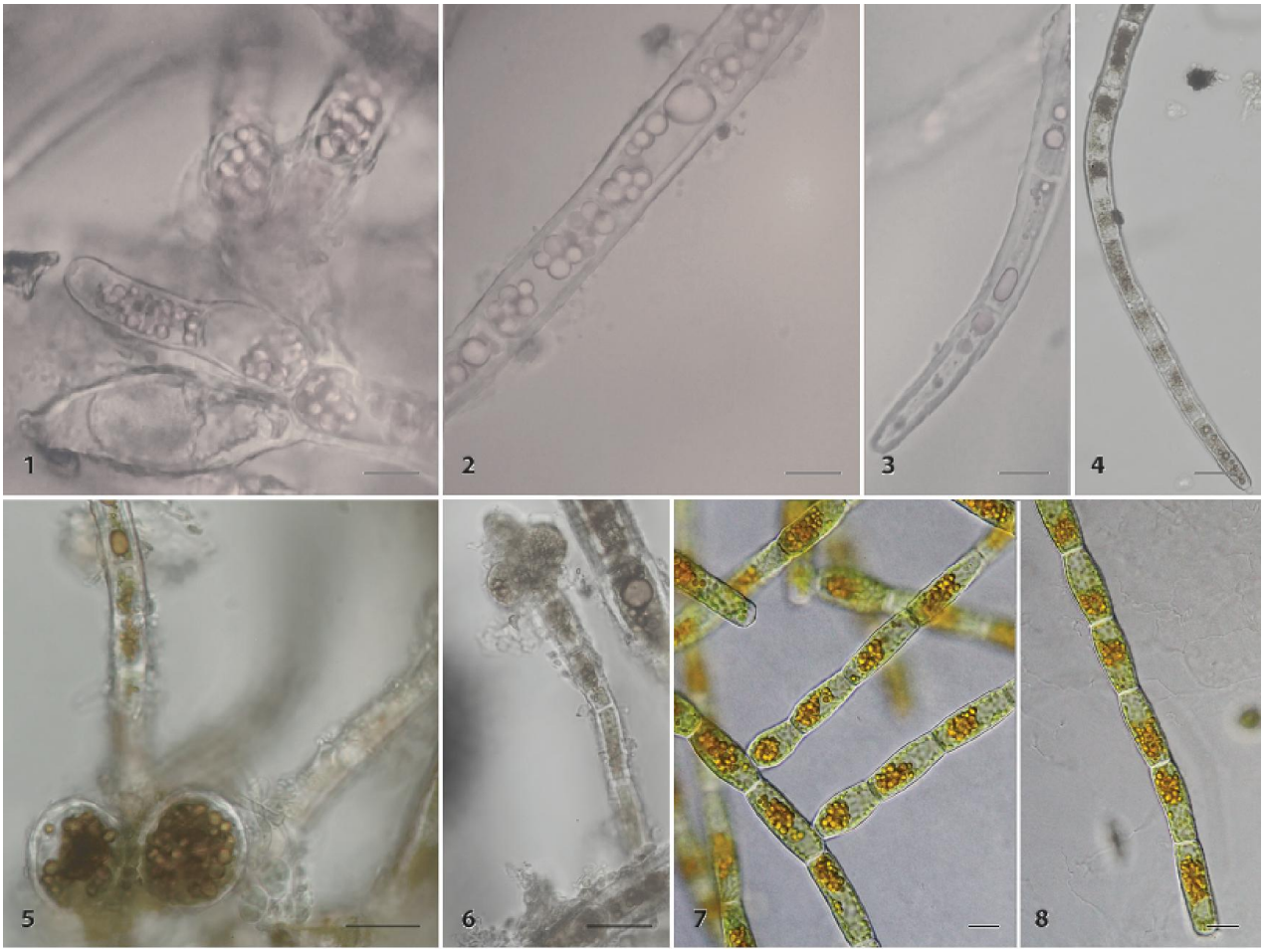


Fig. 30: ***Trentepohlia aurea*****. Scale bar represents 10 µm. 1–3: sample T26; 4–6: sample TA10; 7–8: cultivated sample TA10**

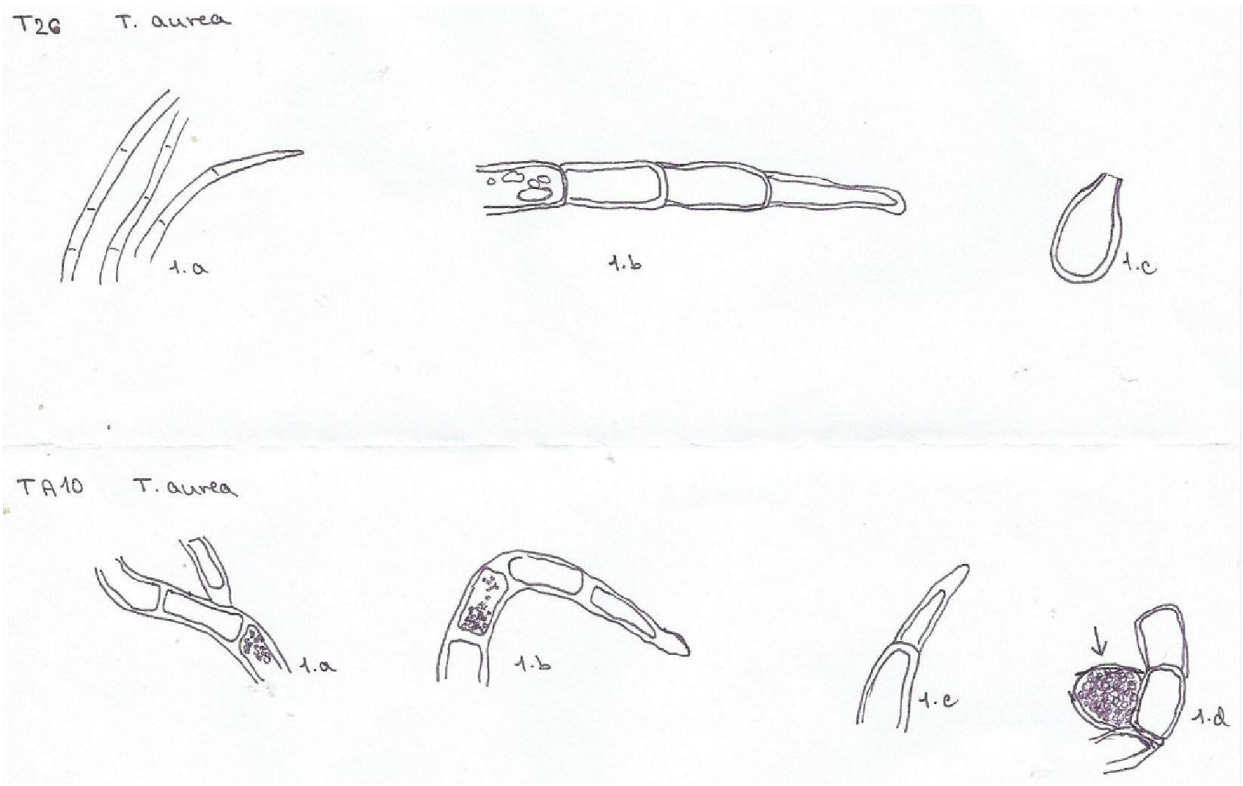


Fig. 31: Hand drawn pictures of *Trentepohlia aurea*. T26 1.a: general appearance of filaments; 1.b: detail of a filament with a tapered tip; 1.c: detail of the bottle-shaped gametangium. TA10 1.a: branching pattern; 1.b–1.c: filament with a tapered tip; 1.d: detail of the globular gametangium situated laterally on a filament.

3.2 Phylogenetic Analyses

Two *rbc*L datasets have been used in phylogenetic analyses. The first one contained the sequences of Trentepohliales and should be considered as the main output of this thesis. The final tree is shown on the figure 32. I have defined thirteen clades on the tree for simplification of the orientation in the tree.

I was able to unambiguously molecularly characterize 13 specimens (T6, T10, T12, T25, T28, T34, T35, T45, T49, TA2, TA5, TA9 and TA17). In these specimens, clonal sequences belonged to only one *Trentepohlia* genotype and I gained more than three such sequences so it was possible to check them mutually for polymeration errors. These sequences are highlighted in bold blue and they occurred in clades 1, 9, 11, 12 and 13.

Secondly, there are 17 sequences from 9 samples (non-bold blue). In some of these samples I was not able to gain satisfactory amount of sequences for polymerase error check (T26, T32, T47, T50 and TA10). The other represented mixtures of various *Trentepohlia* species (T7, T33,

T37 and T51). These sequences are scattered around the tree showing the affinity to various clades. The reason why T33, T51 and T37 are in bold blue is that there were at least three similar genotypes present in mentioned samples. I presumed the most abundant genotype would belong to the most abundant morphology I see in the sample. In the sample T51, also one clonal sequence with different genotype occurred (T51D). The same situation took place in the case of sample T37 (one dissimilar clone T37G3). The situation in the sample T33 is little more complicated. There were three similar genotypes in this sample but also six different ones. No other sample possessed so many different species sequences.

One sequence, T27 (direct seq) was the only one that was obtained in the beginning trials without cloning and was used more out of curiosity.

In the clade 1, there are *T. umbrina* (T6, and T51)*, T. arborum* (T10 and T12) and *T. jolithus* (TA17) clustered together with GenBank sequence of *T. arborum* and some sequences from mixed samples T33 and T7 and T51. Clade 2 represents a well supported cluster of the species of *Printzina*. In the clade 3, two sequences from mixed T47 sample show the affinity to *Printzina*. Neither *Trentepohlia* nor *Printzina* seem to be monophyletic as it can be seen not only in clades 4, 5, 6 and 10. The same result applies to *Cephaleuros* (see clade 7) that is clustered with *Trentepohlia dialepta*. In the clade 8, two single clonal sequences from *T. aurea* samples (T26 and TA10) form a well-supported monophyletic group with *T. aurea* from Galway and the symbiotic *T. aurea* from *Coenogonium*. Clade 9 shows a well-supported assemblage of symbiotic and free living species of *T. abietina* (T28 and the rest). However, *T. abietina* species (TA5 and TA9) occur also in the clade 11 close to *Trentepohlia flava*. The clade 12 represents a well-supported group of *T. umbrina* from Italy (TA2), Portugal (T49) and Czech Republic (T25, T34, T35 and T37). Very close to group of these *T. umbrina* species is *T. jolithus* (T45). The last clade 13 contains *T. annulata* (T33) clustered with further unspecified symbiotic species of *Trentepohlia*. Other sequences from the mixed sample T33 group with clade 1, 8, 10 and 12.

