

## Morphological characteristics and DNA sequence analysis of *Petriella setifera* and *Oidiodendron setiferum* from twigs of diseased oak

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Examination of isolates of *Petriella setifera* and *Oidiodendron setiferum* revealed new diagnostic morphological characteristics. Chlamydoconidia formed by *P. setifera*, isolated from twigs of sessile oak (*Quercus petraea*) showing symptoms of oak decline, are described for the first time. The first pictures of *P. setifera* anamorphs since the publication of its original description in 1912 are presented. Isolates of *O. setiferum*, from sessile oak twigs and from a log of Scots pine (*Pinus sylvestris*), were found to have swollen, hyaline, thin-walled, sterile apices on the non-fertile hairs surrounding the fertile heads of conidiophores. They also had numerous coils formed by thin hyphae in the submerged mycelium in agar culture. The taxonomy of both fungi was confirmed by rDNA sequence analysis.

**Key words:** morphology, *Oidiodendron setiferum*, *Petriella setifera*, rDNA sequence analysis, taxonomy

### INTRODUCTION

During a study of (i) fungal communities from twigs and branches of sessile oak (*Quercus petraea* Liebl.) with symptoms of oak decline in north-west Poland and (ii) fungi on Scots pine (*Pinus sylvestris* L.) timber imported to Poland from Lithuania, in 2002, two distinctive fungi producing setae were recorded after incubation of samples for 3 months in moist chambers. *Petriella setifera* (Schmidt) Curzi appeared on oak twigs whilst *Oidiodendron setiferum* Udagawa et Toyazaki appeared both on oak twigs and sapwood of Scots pine logs.

*Petriella setifera* first produced numerous characteristic fertile synnemata, which emerged from the bark of the twigs. On synthetic low nutrient agar (SNA, Nirenberg 1976) and potato dextrose agar (PDA), this fungus first produced hairy

perithecia with ostioles formed by terminal hairs. The teleomorph was then followed almost immediately by development of *Sporotrichum* and *Graphium* anamorphs.

*Oidiodendron setiferum*, in its mature form was characterized by long (up to 200  $\mu\text{m}$ ), darkly pigmented, mostly smooth conidiophores with symmetrical, penicillate, fertile heads composed of branches ending with arthroconidia originating by basipetal septation and strongly branched sterile hairs surrounding the fertile heads.

In addition to these diagnostic characteristics, both fungi were observed to have distinctive, previously undescribed morphological features. The objective of this paper is (i) to report new records of both fungi, after confirming their identities and phylogenetic relationships by morphological and molecular procedures, and (ii) to present their newly observed morphological characteristics.

## MATERIALS AND METHODS

**Isolation and identification of fungi.** Several 1-cm-diam. twigs of sessile oak, from trees with oak decline symptoms from Smolarz Forest District, north-west Poland (52°54'N, 15°47'E), were placed in a moist chamber at 25°C in a natural day/night cycle. Logs, 20 cm long and 40 cm in diam., cut from a 100-year-old Scots pine that had been felled in early spring 2003 in Lithuania were delivered to the Department of Forest Pathology, Poznań, Poland in May 2003. In the laboratory, all surfaces of the wood were thoroughly washed by scrubbing with a brush in warm, running tap water for 5 min and then rinsed in sterile distilled water. While still wet, samples were completely wrapped in sterile plastic bags and incubated in a moist chamber, in darkness, at 25° and 95% humidity. After 3 months the samples were unwrapped and fungi growing on the surfaces of both sapwood and hardwood were recorded. The fungi were subcultured and identification confirmed by their morphologies on PDA, malt extract agar (MA; Difco), oatmeal agar (OMA) and SNA. For light microscopy, fungal material from SNA was mounted on glass slides in water and photographed using Ilford FP 4+ film.

Axenic cultures of *P. setifera* were obtained from masses of conidia produced on synnemata on the oak-twig bark surface after incubation. Axenic cultures of *O. setiferum* were obtained from fertile heads with conidia produced on the bark of oak twigs and Scots pine logs.

