PRESENCE AND DISTRIBUTION OF *SUILLUS PLORANS* IN THE POLISH TATRA MTS (WESTERN CARPATHIANS)

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ABSTRACT

Suillus plorans is a very interesting mountain ectomycorrhizal fungus, attached to *Pinus cembra*. The paper reports its presence in the Polish Tatra Mts as a new species for Poland, discusses its previous situation among macromycetes of Poland, and its conservation status. It includes detailed descriptions of carpophores and mycorrhizae specimens collected in Poland. Basing on records of both carpophores and mycorrhizae, a map of species' occurrence in the Tatra National Park was prepared and compared with the distribution of *Pinus cembra* in this area. Identity of mycorrhizae was assessed by a morphological study and confirmed by PCR-RFLP patterns analysis of carpophore and mycorrhizal mycelium.

KEY WORDS: *Suillus plorans* (Roll.) Kuntze, *Pinus cembra* L., Tatra Mts, ectomycorrhiza, PCR-RFLP.

INTRODUCTION

The mycorrhizal genus *Suillus* amounts to 50 species, associated with conifers (Hawksworth et al. 1995). Most of them are characterized by a strong specificity of symbiotic host, limited to a particular tree species or to a group of species (e.g. two-needle pines) (Kretzer et al. 1996). Thus, in many cases, the distribution of fungi is strongly dependent on the history of their plant partners and can involve a co-speciation (Wu et al. 2000). Both mycorrhizal symbionts are involved in colonization of new sites and influenced by factors of changing environment (e.g. Pirozynski 1980). These phenomena closely link together their contemporary distribution patterns. It can be well demonstrated for example in case of some *Suillus* representatives which are connected to stone pine (*Pinus cembra*) (Fig. 1). This five-needle pine species is considered as the most interesting and rarest of native trees in Poland. It grows naturally only in the Tatra Mountains in a very specific habitat, as dispersed solitary trees or – in some localities – forming a peculiar *Cembro-Piceetum* association at the upper forest limit (e.g. Środoń 1936, Myczkowski and Bednarz 1974). All three species of *Suillus*, connected by mycorrhizal symbiosis to stone pine in Europe, were found in the Tatra Mts. Several authors report *S. sibiricus* both in Polish and Slovak parts of the massif (Nespiak 1962a, Přihoda 1971, Frejlak 1973), while few stations of *S. placidus* and *S. plorans* were recorded only in its southern (Slovak) part (Nespiak 1962a, Pilàt and Dermek 1974). Striking is the fact, that *S. plorans*, which is considered in Europe as exclusively attached to stone pine (e.g. Breitenbach and Kränzlin 1991, Redeuilh and Simonini 1998) and present on most of its localities, has never been found in the Polish Tatra Mts. Also in the Slovak part of the Tatra Mts the known records are very sparse. It was very interesting then to carry out an observation focused on the potential presence and distribution of this fungus, as well as its frequency in the Polish Tatra Mts.

MATERIALS AND METHODS

Field research

All the most important localities of stone pine (*Pinus cembra*) in the Polish Tatra Mts were examined during