The second dataset represents the main cohabitants present among the Trentepohliales, green algae living on the substrates together with them (and being able to be amplified using the primers designated for *Trentepohlia*). These belong mostly to Trebouxiophyceae. This dataset has been used out of curiosity of the phylogenetic position of cohabitants and the final tree is shown on the figure 33. There was no pressure to gain as many similar sequences as possible to check them mutually for errors. Therefore, only the solitary clonal sequences occur in this phylogenetic tree. We can see the primers designated for *Trentepohlia* fit well to the following species: *Coccomyxa*,

Asterochloris, *Dictyochloropsis*, *Chloroidium* and *Parachloroidium*. There were also some sequences dissimilar to any other one published in the GenBank (identity less than 85 %). It is possible that these sequences belong to the genus *Apatococcus*. I have spotted this alga repeatedly in my samples. Moreover, it is a very abundant subaerial alga inhabiting similar substrates as *Trentepohlia* (Gustavs et al., 2016). Apart from one unverified sequence (KF355935), there are no sequences of the *rbc*L gene of *Apatococcus* in the GenBank (see more in discussion). Therefore, I have placed these sequences into a clade called the *Apatococcus* clade.

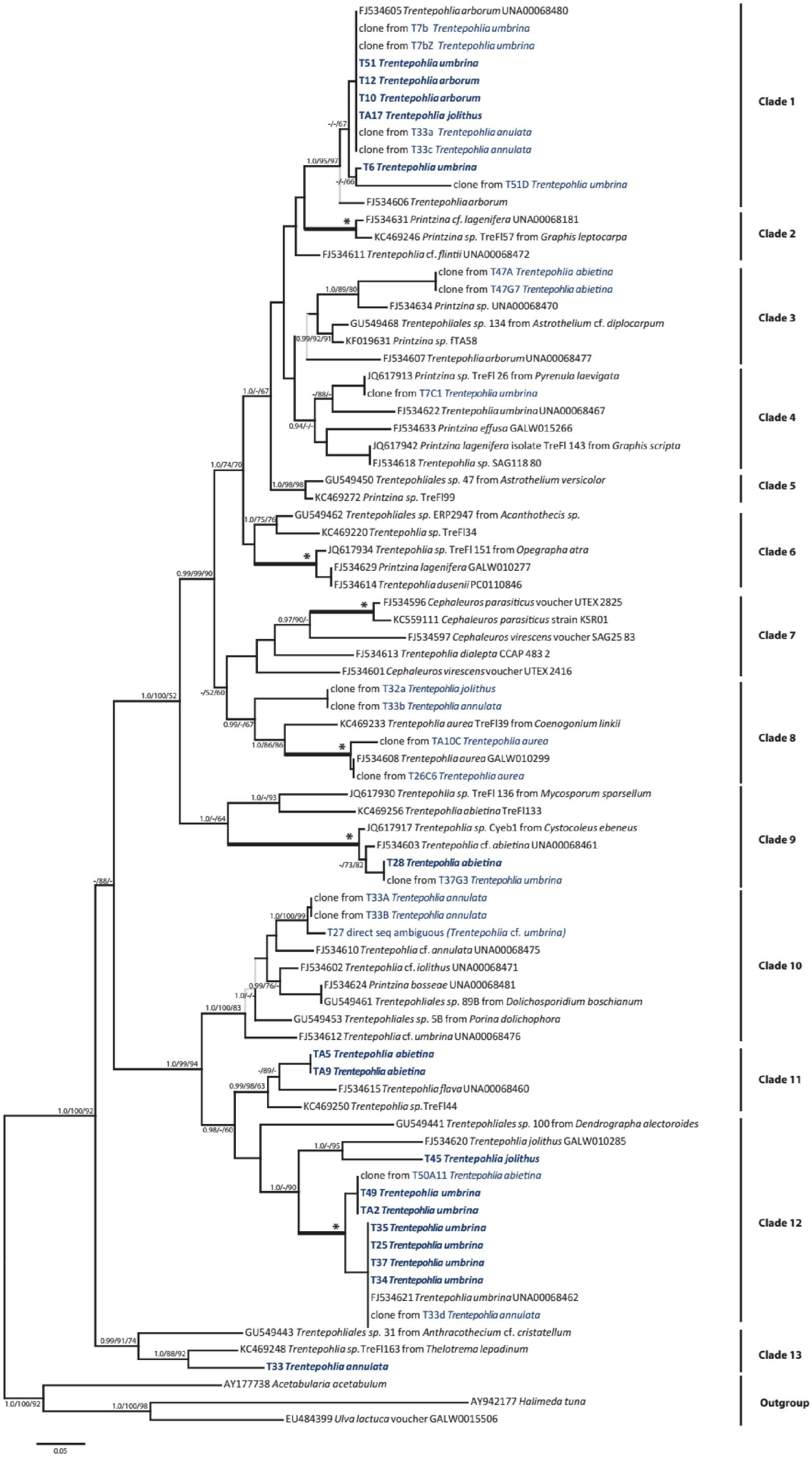


Fig. 32: Bayesian analysis based on the *rbc*L dataset of the Trentepohliales. Values at the nodes represent statistical support of grouping of the taxa that was estimated using MrBayes posterior-node probability (left), maximum parsimony bootstrap (middle) and maximum likelihood bootstrap (right). Asterisks indicate full support. Scale bar shows the estimated number of substitutions per site. Own sequences are coloured in blue, GenBank sequences are black. Bold-blue stands for at least three separate clonal sequences of the same genotypes of particular samples that have been checked mutually for polymeration errors. Non-bold labels indicate solitary clonal sequences of particular samples.

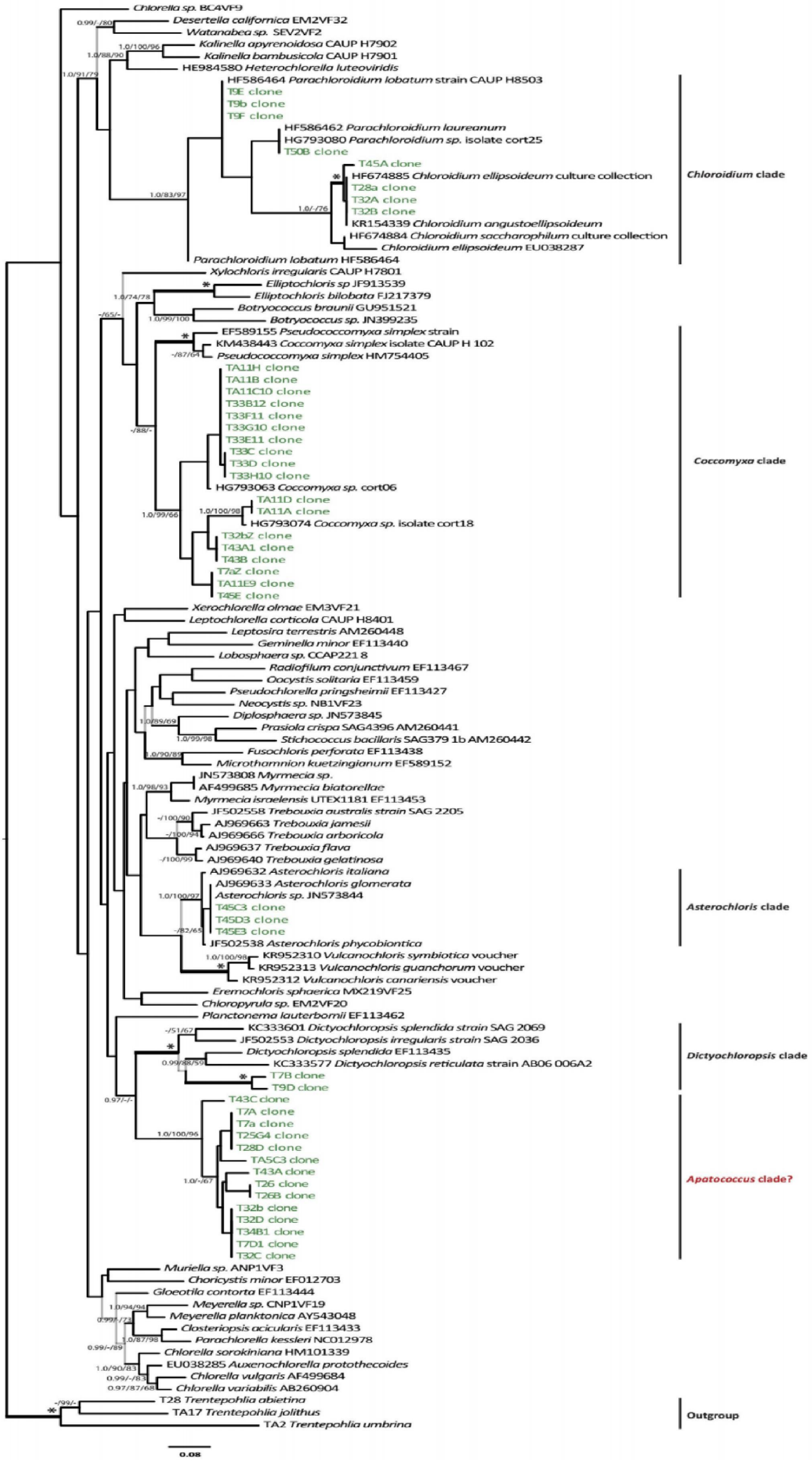


Fig. 33: Maximum likelihood analysis based on the *rbc*L dataset of the Trebouxiophyceae. Values at the nodes represent statistical support of grouping of the taxa that was estimated using MrBayes posterior-node probability (left), maximum parsimony bootstrap (middle) and maximum likelihood bootstrap (right). Asterisks indicate full support. Scale bar shows the estimated number of substitutions per site. Green colour represents own sequences whereas black colour belongs to the GenBank sequences.