**DNA extraction.** For DNA isolation, *P. setifera* and *O. setiferum* were grown in L broth (tryptone, 10 g l<sup>-1</sup>; yeast extract, 5 g l<sup>-1</sup>; sodium chloride 10 g l<sup>-1</sup>) at 25° C for 10 days. The mycelium was separated by vacuum filtration, then freeze-dried in 2 ml tubes and ground using a metal rod to produce the starting material for the DNA extraction. Lysis buffer (50 mM-Tris pH 7.2, 0.4 ml; 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) was added at 0.8 ml to each 0.3 g sample of the ground mycelium in a screw-top tube. The remainder of the method was as described by Lee and Taylor (1990), except for an additional RNaseA digestion followed by phenol extraction and precipitation with isopropanol to remove contaminating RNA.

**rDNA amplification.** Consensus fungal primers ITS 4 (5' TCC TCC GCT TAT TGA TAT GC) and ITS 5 (5' GGA AGT AAA AGT CGT AAC AAG G) (White et al., 1990) were used to amplify ribosomal DNA (rDNA), specifically a region of DNA stretching from the 3' end of the 18S-like gene to the 5' end of the 28S-like gene and including the 5.8S-gene and the two internal transcribed spacer (ITS)

Table 1  
Fungi used for comparative purposes in this study

Name	Strain no.	Origin and depositor	EMBL no.
<i>Chaetomium funicola</i> Cooke	olrim130 <sup>a</sup>	Live stem, xylem of <i>Betula pendula</i> , Lithuania, <i>Lygis, V</i> 2003	AY354233
<i>Ch. globosum</i> Kunze:Fries	Cg3 <sup>b</sup>	<i>Aggarwal, R</i> , 2003	AY429050
<i>Myxotrichum arcticum</i> Udagawa, Uchiyama & Kamiya	UAMH 7565 <sup>c</sup>	Forest soil, Alaska, USA, <i>Hambleton S.</i> , 1988	AF062810
<i>M. carminoparum</i> Robak	UAMH 1597	Wood pulp, Norway, <i>Hambleton S.</i> , 1988	AF062812
<i>M. cancellatum</i> W. Phillips	UAMH 1911	Frozen blueberry pastry, <i>Hambleton S.</i> , 1988	AF062811
<i>Oidiodendron cerealis</i> (Thümen) G.L. Barron	UAMH 7526	Peat soil, Ontario, Canada, <i>Hambleton S.</i> , 1988	AF062788
<i>O. chlamydo sporicum</i> Morrall	UAMH 6520	Soil, ex boreal forest, Saskatchewan, Canada, <i>Hambleton S.</i> , 1988	AF062789
<i>O. citrinum</i> G.L. Barron	UAMH 1525	Soil, ex cedar bog, Ontario, Canada, <i>Hambleton S.</i> , 1988	AF062790
<i>O. echinulatum</i> G.L. Barron	UAMH 8467	Soil ex cedar bog, Ontario, Canada, <i>Hambleton S.</i> , 1988	AF062791
<i>O. flavum</i> Szilvinyi	UAMH 1524	Soil ex cedar bog, Ontario, Canada, <i>Hambleton S.</i> , 1988	AF062792
<i>O. griseum</i> Robak	UAMH 1403	Wood pulp; Sweden, <i>Hambleton S.</i> , 1988	AF062793
<i>O. majus</i> G.L. Barron	E97053 <sup>d</sup>	Root of <i>Rhododendron obtusum</i> var. <i>kaempferi</i> , <i>Usuki, F.</i> , 2002	AB089654
<i>O. periconioides</i> Morrall	UAMH 8527	soil, Saskatchewan, Canada, <i>Hambleton S.</i> , 1988	AF062802
<i>O. pilicola</i> Kobayasi	UAMH 7526	Forest soil, Sweden, <i>Hambleton S.</i> , 1988	AF062787
<i>O. rhodogenum</i> Robak	UAMH 1405	Pulp in sludge strainers, Norway, <i>Hambleton S.</i> , 1988	AF062803
<i>O. scytaloides</i> W. Gams & B.E. Söderström	UAMH 6521	Forest soil, Sweden, <i>Hambleton S.</i> , 1988	AF062804
<i>O. setiferum</i> Udagawa & Toyazaki (ex-type strain)	UAMH 5715	House dust, Japan, <i>Hambleton S.</i> , 1988	AF062805
<i>O. tenuissimum</i> (Peck) S. Hughes	UAMH 8511	Wood pulp; Norway, <i>Hambleton S.</i> , 1988	AF062807
<i>O. truncatum</i> Barron	UAMH 1399	Soil ex mixed woods, Ontario, Canada, <i>Hambleton S.</i> , 1988	AF062809
<i>Petriella setifera</i> (A. Schmidt) Curzi	e	<i>Withuhn R.C.</i> , 1998	AF043596