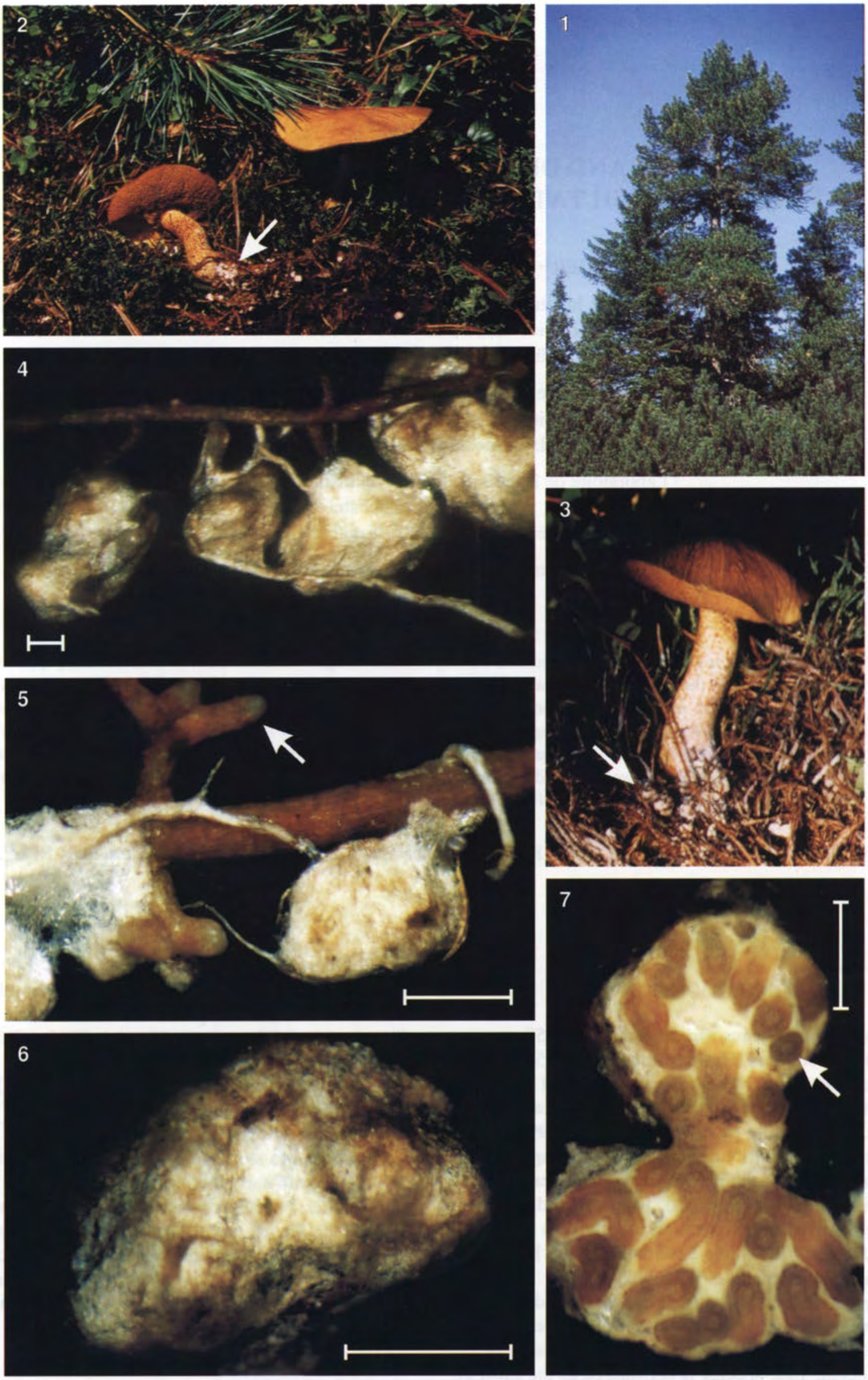


Fig. 1. *Pinus cembra* growing together with *P. mugo* in Dolina Sucha Kasprowa valley, Tatra Mts.
Figs 2, 3. *Suillus plorans* – carpophores with mycorrhizae attached to their bases (arrows).
Figs 4-7. Ectomycorrhizae of *Suillus plorans*.
Figs 4, 5. Tuberculate mycorrhizal systems and foreign ectomycorrhiza accompanying them (arrow), bars = 2 mm.
Fig. 6. Surface of the mature mycorrhizal tubercle, bars = 2 mm.
Fig. 7. Section through mycorrhizal system and individual mycorrhizal tips (arrow), bars = 2 mm.

TABLE 1. Stations of *Pinus cembra* examined in the work.

No of station	Name of region	Description of the station
1	Dolina Rybiego Potoku valley	Northern slopes of Mięguszowiecki Szczyt Wielki (2438 m a.s.l.) and Cubryna (2376 m a.s.l.), at the lowest rocks of their northern walls. Altitude appr. 1600 m. a.s.l. Several individual, young trees.
2	Dolina Rybiego Potoku valley	Above Morskie Oko lake, at the western foot of Żabi Wyżni Szczyt (2259 m). Altitude appr. 1480 m. a.s.l. The spruce forest with admixture of stone pine.
3	Dolina Roztoki valley	Central part of the valley. North-western slopes of Opalone crest (1644 m a.s.l.). Altitude 1290-1350 m. a.s.l. Several concentrations of stone pine (patches of *Cembro-Piceetum*).
4	Dolina Waksmundzka valley	South-eastern slope of Mała Koszysta (2013 m), so-called "Limbowa ubocz". Altitude 1500-1540 m. a.s.l. Concentrations of stone pine amogst the shrubs of dwarf pine (*Pinus mugo*).
5	Dolina Suchej Wody valley	North-western ridge of Żółta Turnia (2087 m). Altitude 1450-1500 m. a.s.l. Individual trees amongst shrubs of dwarf pine.
6	Dolina Sucha Kasprowa valley	Several localities in the valley. Altitudes 1380-1420 and 1550 m. a.s.l. Single, isolated trees within the spruce forest; patches of typical *Cembro-Piceetum* at the upper forest limit.
7	Zawrat Kasprowy ridge	Along the ridge. Altitude 1600 m. a.s.l. The spruce forest with single stone pines and shrubs of dwarf pine, at the upper forest limit.
8	Dolina Bialego valley	Near the tourist track "Ścieżka nad Reglami", northern slope of the Kalacka Kopa Mt., Altitude 1300 m a.s.l., at the upper forest limit.