4. Discussion

4.1 Homogenity of Trentepohlialean Crusts and Presence of Species Mixtures

For many decades, the growths of *Trentepohlia* have been considered homogenous (Hansgirg, 1886; Fischer, 1922; Printz, 1939). In fact, there are no studies to date even suggesting it could be otherwise. The crusts of *Trentepohlia* appear to be homogenous by the naked eye in terms of colour and surface. Under the light microscope, filaments seem to belong only to one species and slight differences can be easily attributed to the great morphological variation existing within this genus (Rindi et al., 2009a; Hametner et al., 2014b). However, some samples seemed to comprise of really different filaments entangled together. Since seeing such samples, I have been bothered by a burning question tackling the pieceful traditional approach for study of *Trentepohlia*. Are the growths of *Trentepohlia* formed really only of one species?

When studying *Trentepohlia*, scientists in the 18th and 19th centuries (Linnaeus, 1753; Gobi, 1827, etc.) used fresh material for morphological observations and species delimitation. In the late 20th century, mainly agar cultures became popular (Lee et al., 1990; Abe et al., 1999). Later, new molecular methods allowed us to obtain DNA sequences from cultured material and better understand phylogeny of organisms (Lopéz-Bautista & Chapman, 2003). After the initial enthusiasm had faded away, it was clear that there was no such a method that would flawlessly solve all the tasks we would demand. With polyphyletic complexes of cryptic species present, we cannot rely only on morphological or molecular methods (Rindi et al., 2009a).

After initial trials with cultivation of *Trentepohlia*, it was quite clear to me, this approach is not without pitfalls. Not only is *Trentepohlia* a slow growing organism in culture, its filaments also often become sterile and change some aspects of their morphology (colour, cell dimensions). This is a serious problem since species delimitation has always been focused on the dimensions and shape of the cells and the features of the reproductive organs in fresh natural samples. With that in mind I decided to follow the approach of the scientists in 18th and 19th centuries of detailed morphological observations with additional drawings of the observed features. Of course, diving into species concepts of algae would be unimaginable without modern molecular methods. Therefore, I decided to combine the old-fashioned

morphological approach with the modern methods of DNA sequencing and new and yet untried path of cloning of *Trentepohlia*.

Results revealed a striking feature of some populations of *Trentepohlia*. There were a few different species of this genus present in a single sample (T7, T33, T37 and T51). One could simply assume possible flaws of my approach. Cross-contamination of the samples during sampling itself or contamination in the laboratory environment comes to mind. With all the respect to these arguments, I can only state all precautions of clean sampling have been upheld and only sterilized tools have been used. Simultaneously, I sampled only very small areas of the overgrown surface only from one spot if possible. During the cloning, strict rules of aseptic work were obeyed (change of gloves, work in FlowBox, sterilization of tools and working space with ethanol and UV-light, watchful handle of samples). Therefore, the mixtures of the species do not result from cross-contamination and my results provide a new and interesting perspective on our algal growths perception. It would be certainly interesting to clone some other filamentous subaerial algae, for example *Klebsormidium*, to find out if this situation happens only in the case of *Trentepohlia* or in general.

4.2 Problems and Benefits Linked to the Inuniformity of *rbc*L Sequences of the Trentepohliales

Thanks to the cloning, I was also able to amplify some common green algae living on the substrates together with *Trentepohlia* that could be amplified using the primers for *Trentepohlia*. For obtaining these results I should be probably grateful to the impossibility to design specific primers that would get on a satisfactory length of *rbc*L gene of all trentepohlialean taxa. The same problem was also recorded by Rindi et al. (2009a). They concluded that *rbc*L datasets of the Trentepohliales tend to have a higher substitution rate than other groups of green algae which is perhaps why difficulties with the primer designation occur.

An interesting dataset of the Trebouxiophyceae was created on this account and showed the affinity of the clones to some trebouxiophycean genera such as *Coccomyxa* or *Chloroidium*. Also, the whole clade of the sequences with no strong affinity to other GenBank sequences occurred in the dataset of green cohabitants. After contemplating all possibilities, the most likely solution is that this species is *Apatococcus*. *Apatococcus* dominates green

algal biofilms in the temperate zones. It grows on both natural and artificial substrates (Gustavs et al., 2016) just like *Trentepohlia*. Apart from one unverified sequence (KF355935), *Apatococcus* sequences are still missing in the GenBank database. The unverified sequence belongs to the culture CCALA 213 and according to BLAST it shows 100% identity with *Chloroidium*. The same organism is kept as well in the SAG culture collection under the code 34.83 with the note that this species was misidentified. This strain is not *Apatococcus* and will be hopefully soon reclassified. Therefore, I think the sequences of *Apatococcus* are not present in the GenBank. It would be benefitial to try to cultivate *Apatococcus* in the future and obtain clear *rbc*L sequences. This goal has been complicated so far due to its slow growth rates and low accumulation of biomass in the cultures (Gustavs et al., 2016).

Speaking of the dataset of green algal cohabitants, some phylogenetic analyses showed a curious aspect. When performing Bayesian and Maximum likelihood analyses with partitioning into the three codon positions of protein-coding *rbc*L gene, some species of *Trebouxia* and *Myrmecia* occurred out of the Trebouxiales. This is not consistent with our current understanding of phylogeny of the Trebouxiophyceae (Gaysina et al., 2013; Neustupa et al., 2013; Vančurová et al., 2015). Therefore, these analyses with changed settings were performed repeatedly and in the end one of them, which reflected the current phylogeny of the Trebouxiophyceae the best, was chosen. Sometimes protein-coding genes are affected with high substitutional saturation at the third codon positions and these positions are therefore excluded from the analyses (Nakada et al., 2008). However, this was not helpful in case of my dataset. Even without third codon positions or without position partitioning itself, most of the analyses showed the displeasing pattern mentioned above. This problem could be caused by a lack of phylogenetic signal in the mentioned dataset or perhaps by the presence of the new clade of *Apatococcus*. Fučíková et al. (2014) also reported some problems with the *rbc*L phylogeny and conflicts of signals between 18S and *rbc*L data not only in the Trebouxiophyceae but also in the whole lineage Chlorophyta. It seems that sometimes phylogeny based on *rbc*L is problematic and should be perhaps appendixed by other gene analyses.

4.3 Morphology Versus Phylogeny

From the dask of research of *Trentepohlia*, species concept has been built on morphology (Linnaeus, 1753; Gobi, 1827; Hariot, 1889; etc.). Since the first molecular data of Trentepohliales became available, it has been more than clear this concept stands on unstable foundations (Lopéz-Bautista & Chapman, 2003; Lopéz-Bautista et al., 2006; Rindi et al., 2009a). Latest molecular revision based on 18S rDNA as well as *rbc*L carried out by Rindi (2009a) brought solid evidences for several thought-provoking facts that correspond well with the results of this study.

"Some of the traditional morphological characters used for taxonomy in species of Trentepohlia are not phylogenetically significant." (Rindi et al., 2009a)

I have delimited my samples using the traditional morphological approach. However, the resulting phylogeny was largely in contrast with these delimitations.

In my results, *T. umbrina*, *T. jolithus* and *T. arborum* clustered together in the clade 1. *T. umbrina* and *T. jolithus* even occurred together in the clade 1 and also close to each other in the clade 12. I have already described the morphological resemblance of the prostrate part of *T. jolithus* (TA17) to the specimens of *T. umbrina*. When I saw the great abundance of *T. umbrina* in temperate regions, one prevailing thought came to mind. Is it possible that the typical *umbrina*-like morphology of fragmented filaments with almost globular cells that we see all around us would be just an ontogenetic stage capable of growing firm filaments only in the right conditions? Gobi (1827) saw *umbrina*-like prostrate cells in *T. uncinata*, from which a number of filaments with zoosporangia arose. He also stated these cells resembled *T. umbrina* greatly. This is also apparent from some drawings of *T. jolithus* (Cribb, 1958; 1989). If the *umbrina*-like morphology is just an ontogenetic state of otherwise fully developed forms it would explain some inconsistencies between the morphologically and molecularly delimited species.

On the other hand, the samples T10 and T12 showed no resemblance to *T. umbrina* whatsoever. However, I am afraid clustering of T10 and T12 in the clade 1 with *T. umbrina* and *T. jolithus* could be caused by decreased qaulity of the samples. I received this material from my friend who travelled to Laos and the samples were quite old and damaged when they reached me, which could finally insert some bias into the phylogenetic analyses.

The two independent clades with *T. umbrina* (clade 1 and 12) confused me. There was almost no morphological difference among the *T. umbrina* species in the clades 1 and 12

(apart from T6 having smaller cell dimensions than the others). The same related to both *T. jolithus* (T45 and TA17) occurring in the clades 1 a 12. Their morphology was also similar (TA17 only had smaller cell dimensions). *T. abietina* (T28, TA5 and TA9) appeared also in both clades 9 and 11. T28 would show some morphological differeces from TA5 and TA9 (almost no gametangia present, the filaments quite short and without swollen terminal cells).

One would presume that the clades perhaps cluster according to the locality of their origin or the substrate they grew on. Sadly, I have seen no such differentiation and clustering of the species seemed to be independent on these factors.

The only species that appeared to be monophyletic was *T. aurea*. All solitary clonal sequences of this species are present in the clade 8 close to the *Cephaleuros* clade. Rindi et al. (2009a) recently stressed the importance of recovering the phylogenetic position of *T. aurea* since it is type species of the genus *Trentepohlia* and its position also affects the genus-level classification in the Trentepohliales.