Explanations:

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regions. This region is highly variable and therefore suitable for studying relationships within and between species. Each 25  $\mu$ l PCR mixture contained 25 pmol of each primer, 0.25 units of MBI *Taq* polymerase (MBI Fermentas, St. Lwon-Rot, Germany), buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P-40, 0.1 mg ml<sup>-1</sup> BSA, 1.5 mM MgCl<sub>2</sub>), 0.2 mM deoxyribonucleoside triphosphates (dNTPs) and 100 ng fungal DNA. Cycling conditions were an initial denaturation step at 94° for 10 min, followed by 30 cycles of 94° for 30 s, 42° for 30 s and 72° for 2 min. This was followed by a final 10 min at 72°. Negative controls (no DNA) were included in each PCR experiment.

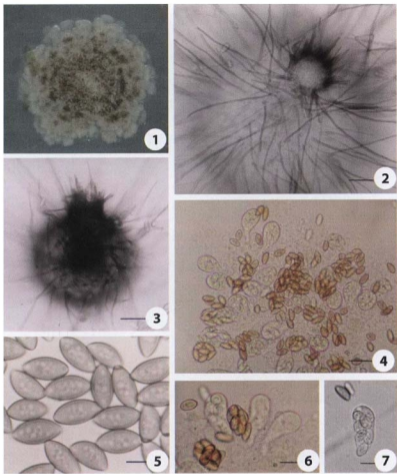
**Sequencing of the ITS rDNA region.** Amplicons generated using ITS4 + ITS5 were purified using the MinElute PCR Purification Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. DNA sequences were determined using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (version 3.1, Applied Biosystems, Foster City, CA 94404, USA) with primers ITS4, ITS5 and *ewfitsrev1* (5' TCC TCC GCT TAT TGA TAT GCT T). Reactions were run at the DNA sequencing Facility, Oxford University, UK (<http://polaris.bioch.ox.ac.uk/dnaseq/index.cfm>).

**DNA sequence data analysis.** The ITS sequence of *Oidiodendron setiferum* was compared with ITS sequences of *O. setiferum* (AF062805) and another 14 species of *Oidiodendron* and three species of *Myxotrichum*, and the ITS sequence of *Petriella setifera* was compared with ITS sequences of *P. setifera* (AF043596) and two species of *Chaetomium* obtained from EMBL/Genbank (Table 1). DNA sequences were assembled using the STADEN package (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK). DNA sequence analyses were performed using the programs BLAST, FASTA, SEQUED and PILEUP available in the GCG (Wisconsin) package (Genetic Computer Group 1994). Phylogenetic analyses were carried out using programs in PHYLIP version 3.6 (Felsenstein 2004). DNA sequences were aligned using the GCG programs PILEUP and CLUSTALX (Thompson et al. 1997) and edited manually using GeneDoc (Nicholas and Nicholas 1997). Genetic distances between pairs of fungi were calculated with the program DNADIST using the Kimura 2-parameter method.