September 2000, regarding the presence of *Suillus plorans* (Table 1).

Carpophores were collected together with the ectomycorrhizae in their vicinity. In case of lack of fruitbody, samples of soil and organic debris with mycorrhizal roots of stone pine were collected.

Determination of carpophores and mycorrhizae

The material was analysed in the laboratory, soon after collecting in the field. Primary determination of specimens was confirmed using keys (e.g. Moser 1983; Alessio 1985) and compared with other descriptions and iconography available in the literature (e.g. Favre 1945; Marchand 1974). Identification was based on numerous specimens covering various stages of carpophores' development.

Mycorrhizae were identified by tracing the rhizomorph connections to the fruitbodies and compared with description and illustrations by Treu (1990a, b).

PCR-RFLP analyses of mycelium

The carpophores and mycorrhizae from respective localities were compared by means of PCR-RFLP technique. Additionally, mycorrhizae from stations devoided of fruitbodies in the time of observation were analyzed in order to support the morphological observations and confirm their identity.

Mycorrhizae (1-2 systems), and fruitbody tissue (fragment of hymenophore) were stored in 300 µl of CTAB buffer. The DNA isolation procedure, PCR amplification and PCR-RFLP analysis followed Gardes and Bruns (1993) and Agerer et al. (1996).

The material in CTAB buffer was crushed, incubated in 65°C, and mixed with equal volume of cold chloroform. After centrifugation, the upper phase containing DNA was transferred to a new Eppendorf tube, mixed with isopropanol, and centrifuged. The pellet was washed with sodium acetate-ethanol solution, and afterwards with 80% ethanol. Freshly isolated DNA was suspended in 35-50 µl of TE buffer (pH 8).

The amplification was performed using ITS1/ITS4 universal primers, which amplify the DNA fragment within ITS region of the ribosomal nuclear DNA (Gardes et al. 1991, Henrion et al. 1992). The reaction mix (final volume 20 µl) contained: PCR buffer (10×, without magnesium; EMBI Fermentas) – 1/10 of the final volume, $MgCl_2$ (25 mM) – 1.5 mM, dNTP (2 mM; EMBI Fermentas) – 1.25 mM (of each: dATP, dCTP, dGTP, dTTP), primers (10 µM; EMBI Fermentas) - 0.5 µM (of each: ITS1 and ITS4), Taq polymerase (5000 U/ ml; EMBI Fermentas) - 0.4 U/20 µl, DNA solution – 2 µl, H_2O – to 20 µl. DNA solution from carpophores was diluted 20×. The annealing temperature was 54°C, and 43 cycles were run during amplification.

For the restriction analysis, enzymes AluI, EcoRI, HinfI (EMBI Fermentas) were used. The amplified DNA was incubated with enzymes according to manufacturer's instructions, and the products were separated on 1.5% agarose gel during electrophoresis.

RESULTS AND DISCUSSION

Descriptions of carpophores and mycorrhizae of S. plorans from the Polish Tatra Mts

Carpophores (Fig. 2, 3): Cap 2-10 cm in diameter, yellow-brown, slimy and slightly fibrillous. Stipe 4-12 cm long and 0,7-2 cm in diameter, cylindric, sulphur-yellow to copper-yellow, covered with bright at first, then dark brown glands; without ring. Flesh orange-yellow, not turning blue on cut. Taste mild. Tubes adnate or slightly decurrent, yellow-fox coloured. Pores angular, up to 2 mm in diameter, in young specimens excreting watery-white juice. Spores cylindric 8-11×3-4 µm, with oil drops. Basidia slightly clavate, 4-spored, 28-31×7.2-8.5 µm, present also on the stipe surface. Cheilocystidia clavate, in tufts or concentrations, 60-70×8.5-9.6 µm, pleurocystidia clavate, not numerous, in tufts, 54-60×7.2-8.4 µm, caulocystidia clavate, dark brown, in big concentrations, in the glandular dots, 51.5-76×8.5-9 µm. Pileipellis an intricate trichoderm, composed of hyphae 4.8-7 (9.6) µm across, submerged in gelatinous substance. Hyphae, cystidia and basidia clampless.