"Classification on the genus level is chaotic and will need major modifications." (Rindi et al., 2009a)

This is true mainly for *Trentepohlia* and *Printzina* that are clearly not monophyletic. Morphological circumscriptions of these two genera overlap greatly and reasons for their division postulated by Thompson & Wujek (1992) are not strong enough to keep *Trentepohlia* and *Printzina* separated (Lopéz-Bautista et al., 2006; Rindi & Lopéz-Bautista, 2007).

My results support the idea that these genera are not monophyletic. I feel that there is enough evidence to unify both genera into *Trentepohlia* again. In the case of *Cephaleuros*, 18S rDNA data of Rindi et al. (2009a) proved it to be monophyletic whereas in their *rbc*L data, strain of *T. dialepta* from CCAP made it paraphyletic. My results corroborated the paraphyletic nature. Accordingly, Rindi et al. (2009a) suggested the transfer of *T. dialepta* to *Cephaleuros.*

"Species T. umbrina, *T. arborum* and *P. lagenifera represent polyphyletic morphotaxa forming complexes of cryptic species.*" (Rindi et al., 2009a)

Bickford (2007) described the cryptic species as distinct species erroneously classified under a single species due to a lack of clear morphological differences. My results are in agreement with the existence of the cryptic species mentioned by Rindi et al. (2009a).

5. Conclusions

5.1 Species Concept in *Trentepohlia*. Is There a Way out from This Mess?

In despair we ask: how to proceed in the study of the Trentepohliales with all that chaos in the current taxonomy? Should we sample and sequence the species from the type localities and characterize their morphology in detail? That could be definitely a good way to start but since the species were described centuries ago, I am afraid we would not find them at the type localities anymore. Anyway, it would be certainly benefitial to search the type localities and when finding the species, circumscribe them precisely, compare our circumscriptions with the oldest references, maintain their cultures and define them molecularly. It would also be benefitial to sample some localities with *T. jolithus* and *T. umbrina* repeatedly during a longer period to reveal their true relationship.

At the genus level, I feel that major rearrangements will be necessary in the Trentepohliales. The unification of *Trentepohlia* and *Printzina* seems to be inevitable. The unification at the species level would perhaps be helpful as well. Based on the literature examination, I think the synonymization of *T. uncinana* with *T. arborum* and also the synonymization of *T. odorata* with *T. jolithus* should take place. In other species, strict narrowing of circumscriptions with regard to molecular results might help to clear the chaotic situation.

5.2 Future Plans

Currently, the taxonomy of *Trentepohlia* is chaotic and foundations of species delimitations wobble with every other new phylogenetic study. The more our knowledge of *Trentepohlia* grows the more cryptic species, species with unclarified morphological boundaries and disorder in classifications we discover. Additionally, this problem stretches out to every related higher taxonomical unit which we focus our attention on: the order Trentepohliales (Lopéz-Bautista et al., 2006), the class Ulvophyceae (Friedl & O'Kelly, 2002), the lineage Chlorophyta (Lewis & Flechtner, 2004), green algae (Pröschold & Leliaert, 2007) red algae (Saunders, 2008; Payo et al., 2013), etc.

"It is a remarkable testament to humanity's narcissism that we know the number of books in the US Library of Congress on 1 February 2011 was 22,194,656, but cannot tell you - to within an order-of-magnitude – how many distinct species of plants and animals we share our world with." (May, 2011)

Great deal of scientific energy has been commited to the postulation of adequate species concepts definitions (Mayden et al., 1997) and their repeated unification (Queiroz, 2007). Species concepts in algae are often considered to be difficult. Afterall, what chances of sorting out this confusion we have if we use different perceptions of the term species concept, moreover, if we are not even able to describe generally what an alga is (Guiry, 2012)? It is not in one's strengths to solve and sort out all this disorder. On the contrary, the whole phycological community could participate in the solution if each of the taxonomists put their own house in order, i.e. if one sorts out even the smallest task (relationships in two species, two genera, whole groups and so on).

To fulfill my part in this challenging task, in my Ph.D. thesis I would like to focus on the class Ulvophyceae, especially the order Ulvales/Ulotrichales and the genus *Ulothrix*. Phylogenetic position of some ulvophycean orders has been revised lately (Škaloud et al., 2013a). Nevertheless, satisfying clarification of the phylogenetic position in majority of the taxa in this order is still absent in the current state of knowledge. Very confusing is the situation in the genus *Ulothrix* itself. Some phycologists have comprised genera *Ulothrix* and *Uronema* in the past (Mattox & Bold, 1962), whereas other kept them separated (Zarina et al., 2005). Pröschold & Leliaert (2007) proved these two genera to be distinct. Therefore, I would like to perform complete revision of the genus *Ulothrix.* Simultaneously, I will focus on the extra-marine taxa of the Ulvales/Ulotrichales and carry out their detailed morphological as well as molecular revision. Hopefully, I will be able to reassess the boundaries between these two close orders and elucidate the phylogenetic position of their freshwater and terrestrial members.

6. References

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7. Appendix

7.1 The Delimitation Key of the European *Trentepohlia* Species

Table 1: Overview of the main morphological features of *T. annulata*

Morphological character	***Trentepohlia annulata***
Thallus organization	**prostrate part developed**
Texture of thallus	**moss-like, up to 1 mm high;** up to 1.5 mm (Printz, 1939)
Shape of cells (erect)	**cylindrical, barrel shaped**
Width of cells (erect)	**12–17 µm;** 9.5–max 24 µm (Printz, 1939)
Length of cells (erect)	1.5–3 × width (Printz, 1964)
Shape of cells (prostrate)	**moniliform-branched or pseudoparenchymatic;** swollen in the middle (Printz, 1939)
Width of cells (prostrate)	up to 33 µm (Printz, 1939)
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	xxx
Width of cells (non-differentiated)	xxx
Length of cells (non-differentiated)	xxx
Branching pattern	**richly branched;** seldom branched (Ettl & Gärtner, 1995)
Cell wall	**smooth, terminal cellulose caps;** in layers (Printz, 1939)
Shape of gametangia	**globular to elliptical, thick membrane**
Width of gametangia	**16–30 µm × 29–52 µm**
Arrangement of gametangia	**lateral or terminal;** on prostrate filaments (Printz, 1939)
Presence of beak in gametangium	xxx
Shape of sporangia	**young globular, mature transverse oval, "Trichtersporangia"**
Width of sporangia	**22 µm × 44 µm;** 17–38 × 30–58 µm (Printz, 1939)
Arrangement of sporangia	**terminal**
Shape of cell supporting sporangium	**with 1–2 funnel-like cellulose rings**
Shape of terminal cells	**either bearing generative organs or cellulose caps**
Position of ostiole in sporangium	**on downwards inclined end of sporangium**
Colour	**yellow-green to brown**
Substratum	**tree bark, horizontal surface of treestumps, rock;** able to grow on horizontal substrates (Brand, 1902)
Environment	**humid and shady;** mainly in mountains (Printz, 1939)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Brand (1902)**
Type locality	**Germany, Oberbayern (Benediktenwald), *Picea* stump**
Own experience	Yellow moss-like sample found in Krkonoše mountains (Czech Republic) in more than 800 h. a. s. l. on vertical side of tree stump in forest. Trichtersporangia positioned terminally on long branches, could be easily

	torn off, cellulose rings present.

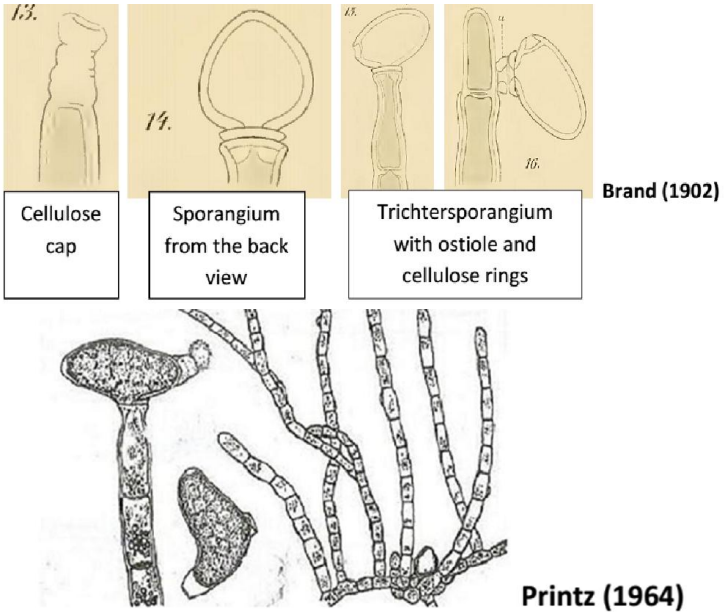
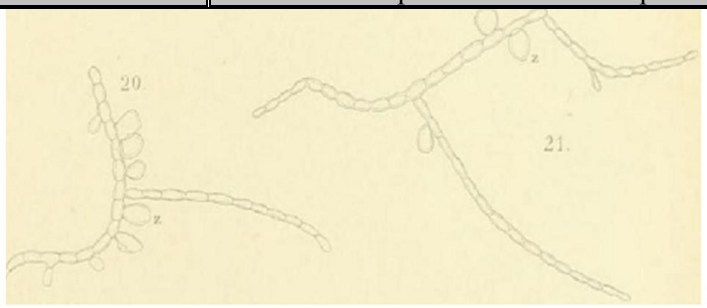


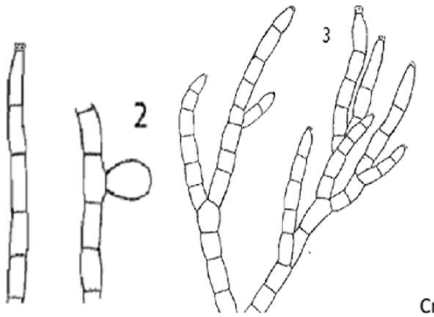
Table 2: Overview of the main morphological features of *T. abietina*