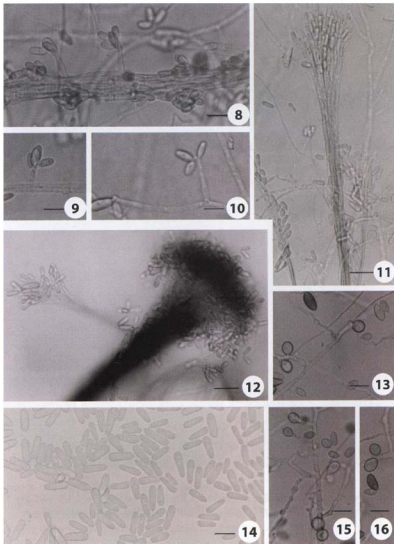
Phylogenetic trees were constructed using the distance method NEIGHBOUR and the maximum likelihood method DNAML using the original data set and 100 bootstrap data sets generated from this by the program SEQBOOT. Consensus trees were generated by the program CONSENSE. Trees were displayed by means of the program TREEVIEW (Page 1996).

## RESULTS

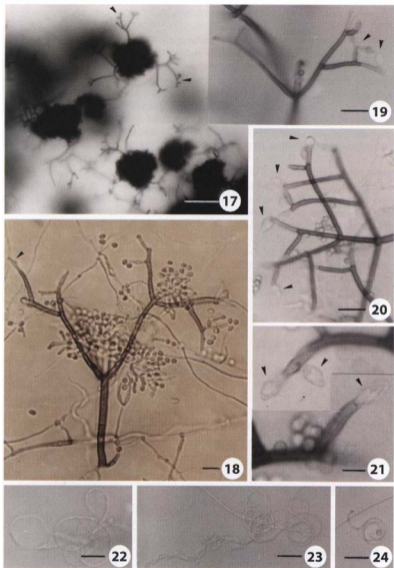
*Petriella setifera* and *Oidiodendron setiferum* were found in Poland on twigs of sessile oak with symptoms of oak decline and on bark of Scots pine logs imported from Lithuania. On oak twigs, both fungi were usually accompanied by two species of *Phomopsis* and *Rosellinia aquila* (Fr.) de Not. On the Scots pine log, *O. setiferum* was accompanied by *Chloridium virescens* var. *chlamydosporum* (v. Beyma) W. Gams et Hol.-Jech., *Ceratocystis piceae* (Münch) Bakshi, *Penicillium minioluteum* Dierckx, which caused sapstain, and *Phlebiopsis gigantea* (Fr.:Fr.) Jülich, which caused the initial decay of the wood.



Figs 1-7. *Petriella setifera*. Fig. 1. Two-week old colony on PDA. Fig. 2. Upper part of perithecium with an ostiole created of terminal hairs. Fig. 3. Perithecium. Fig. 4, 6-7. Asci. Fig. 5. Ascospores. Bars = 20  $\mu$ m (Figs 2, 4); 35  $\mu$ m (Fig. 3); 5  $\mu$ m (Fig. 5); 10  $\mu$ m (Figs 6-7).



Figs 8-16. *Petriella setifera*. Figs 8-10. Conidiophores with conidia of the *Sporotrichum*-anamorph. Figs 11-12. Synnemata of the *Graphium*-anamorph. Figs 13, 15-16. Chlamydospores. Fig. 14. Conidia. Bars = 10  $\mu$ m (Figs 8-10; 13-16); 20  $\mu$ m (Figs 11-12).



Figs 17-24. *Oidiiodendron setiferum*. Fig. 17. Sterile hairs growing from fertile heads. Fig. 18. Conidiophore with fertile head and dichotomously branched sterile hairs. Figs 20-21. Sterile apices at ends of branched hairs. Figs 22-24. Coils of hyphae submerged in medium. Bars = 100  $\mu\text{m}$  (Fig. 17); 10  $\mu\text{m}$  (Figs 18-20, 22-24); 5  $\mu\text{m}$  (Fig. 21).