Carpophores from Polish sites resembled the illustrations of ssp. *plorans* recorded in Slovakia, in the vicinity of Popradské Pleso (Pilàt and Dermek 1974). In the literature concerning populations in the Alps, an important variability of cap colours is underlined (e.g. Favre 1945). Specimens found in all the stations from the Polish Tatra Mts were rather morphologically uniform and relevant to the typical form illustrated by Favre (1945).

Mycorrhizae. Morphological characters (Fig. 4-7): Mycorrhizal systems tubercle-like, round in shape, sometimes slightly flattened and irregular (especially bigger and older specimens), silvery, whitish-ochraceous when young, turning dark ochraceous with age, covered with pinkish or brown-reddish veins. Mycorrhizal systems up to 10 mm in diameter; the surface of mycorrhizal tubercles covered with extramatrical hyphae. Mycorrhizal tips appr. 0.6 µm in diameter, up to 1.5 µm long. The rhizomorphs concolourous with the tubercles, round in shape, branched frequently, form sometimes an interconnected net, covered with abundant extramatrical mycelium. They are formed abundantly, mostly at the base of the system, and originate from beneath hyphal sheath covering the system.

Anatomical characters of mantle in plan views (Fig. 8--17): The outermost layer of the hyphal sheath covering the system consists of both thick- and thin-walled hyphae. Especially in older mycorrhizal systems, the hyphae become immersed in the abundantly produced pigment. The thick-walled hyphae are usually covered with drops of pigment, and sometimes with suilloid crystals and roundish warts. These hyphae are characterised by the very distinctive, knee-like swellings at the septa. Hyphae of the sheath 3.5-4 µm in diameter, with the walls 0.8-1.2 µm thick. Down the sheath, thin-walled hyphae, 3.5-5 µm in diameter, can be found; some of them form knee-like swellings at the septa. Suilloid crystals become also more abundant. The mantle of individual hyphal tips of type F (according to Agerer 1991); the outermost layer formed by hyaline, thin-walled hyphae, 2-7.5 µm in diameter, covered with suilloid crystals and only occasionally drops of the pigment, with simple septa. This layer of the mantle is loose, and connected to the hyphal sheath. In some parts of the mantle the hyphal segments become swollen, and often globular or fusiform in shape, 7-15×7.5-38 µm. They are usually formed in larger groups. Middle part of the mantle is a transitional layer to the inner part, thus more compact and devoided of the hyphal swellings. The hyphae often grow in parallel groups. Inner mantle layer compact, composed of intermixed, usually bent or curved, thin-walled hyphae 2.5-7 µm in diameter, forming simple septa. The hyphal connections in form of short, open bridges, rarely closed by the septum. Backwardly growing hyphae present. Hyphal branches growing out usually close to septa.

Anatomical characters of rhizomorphs: Rhizomorphs of type F (according to Agerer 1991), highly differentiated, with centrally arranged vessel-like hyphae. Rhizomorphs up to 5 mm in diameter, with nodes in places of ramification. Hyphae of the rhizomorph surface similar to those of the common hyphal sheath of the tuberculate mycorrhizal system; usually covered with warts, crystals and brownish pigment. Non vessel-like hyphae of the rhizomorph thin walled, 2-6.5 µm in diameter, with simple septa. Central vessel-like hyphae with thick, up to 1.5 µm walls, mostly with dissolved septa, sometimes filled with granular contents, or brownish crystalloids. Some hyphae of the rhizomorphs filled with crystals brightly shining in polarised light.

Colour reactions in chemicals: KOH 15% – pigment on the surface of common sheath and mantle turns purplish or brown-purplish.

The mycorrhizae of *Suillus plorans* and *Pinus cembra* were described by Treu (1990a) from Berchtesgaden National Park in Germany. The description fits well the characteristics of the specimens found in the Tatra National Park in Poland. Some differences concern the colour of the pigment excreted by the hyphae of the outer hyphal sheath and hyphae of the outer mantle. According to Treu (1990a), the colour of the pigment is reddish. Indeed, the colour of the fresh pigment was reddish also in case of the Polish specimens, but it used to turn more brownish when mycorrhizae were kept in water for some time, or when older material was observed. Slight differences in dimensions of hyphal structures can also be found.