Morphological character	***Trentepohlia abietina***
Thallus organization	prostrate part developed (Rindi et al., 2006b)
Texture of thallus	**small cushions**
Shape of cells (erect)	cylindrical (Rindi et al., 2006b)
Width of cells (erect)	5–10 µm (De Wildeman, 1891); 7–10 µm (Rindi et al., 2006b)
Length of cells (erect)	2–3 × width (De Wildeman, 1891); 1.5–5 × width (Rindi et al., 2006b)
Shape of cells (prostrate)	round to elliptical (Rindi et al., 2006)
Width of cells (prostrate)	4–10 µm (Prinzt, 1939); 8–12 µm (Rindi et al., 2006b)
Length of cells (prostrate)	1–3 × width (Printz, 1939)
Shape of cells (non-differentiated)	cylindrical or swollen in the middle (Hansgirg, 1886)
Width of cells (non-differentiated)	6–9 µm (Hansgirg, 1886); 4–10 µm (Hariot, 1890)
Length of cells (non-differentiated)	1–3 × width (Hansgirg, 1886); 12–48 µm (Hariot, 1889)
Branching pattern	**branched** in various ways, branches curved (Printz, 1920)
Cell wall	divergent layers, in living specimen hard to recognize; thick on the top of branches (Printz, 1939)
Shape of gametangia	globular (Hansgirg, 1886)
Width of gametangia	14–25 µm (De Wildeman, 1891); up to 30 µm (Hansgirg, 1886)
Arrangement of gametangia	terminal or lateral (Hansgirg, 1886)
Presence of beak in gametangium	xxx
Shape of sporangia	round to elliptical (Brand, 1902; Fischer, 1922; Printz, 1964)
Width of sporangia	10–20 µm (Brand, 1902; Fischer, 1922; Printz, 1964)
Arrangement of sporangia	xxx

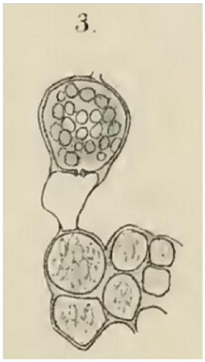
Shape of cell supporting sporangium	occur rarely, hook-formed (De Wildeman, 1891); can be hook-formed (Karsten, 1891), he was not sure if the sample was indeed *T. abietina*
Shape of terminal cells	xxx
Position of ostiole in sporangium	xxx
Colour	orange to yellow; red to gold (Hansgirg, 1886)
Substratum	**bark of trees;** mainly coniferous (Hansgirg, 1886); occasionally also bark of deciduous trees (Printz, 1939)
Environment	higher situated mountain regions (Hansgirg, 1886); humid, mainly shaded forests (Printz, 1939)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Flotow ex Kützing (1845) as Chroolepus abietinum**
Type locality	**Hirschberg, bark of *Abies* tree**
Own experience	On bark of deciduous trees, formed conspicuous bright orange fluffy mats. Prostrate and erect axes, quite thin, gametangia occur mostly laterally, sometimes terminally. Printz (1920) and others hesitate about separation of *T. abietina* from *T. aurea*. Morphological characters of both species tend to overlap with uncertain boundaries.



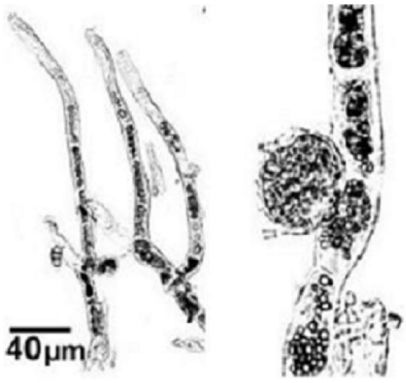
De Wildeman (1891)



Cribb (1958)



Karsten (1891)



Rindi et al. (2005)

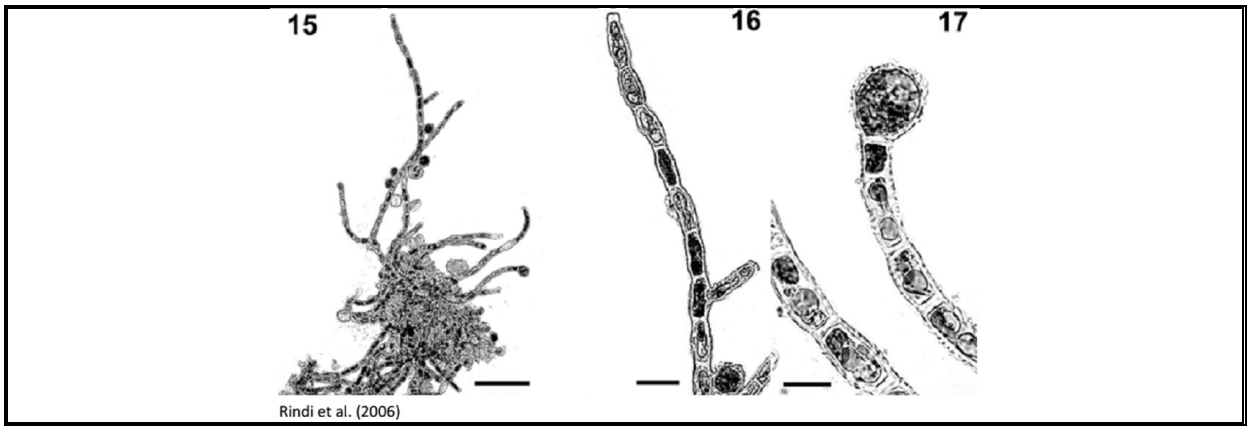
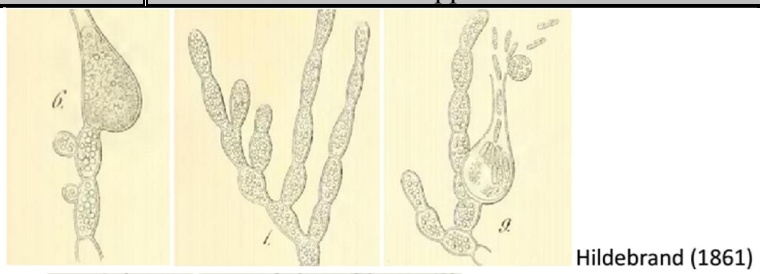


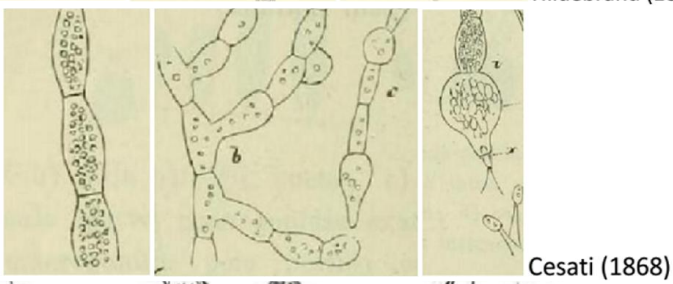
Table 3: Overview of the main morphological features of *P. lagenifera*

Morphological character	***Printzina lagenifera***
Thallus organization	prostrate part often not developed (Thompson & Wujek, 1992); almost no difference between prostrate and erect part (Ettl & Gärtner, 1995)
Texture of thallus	xxx
Shape of cells (erect)	xxx
Width of cells (erect)	xxx
Length of cells (erect)	xxx
Shape of cells (prostrate)	xxx
Width of cells (prostrate)	xxx
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	**swollen**, barrel-shaped, globular or cylindrical
Width of cells (non-differentiated)	7–10 µm (De Wildeman, 1891); often 3–6 µm, can be up to 19 µm (Thompson & Wujek, 1992); width and form is various (Ettl & Gärtner, 1995)
Length of cells (non-differentiated)	1–4 ×, occasionally up to 8 × width (Thompson &Wujek, 1992)
Branching pattern	extremely irregular (Thompson&Wujek, 1992)
Cell wall	smooth (Thompson & Wujek, 1992)
Shape of gametangia	**flask-shaped, bigger than vegetative cells**
Width of gametangia	9–24 µm (De Wildeman, 1891; Thompson &Wujek, 1992)
Arrangement of gametangia	terminal, occasionally intercalar (Thompson &Wujek, 1992); by one or two in a row (Ettl & Gärtner, 1995)
Presence of beak in gametangium	**yes, can be very long**
Shape of sporangia	oval or nearly globular (Thompson &Wujek, 1992); seldom hook-formed (Hakensporangia; Printz, 1939)
Width of sporangia	15.7–17.8 µm × 17.5–20 µm (Thompson & Wujek, 1992)
Arrangement of sporangia	occur only seldom, on special branched filament (Thompson & Wujek, 1992)
Shape of cell supporting sporangium	cylindric and bent (Thompson & Wujek, 1992)
Shape of terminal cells	xxx
Position of ostiole in sporangium	xxx

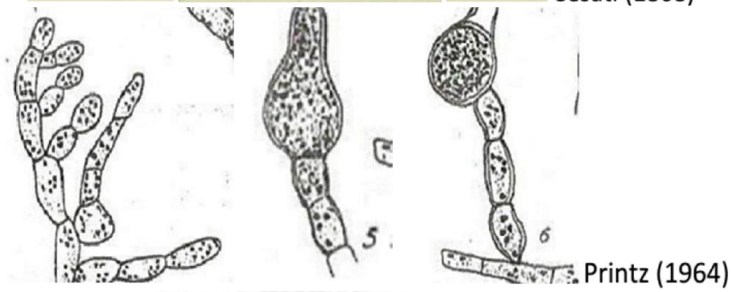
Colour	yellow to olive-green, gold to orange-gold (Thompson & Wujek, 1992)
Substratum	**bark of trees**
Environment	deep-shaded with intensive humidity (Thompson & Wujek, 1992); tropical or subtropical, in temperate regions only in glasshouses on walls, plants or ground (Printz, 1939)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Hildebrand (1861)**
Type locality	**Germany, Bonn, Botanical Garden - on bark of lianas (Schlinggewächse)**
Own experience	often appears as red crust on treebark



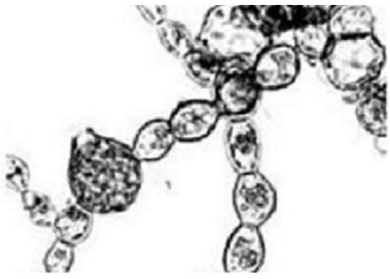
Hildebrand (1861)



Cesati (1868)



Printz (1964)



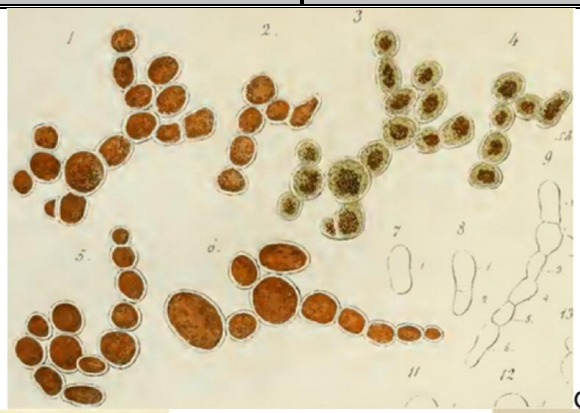
Rindi et al. (2005)

Table 4: Overview of the main morphological features of *T. umbrina*

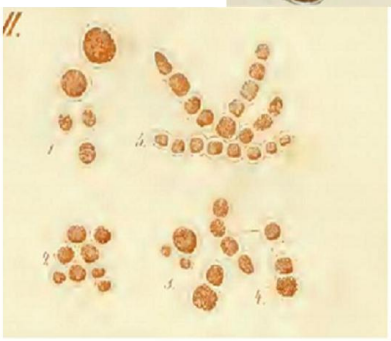
Morphological character	*Trentepohlia umbrina*
Thallus organization	usually without prostrate and erect part differentiation, cells mostly lay in irregular rows in large heaps, frequently in several layers (Printz, 1920)

Texture of thallus	crusty (Karsten, 1891); seldom up to 2 mm thick crusts, easily breaks into pieces (Fischer, 1922)
Shape of cells (erect)	xxx
Width of cells (erect)	xxx
Length of cells (erect)	xxx
Shape of cells (prostrate)	xxx
Width of cells (prostrate)	xxx
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	short, irregular, globular or elliptical (Kützing, 1845)
Width of cells (non-differentiated)	16–27 µm (De Wildeman, 1891); varying from small up to 35 µm in diameter, average size is 15–25 µm (Printz, 1920)
Length of cells (non-differentiated)	young 3–4 × width, old more round (Printz, 1920–3); 1–2 × width (Printz, 1939)
Branching pattern	irregular (Gobi, 1827)
Cell wall	young thin, old thicker with a distinct straticifation; thickness varies from 3–4 µm up to 7 µm (Printz, 1920)
Shape of gametangia	round, after wetting the sample release of swarmers can be observed, swarmers swim for a while and then settle down, become round in shape and obtain cellulose membrane. Gametes do not fuse, they usually act as spores. (Karsten, 1891).
Width of gametangia	do not differ very much from vegetative cells either in form or size (Printz, 1920)
Arrangement of gametangia	terminal or intercalar
Presence of beak in gametangium	sometimes (Ettl & Gärtner, 1995)
Shape of sporangia	can be also hook-formed (Hakensporangia, Brand, 1902)
Width of sporangia	xxx
Arrangement of sporangia	xxx
Shape of cell supporting sporangium	xxx
Shape of terminal cells	xxx
Position of ostiole in sporangium	xxx
Colour	brown-red (Kützing, 1845); also gold or darkred (Gobi, 1827)
Substratum	**bark of trees;** mainly deciduous (Gobi, 1827); also rocks but allways verctical substrates (Brand, 1902); wood (Ettl & Gärtner, 1995)
Environment	humid, oriented towards west or northwest (Karsten, 1891)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Kützing (1843) as *Chroolepus umbrinus***
Type locality	**bark of *Fagus sylvatica***
Own experience	Grows on tree bark and concrete. Usually breaks easily into small groups of cells. Gametangia are difficult to recognize for their look resemble the vegetative cells. Sometimes swarmers can be seen to swim and then settle down changing their shape from

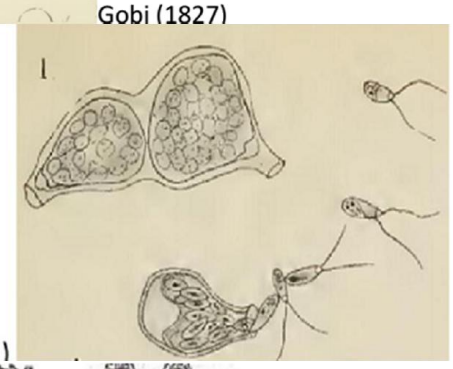
	elliptical to globular thus creating new cells and filaments. Printz (1939): this species occurs often nearby *T. odorata*.



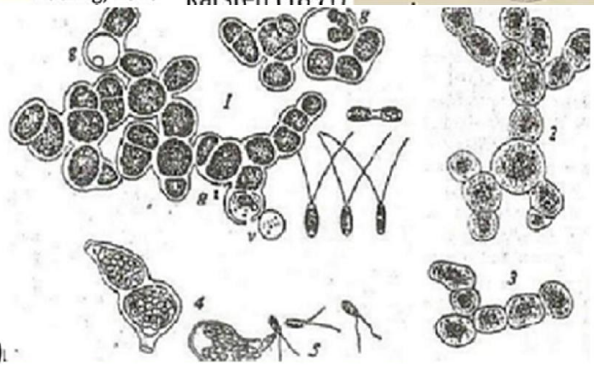
Gobi (1827)



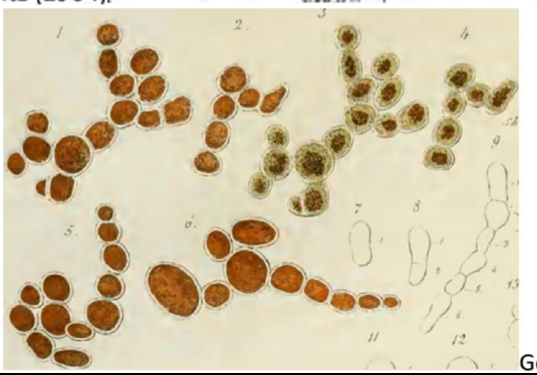
Kützing, 1843



Karsten (1891)



Printz (1964)



Gobi (1827)

Table 5: Overview of the main morphological features of *T. odorata*

Morphological character	***Trentepohlia odorata***
Thallus organization	prostrate part developed (Fischer, 1922)
Texture of thallus	**filamentous**; crusty of tufty (Hariot, 1890)
Shape of cells (erect)	xxx

Width of cells (erect)	xxx
Length of cells (erect)	xxx
Shape of cells (prostrate)	xxx
Width of cells (prostrate)	xxx
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	almost rectangular (Hariot, 1889); globular, ovoid, elliptical or cylindrical (Hariot, 1890)
Width of cells (non-differentiated)	10–16 µm (Hariot, 1889); 10–31 µm (Hariot, 1890); 15–30 µm (Printz, 1939)
Length of cells (non-differentiated)	16–20 µm (Hariot, 1889); 1–2 × width (Printz, 1939)
Branching pattern	branched; branches parallel to axes (Hariot, 1890)
Cell wall	in stripes or layers (Hariot, 1890); thick (Printz, 1939)
Shape of gametangia	almost globular to elliptical (Fischer, 1922)
Width of gametangia	30–40 µm (Fischer, 1922); 15–50 µm (Printz, 1939)
Arrangement of gametangia	lateral, terminal or intercalar (Fischer, 1922)
Presence of beak in gametangium	xxx
Shape of sporangia	ovoid (Hariot, 1889); globular (Hariot, 1890)
Width of sporangia	20–30 µm (Hariot, 1890)
Arrangement of sporangia	lateral, terminal or intercalar (Hariot, 1890); similar to gametangia (Printz, 1939)
Shape of cell supporting sporangium	hook-formed, terminal (Hariot, 1890)
Shape of terminal cells	xxx
Position of ostiole in sporangium	xxx
Colour	red, orange or gold-red (Fischer, 1922)
Substratum	**bark of trees;** mainly *Betula, Populus* (Hansgirg, 1886)
Environment	temperate climate (Ettl & Gärtner, 1995), **smells like violets or roots of *Iris***
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Wiggers (1780)**
Type locality	**Denmark, Viehburg, on bark of *Fagus* and *Betula***
Own experience	There have been hesitations (Gobi, 1827; Cribb, 1958) about the separation of *T. odorata* and *T. umbrina*. Some authors consider them to be the same species. If the two forms *elongata* and *umbrina* are considered following Fischer (1922), there is also a possibility *of T. odorata* f. *elongata* to be *T. iolithus* and *T. odorata* f. *umbrina* to be *T. umbrina*. My suspition grows stronger reading Cribb's notes (1989). His sample of *T. odorata* var *odorata* from Tasmania seems to fit the *T. iolithus* circumscription very well. Cribb himself admitts this possibility. Also reading the circumscription of *T.odorata* var. *elongata* (Printz, 1939) makes me more certain these species are the same.