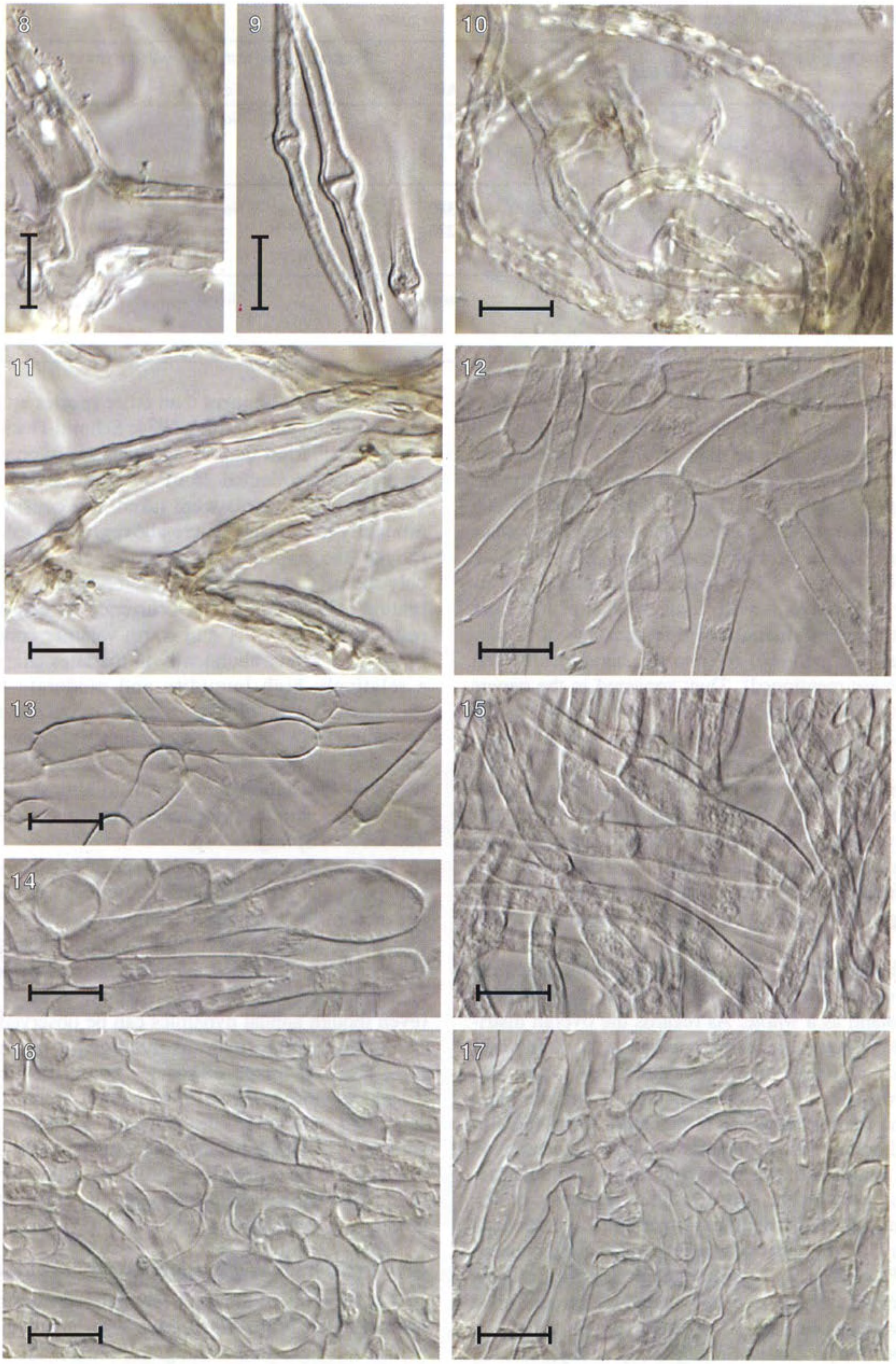
The tubercle-like mycorrhizae are not common (reviewed in Haug et al. 1991; Massicotte et al. 1992). The only ectomycorrhiza very similar to the mycorrhiza of *S. plorans* is formed by *S. variegatus,* a species also occurring in subalpine zone, but attached there exclusively to *Pinus mugo* (two-needle pine). Both species of fungi can be found often in close vicinity, if their host plants are growing together. Mycorrhizae of *Suillus plorans* and *S. variegatus* share the tuberculate mycorrhizal systems, plectenchymatous mantle with groups of hyphal swellings, features of the hyphae forming the hyphal sheath, mantle and rhizomorphs, (especially knee-like swellings), the pigment and crystals, as well as colour reactions (investigations of the *S. variegatus/P. mugo* mycorrhizae from the Tatra Mountains, and *S. variegatus/P. sylvestris* mycorrhizae, not included here). Although small differences in dimensions of anatomical structures can be found, these features can not be taken to distinguish the mycorrhizae. The most profound anatomical difference seems to be the structure of the middle mantle layer, which may be pseudoparenchymatous in case of *Suillus variegatus*.