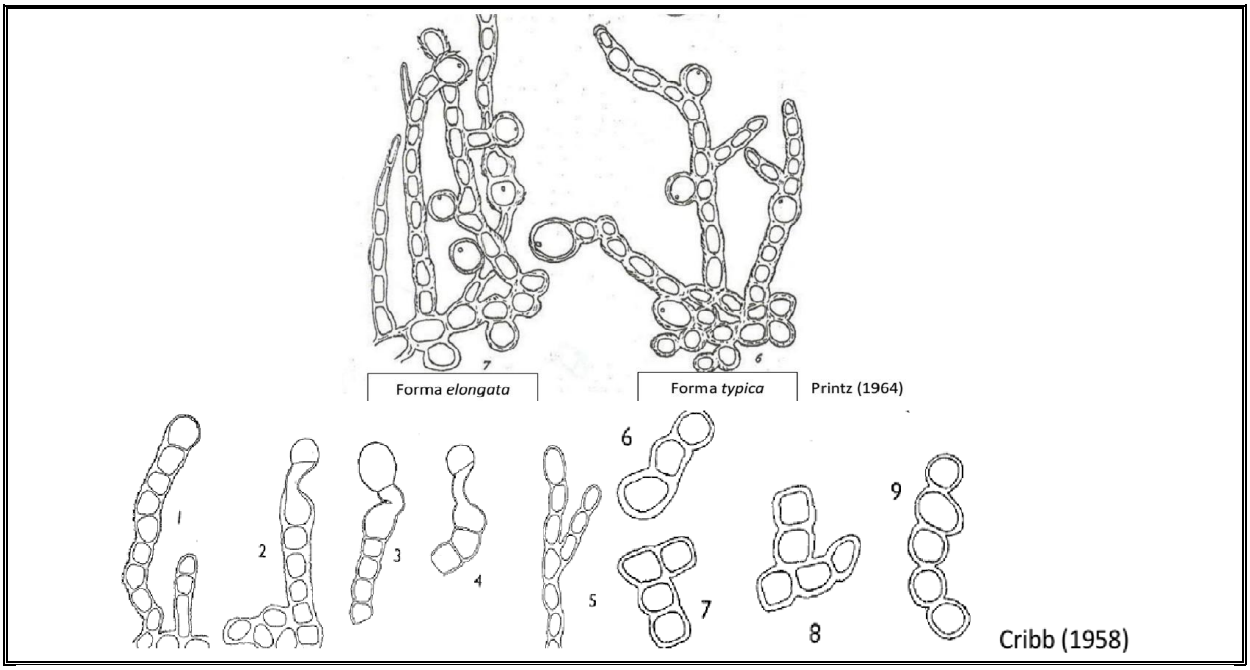
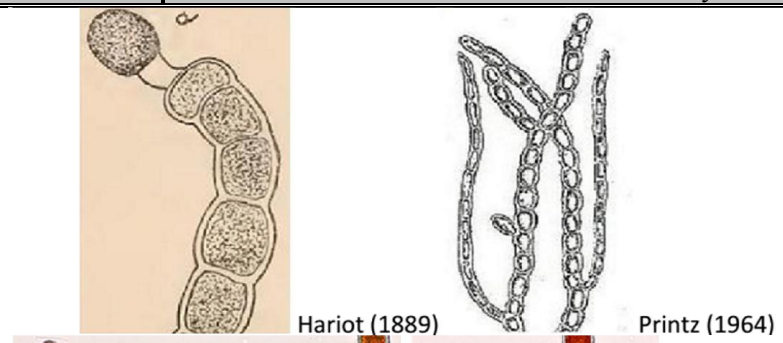


Table 6: Overview of the main morphological features of *T. jolithus*

Morphological character	*Trentepohlia jolithus*
Thallus organization	prostrate part slightly developed (Hariot, 1889; 1890); there is prostrate and erect part differentiation, but the cells of both parts look quite similar (Printz, 1939)
Texture of thallus	tufts (Rabenhorst, 1852; Hansgirg, 1886), **smells like violets**; up to 2 mm high tufts (Printz, 1939)
Shape of cells (erect)	thin, swollen in the middle (Hansgirg, 1886); cylindrical (Hariot, 1889; 1890)
Width of cells (erect)	14-24 µm (Hansgirg, 1886); 14-35 µm (Hariot, 1890)
Length of cells (erect)	1–2 ×, max 6 × (terminal cells) width (Printz, 1939); 24–50 µm (Hariot, 1890)
Shape of cells (prostrate)	xxx
Width of cells (prostrate)	xxx
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	xxx
Width of cells (non-differentiated)	xxx
Length of cells (non-differentiated)	xxx
Branching pattern	richly and irregularly branched (Printz, 1964); branches do not fall apart, branches mostly parallel to main axes (Printz, 1939)
Cell wall	thick (Hansgirg, 1886); in stripes or layers (Hariot, 1890); funnel-like layers (Ettl & Gärtner, 1995)
Shape of gametangia	xxx
Width of gametangia	36–42 µm (Printz, 1939)
Arrangement of gametangia	xxx
Presence of beak in	xxx

gametangium	
Shape of sporangia	sphaerical (Hariot, 1889); hook-formed (Hakensporangia, Brand, 1902)
Width of sporangia	sphaerical 20–48 µm or more elliptical in range 36–40 × 45–54 µm (Hariot, 1889)
Arrangement of sporangia	lateral, intercalar or terminal (Hariot, 1890)
Shape of cell supporting sporangium	hook-formed (Hariot, 1890)
Shape of terminal cells	can be prolonged, up to 6 × width (Hariot 1889; 1890)
Position of ostiole in sporangium	xxx
Colour	**red**; brown-red (Hansgirg, 1886); rusty red (Ettl & Gärtner, 1995)
Substratum	rocks (Hansgirg, 1886; Hariot, 1889); mainly silica or chalk rocks (Ettl & Gärtner, 1995); bark of *Betula* (Printz, 1964); able to grow on horizontal substrates (Brand, 1902)
Environment	humid, often near rivers or waterfalls (Hansgirg); smells like violets (Linnaeus, 1753; Fischer, 1922; Printz, 1939), nickname Veilchenmoos or Veilchenstein (violetmoss or violetrock)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Linnaeus (1753) as *Byssus jolithus*;** Wallroth (1833)
Type locality	**Europe;** Czech Republic, Krkonoše mountains - on rocks in higher altitude (Hansgirg, 1886)
Own experience	prostrate part developed, filaments hold together and it is difficult to separate them, grows mostly on rocks, can make bloodred or brown tufts or crusts. Sporangia on hook-formed suffultory cell. When scraping down the crust from substrate it may smell like violets



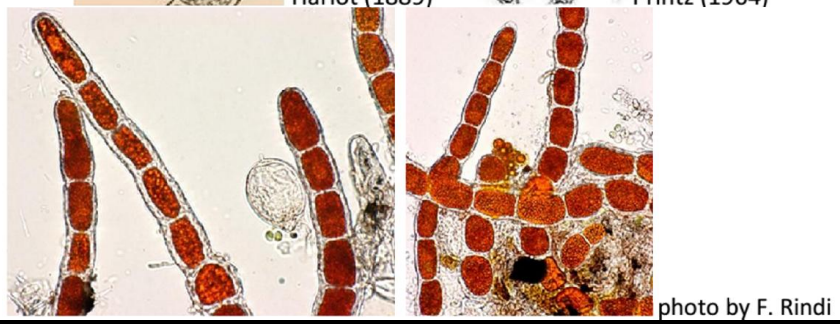
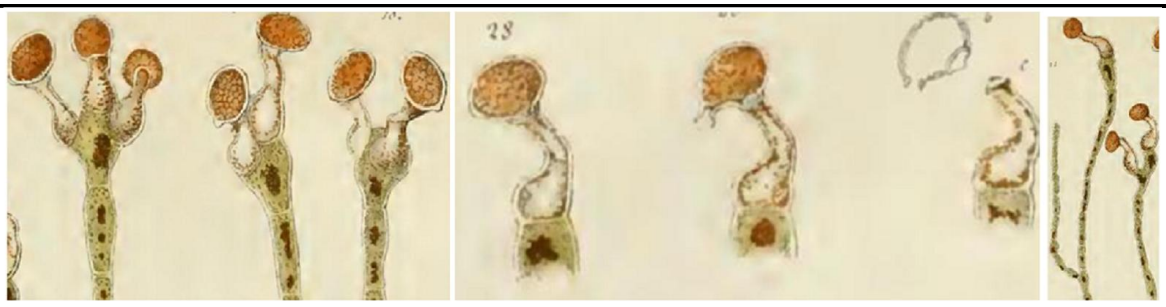


Table 7: Overview of the main morphological features of *T. uncinata*

Morphological character	***Trentepohlia uncinata***
Thallus organization	**prostrate part developed**

Texture of thallus	**little piles or tufts**
Shape of cells (erect)	**cells form longer branched axes**
Width of cells (erect)	**thinner than cells of the prostrate part**, 12–27 µm (Hansgirg, 1886)
Length of cells (erect)	**4 × width**, 1–1.5 × width (Hansgirg, 1886)
Shape of cells (prostrate)	**round to cylindrical**
Width of cells (prostrate)	**wider than cells of the erect part**
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	xxx
Width of cells (non-differentiated)	xxx
Length of cells (non-differentiated)	xxx
Branching pattern	**usually short filaments without branching pattern, some filaments long and branched**
Cell wall	xxx
Shape of gametangia	xxx
Width of gametangia	up to 50 µm (Ettl & Gärtner, 1995)
Arrangement of gametangia	on prostrate and erect filaments (Ettl & Gärtner, 1995)
Presence of beak in gametangium	xxx
Shape of sporangia	**bound to the subsporangial cell with pieces of membrane**, almost round or elliptical (Hansgirg, 1886); in clusters of one or two (Ettl & Gärtner, 1995)
Width of sporangia	up to2 × width of vegetative cells (Hansgirg, 1886); 15–24 × 24–28 µm (Ettl & Gärtner, 1995)
Arrangement of sporangia	**terminal, often in groups of 2 or 3, each on its own subsporangial cell**
Shape of cell supporting sporangium	**terminally positioned, flask-shaped with thin bent neck**
Shape of terminal cells	xxx
Position of ostiole in sporangium	**on the downwards inclined side of sporangium**
Colour	**yellowgreen, red pigments spotted**, brown, gold-red (Hansgirg, 1886)
Substratum	**bark of deciduous trees**, bark of tree stumps (Hansgirg, 1886); on upper parts of tree roots, branches of deciduous trees and conifers (Ettl & Gärtner, 1995)
Environment	**humid**; mostly in mountains (Ettl & Gärtner, 1995)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Gobi (1827)**
Type locality	**Petersburg, bark of *Acer, Populus tremula, Tilia***
Own experience	**Gobi thinks that it could be a more developed form of *T. umbrina***, however, from his pictures the species seems to be rather *T. arborum*