In the field, the possibility of distinguishing between the mycorrhizae formed by these two species is provided rather by the morphology of host tree roots; in case of *P. cembra* they are covered by hairs, while in *P. mugo* – smooth.

PCR-RFLP analysis of ITS region enables a short-cut differentiation between both species, however, only when the results obtained using Alu I restriction enzyme are considered. EcoR I does not cut the amplified DNA fragment, and digestion with Hinf I results in three bands of similar size (Table 2).

Presence and distribution of Suillus plorans in Poland

S. plorans is a widespread species in Europe, present especially in all regions of the Alps (e.g. Favre 1945; Schmid-Heckel 1985; Breitenbach and Kränzlin 1991; Krieglsteiner 1991), and also known from the Slovak Tatra



Figs 8-17. Anatomical structures of *Suillus plorans* mycorrhizae, bars = 10 μm.
Fig. 8. Optical section through young rhizomorph with centrally arranged hypha of increased diameter.
Fig. 9. Extramatrical hyphae swollen at the septae.
Fig. 10. Suilloid crystals on the surface of the mycelium forming common sheath, brightly shining in polarized light.
Fig. 11. Extracellular pigment material cementing hyphae of common sheath.
Figs 12, 13. Hyphae of the outer mantle layer with swollen segments.
Fig. 14. Round and club-shaped hyphal cells in the outer mantle layer.
Fig. 15. Plectenchymatous outer mantle; hyphae of appr. uniform diameter.
Fig. 16. Middle mantle layer.
Fig. 17. Inner mantle layer.

TABLE 2. Results of PCR-RFLP analysis of *Suillus plorans* and *S. variegatus*.

Fragment types → / Samples ↓	Uncut fragment	Fragments after restriction with enzymes: AluI	EcoRI	HinfI
S. plorans – mycorrhizae	730	369 275	No cut	242 214 154
S. plorans – carpophores	725	380 270	No cut	239 211 154
S. variegatus – carpophores	707	No cut	No cut	250 225 173

Mts (Pilàt and Dermek 1974). The status of *S. plorans* among macromycetes of Poland was very ambiguous. It was considered as present in the territory of the country, however this conviction was based exclusively on the informations given by Nespiak (1962a, 1962b, 1966), which seems to be incorrect and needs a discussion. The author recorded once *Suillus plorans* in the Tatra Mts (Nespiak 1962a), but this station – Batizovska Dolina valley – is located totally in Slovakia, on the southern side of the Tatra range. All further informations on the presence of *S. plorans* (Nespiak 1962b, 1966) refer to the same locality (no other records are mentioned). They appeared in the papers concerning the Tatra National Park in Poland, but in the chapters treating mycological curiosities in whole massif, which could cause this misinterpretation.

Interestingly, the mycorrhizae of *Suillus plorans* have already been observed by Dominik and Nespiak (1953) during their investigation on the mycorrhizal status of plants in subalpine belt. They were classified as morphological type C (tuberculate mycorrhizae) on the roots of *P. cembra*, but the fungus species forming these structures was not identified.

Data included in this work report then for the first time the occurrence of this fungus in the Polish Tatra Mts as well as in Poland as a whole. As regards the overall distribution of stone pine, the Tatra population of *S. plorans* should be considered as one geographical unit which was already known basing on Slovak records. Then, the main interest of the findings reported in this work, from the biogeographical point of view, consists in a doubtless and well documented presence of this fungus in Poland. Another very interesting conclusion concerns the frequency of the species in this part of distribution range. What is particularly noteworthy, the fungus occurred not only in big concentrations of *P. cembra*, but accompanied also the solitary, isolated specimens of the tree surrounded by spruce forest (as for several sites in Sucha Dolina Kasprowa valley) or dwarf pine bushes (in Dolina Suchej Wody valley), or even growing on steep slopes above timberline in Morskie Oko valley. Therefore, *S. plorans* is confirmed to be strictly attached to stone pine and exactly follows its local distribution pattern in the area of observations. In all likelihood, the same situation should be stated all over the massif. In the light of these observations, the fungus should be considered no more as a rarity, but as a constant partner of its host tree. It is congruent with the opinion, that *S. plorans* is a typical and probably the most important mycorrhizal fungus of subalpine *P. cembra* forests in the Alps (Mikola 1969) and more frequent than other species attached to this tree (e.g. Pilàt and Dermek 1974; Schmid-Heckel 1985).

A distribution map of *S. plorans* was prepared basing on the data collected from observation sites (Fig. 18). Two types of records were taken into consideration. Additionally to the sites where aboveground structures of the fungus (the carpophores) occurred, also the places where only the mycorrhizae were developed in the time of observations were included. Occurrence of carpophores was restricted by very dry and warm weather conditions. The fungi grew rather abundantly in the sites characterized by a relatively high humidity and moderate temperature (northern slopes of Mięguszowiecki Szczyt Wielki in Morskie Oko valley, Sucha Dolina Kasprowa valley). As concerns the mycorrhizae, they were developed abundantly in all the stations and found in almost all samples of rhizosphere, the most frequently on roots penetrating humid tufts of *Sphagnum* sp.

In fact, in case of several species of ectomycorrhizal macromycetes their symbiotic structures are very well defined and make possible a doubtless taxonomic classification. Thus, they can serve for a distribution study with equal confidence as scoring the occurrence of carpophores and are less dependent on the seasonal weather conditions (even though annual dynamics can be observed). This kind of approach is then more likely to give a much more complete and real image of species spatial distribution. Data based on morphological considerations can be strongly supported by appropriate molecular analyses, as comparisons of PCR-RFLP patterns. Obviously, such kind of observations can be especially useful in practice for cases where much is known about the symbiotic status and host specificity of fungal species.

Conservation status of Suillus plorans in Poland

On the basis of the unclearly defined informations (Nespiak 1962b, 1966), *S. plorans* was included in the "red list" of threatened macrofungi in Poland, where it was classified as an endangered taxon – "E" category (Wojewoda, Ławrynowicz 1992). In the light of new data provided in this paper, as well as recently modified categories of the Red List (IUCN SSC, 1994), the status of *S. plorans* in the «Red list of threatened macrofungi in Poland» needs revision. The frequent presence of the species (carpophores and/or mycorrhizae) in all the observation areas investigated in the Polish Tatra Mts suggests, that it is rather abundant in suitable habitats. As all – not numerous – natural stations of *Pinus cembra* are limited in Poland to the pro-