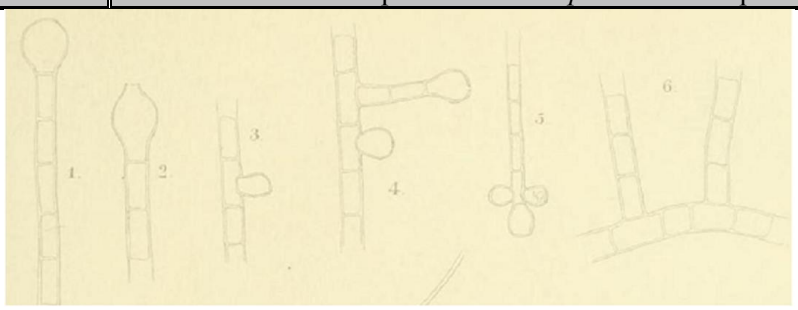
Gobi (1827)

Hansgirg (1886)

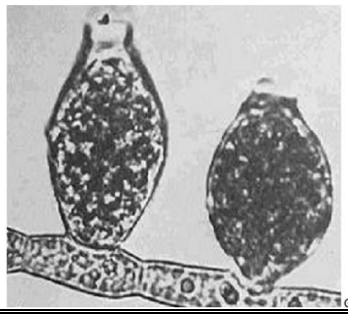
Table 8: Overview of the main morphological features of *T. aurea*

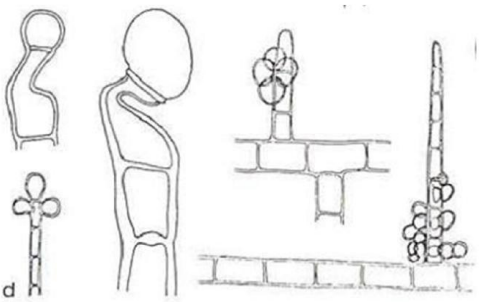
Morphological character	***Trentepohlia aurea***
Thallus organization	prostrate part developed (Printz, 1964)
Texture of thallus	**soft hairy cushions;** conspicuous pillows (Ettl & Gärtner, 1995)
Shape of cells (erect)	cylindrical (Printz, 1939)
Width of cells (erect)	12–16 µm (Hariot, 1889); 9–20 µm (De Wildeman, 1891); 8–30 µm (Printz, 1939)
Length of cells (erect)	32–40 µm (Hariot, 1889); 1,5–3 × width (Printz, 1964)
Shape of cells (prostrate)	swollen or cylindrical (Printz, 1939)
Width of cells (prostrate)	xxx
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	xxx
Width of cells (non-differentiated)	xxx
Length of cells (non-differentiated)	xxx
Branching pattern	**partly plain, partly branched;** richly branched (Ettl & Gärtner, 1995)
Cell wall	in divergent layers, terminal cells often with cellulose cap, often positioned to the side due to growth, cap width can be 10 µm and length 30 µm (Printz, 1939)
Shape of gametangia	globular to ovoid (De Wildeman, 1891); can be flask-shaped (Brand, 1902)
Width of gametangia	18–32 µm (DeWildeman, 1891); 9–40 µm (Fischer, 1922)
Arrangement of gametangia	on prostrate and erect filaments laterally or terminally, seldom intercalarly (Martius, 1817)
Presence of beak in	xxx

gametangium	
Shape of sporangia	ovoid (De Wildeman, 1891)
Width of sporangia	25–30 × 27–40 µm (De Wildeman, 1891)
Arrangement of sporangia	solitarily, seldom in clusters of 2 on suffultory cell (De Wildeman, 1891)
Shape of cell supporting sporangium	swollen basal part and bent neck (Printz, 1939); bent (Ettl & Gärtner, 1995)
Shape of terminal cells	xxx
Position of ostiole in sporangium	xxx
Colour	**saffron**; gold-yellow (Martius, 1817); to orange-red (Hansgirg, 1886)
Substratum	**rocks**; mainly chalk or silica, old wood, bark of trees, in Europe the most abundant species (Printz, 1964); walls and sandstone rocks (Hansgrig, 1886)
Environment	shady and humid, can be found in mountains (Hansgirg, 1886)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Linnaeus (1753)**
Type locality	**Italy**
Own experience	*Trentepohlia aurea* is the type species of the genus. *T. aurea* and *T. abietina* are quite similar to each other. According to Printz (1920) this two species are difficult to distinguish. *T. abietina* tends to have thinner and more elliptical cells. Cells of *T. aurea* should be wider and more cylindrical. Printz suggested this species to be the most abundant in Europe. This is in contrast to what I have observed. In my experience the most abundant species of *Trentepohlia* in Europe is *T. umbrina*.



De Wildeman (1891)


Graham & McBride (1975)

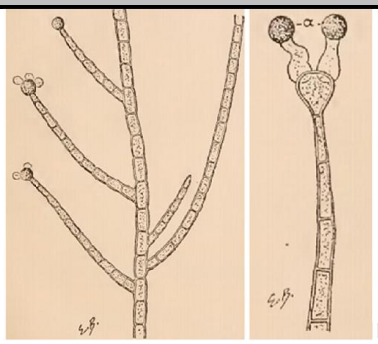


Ettl & Gärtner (1995)

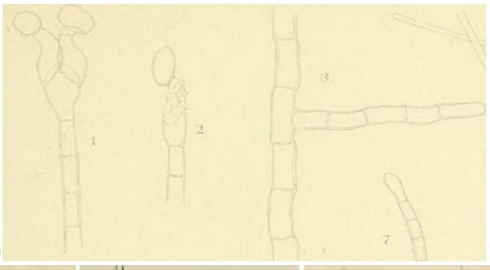
Table 9: Overview of the main morphological features of *T. arborum*

Morphological character	*Trentepohlia arborum*
Thallus organization	prostrate part developed (Ettl & Gärtner, 1995)
Texture of thallus	fluffy patches (Rindi et al., 2005)
Shape of cells (erect)	cylindrical (Rindi et al., 2005)
Width of cells (erect)	16–28 µm (Hariot, 1889); 13–28 µm (Printz, 1939)
Length of cells (erect)	40–60 µm (Hariot, 1889); 1–3 × width (Printz, 1964)
Shape of cells (prostrate)	xxx
Width of cells (prostrate)	xxx
Length of cells (prostrate)	xxx
Shape of cells (non-differentiated)	xxx
Width of cells (non-differentiated)	xxx
Length of cells (non-differentiated)	xxx
Branching pattern	in the angle of 90° (Printz, 1939)
Cell wall	thin (Karsten, 1891); roughened, in layers, or smooth (Printz, 1939)
Shape of gametangia	globular (Karsten, 1891)
Width of gametangia	xxx
Arrangement of gametangia	In clusters of 2 or 3 laterally or on a side branch (Prinzt, 1964); in groups of 2–3 laterally or on short sidebranches (Ettl & Gärtner, 1995)
Presence of beak in gametangium	xxx
Shape of sporangia	globular or elliptical (Hariot, 1889); hook-formed (Hakensporangia, Brand, 1902)
Width of sporangia	18–24 × 24–35 µm (Fischer, 1922)
Arrangement of sporangia	mostly in groups of 2–7 on a swollen terminal cell (Hariot, 1889), Hariot mentions that this arrangment of sporangia resembles *T. uncinata*; in groups of 1–2, rarely 2–7 on terminal cells (De Toni, 1889); globular to elliptical (Printz, 1939)
Shape of cell supporting sporangium	swollen (Ettl & Gärtner, 1995)
Shape of terminal cells	prolonged and tip-shaped (Printz, 1939)
Position of ostiole in sporangium	opposite to the attachment site (Printz, 1939); basally, near the end of attachment (Rindi et al., 2005)
Colour	gold, yellow (Karsten, 1891)
Substratum	**tree bark**; rocks (De Wildeman, 1891)

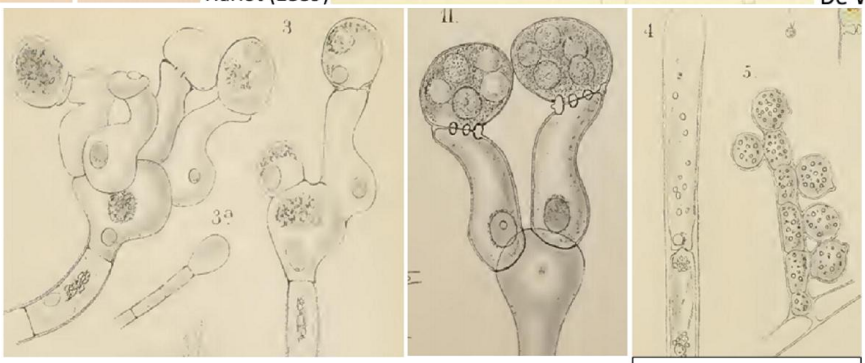
Environment	mostly in tropics on rocks and tree bark, in Europe in glasshouses on walls and plants (Printz, 1939)
Presence of hair-like cells	xxx
Presence of a brown membrane on apical cells	xxx
Original publication	**Agardh (1824) as *Conferva arborum***
Type locality	**bark of trees at Mariana Islands**
Own experience	*T. arborum* has been called variously in the past, for example: De Toni (1889), De Wildeman (1891): *T. plejocarpa*; Karsten (1891): *T. bisporangiata*. I have never seen the typical hook-formed sporangia in groups on terminal cells. On contrary, thallus was sterile consisting only of few branches (angle of branching was always 90°). According to Printz (1939), this species is hard to distinguish from *T. aurea* unless characteristical "Stielsporangia" are present (Printz, 1939).



Hariot (1889)

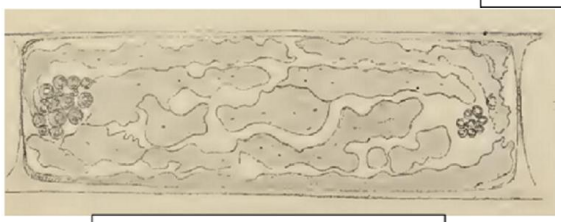


De Wildeman (1891)



(Karsten, 1891)

Globular gametangia



Haematochrom