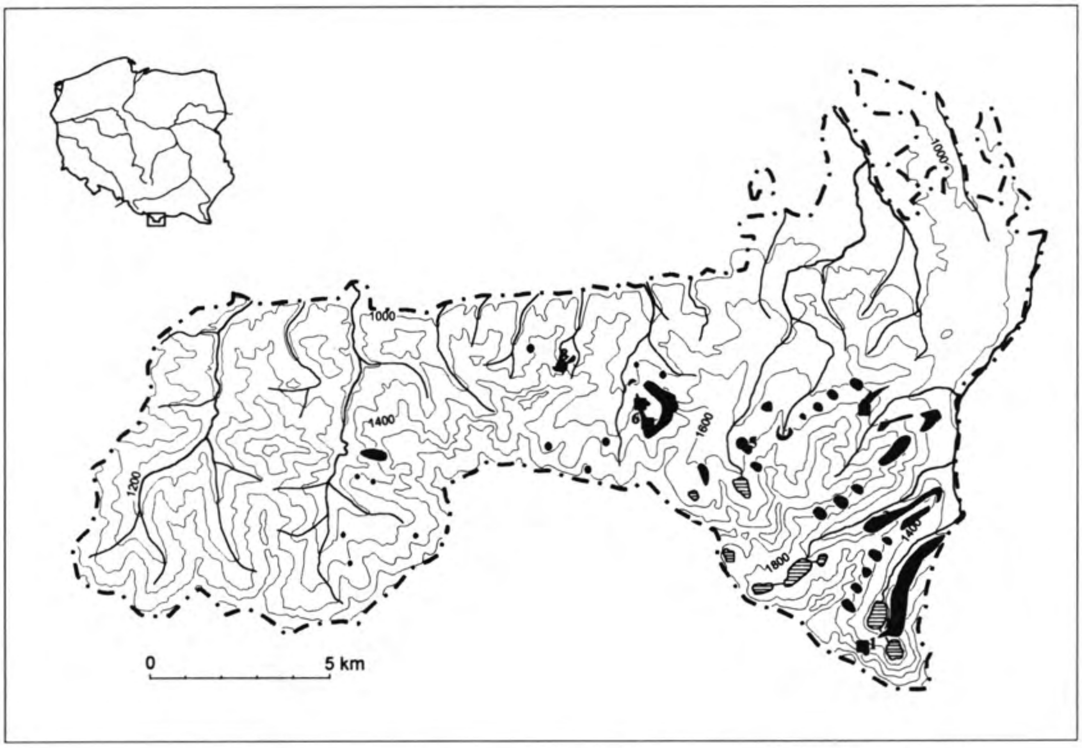


Fig. 18. Map of *Suillus plorans* localities in the Polish Tatra Mts compared to distribution of *Pinus cembra* (natural stations). Broken line – border of the Tatra National Park, grey colour – distribution of *Pinus cembra* (after Chmiel 1996); squares – localities where both carpophores and mycorrhiza were stated; dots – localities based only on mycorrhiza record. Numbers of localities refer to Table 1.

tected area of the Tatra National Park, *S. plorans* should be probably accounted as not threatened (LR – «Lower Risk» category) but only in case if the protection of its habitat remains undisturbed (cd – «conservation dependent» subcategory).

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OBECNOŚĆ I ROZMIESZCZENIE *SUILLUS PLORANS* W POLSKICH TATRACH (KARPATY ZACHODNIE)

STRESZCZENIE

Suillus plorans jest bardzo interesującym, górskim grzybem ektomikoryzowym związanym z limbą (*Pinus cembra*) (Ryc. 1). Na obszarze Tatr był on dotychczas znany jedynie z kilku stanowisk, położonych w obrębie części słowackiej. Grzyb ten był podawany także jako obecny w Polsce oraz umieszczony na Czerwonej Liście, w oparciu o niejasno sformułowane doniesienia Nespiaka (1962a, 1962b, 1966) dotyczące w rzeczywistości stanowiska w Tatrach słowackich. Niniejsza praca przedstawia pierwsze znaleziska tego gatunku z polskiej części Tatr i zarazem nowe dla terytorium Polski, podaje również szczegółową charakterystykę owocników (Ryc. 2-3) i mikoryz (Ryc. 4-17) z tego obszaru. Odwiedzono wszystkie najważniejsze skupiska limby w Tatrzańskim Parku Narodowym (tab. 1) obserwując obecność owocników i/lub mikoryz *S. plorans*. Przynależność taksonomiczna mikoryz – po określeniu na podstawie cech morfologicznych – została potwierdzona za pomocą analizy metodą PCR-RFLP (tab. 2). Stwierdzono obecność grzyba we wszystkich badanych miejscach; mikoryzy występowały obficie w próbach korzeni z każdego z nich, natomiast owocniki wykształcone były w okresie prowadzenia obserwacji tylko na części stanowisk (Ryc. 18). Co ciekawe, grzyb towarzyszył nawet izolowanym, pojedynczym okazom limby rosnącym w obrębie lasu świerkowego, kosodrzewiny, względnie powyżej granicy lasu. Występowanie *S. plorans* w polskich Tatrach pokrywa się ściśle z występowaniem partnera symbiotycznego (Ryc. 18). O ile jego występowanie jest przez to bardzo ograniczone przestrzennie, to na właściwych siedliskach jest liczny i nie wydaje się zagrożony. W świetle tych danych oraz niedawno zmodyfikowanych kategorii czerwonej listy celowa wydaje się rewizja kategorii zagrożenia tego gatunku z «E» (endangered) na «LR, cd» (Lower Risk, conservation dependent).

SŁOWA KLUCZOWE: *Suillus plorans* (Roll.) Kuntze, *Pinus cembra* L., Tatry, ektomikoryza, PCR-RFLP.