## TAXONOMY

*Petriella setifera* on PDA and SNA produced brownish-grey, concentrically zonate colonies. The *Sporotrichum*- and *Graphium* anamorphs appeared respectively after 2 and 5 days of incubation. Perithecia started to form after 7 days and chlamydospores after 14 days of incubation. Chlamydospores were single, globose to oval, smooth-walled, brown to greyish, 4-8 (-10)  $\mu\text{m}$  diam (Figs 1-16). This is the first record of chlamydospores formed by *P. setifera*.

Specimen studied: Poland, Smolarz 52°54'N, 15°47'E, isolated from branches of *Quercus petraea*, 2002, H. Kwaśna (KFL C11, RR 225 = Rothamsted Research Fungus Culture Collection, IMI 391618, CBS 114629). The sequence of *P. setifera* has been deposited at EMBL as No. AJ784398.

*Oidi dendron setiferum* on MA and OMA produced restricted colonies that were plain, thin, brown to brownish grey, powdery at maturity, 13-15 mm diam after 14 d at 25°. The fungus produced characteristic conidiophores with the fertile head surrounded by (1-) 2-4 setiform hairs. The hairs were usually mono- and, more rarely, biseriate, monopodially or dichotomously branched, dark brown, smooth and rarely roughened, recurved. They matured from the base, below the fertile head, and terminated with swollen, often dichotomously branched, hyaline, thin-walled, sterile apices. The latter are observed for the first time. The submerged vegetative mycelium was delicate, hyaline 0.5-3  $\mu\text{m}$  wide, distinctly sinuous. The fungus produced numerous coils formed by thin hyphae in the submerged mycelium (Figs 17-24). They have not been recorded previously (Udagawa and Toyazaki 1987; Pagano and Zucconi 1994).

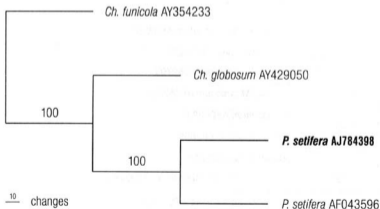


Fig. 25. Phylogenetic relationship of *P. setifera* derived from ITS sequences. Neighbour-joining analysis of an ITS alignment of 549 sites. Genetic distances were computed according to the Kimura-2-parameter model. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. Bootstrap values above 50% (from 1000 replicates) are indicated above the branches.



Specimens studied: Lithuania, isolated from *Pinus sylvestris* wood, 2002, H. Kwaśna (KFL O34, RR 238; IMI 391829, CBS 114896); Poland, Smolarz 52°54'N, 15°47'E, from branches of *Quercus petraea*, 2003, H. Kwaśna (KFL O35). Sequence of *O. setiferum* (KFL O34) has been deposited at EMBL under No. AJ784399.

DNA fragments of *P. setifera* and *O. setiferum* were successfully sequenced. The alignment of rDNA sequences of *P. setifera* and two species of *Chaetomium* included 549 nucleotide positions. In the neighbour-joining tree, the *P. setifera* being studied grouped with *P. setifera* (AF043596) with 100% bootstrap support (Fig. 25). The alignment of rDNA sequences of *O. setiferum* and other 17 *Oidiodendron* and *Myxotrichum* species included 535 nucleotide positions. In the neighbour-joining tree, the *O. setiferum* being studied grouped with *O. setiferum* (AF062805) with 98% bootstrap support (Fig. 26).

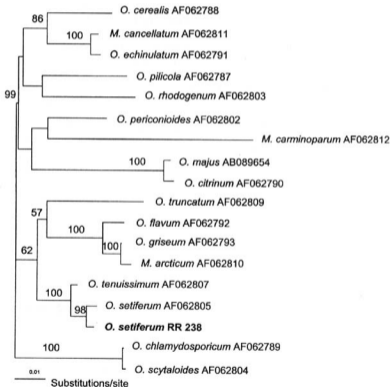


Fig. 26. Phylogenetic relationship of *O. setiferum* derived from ITS sequences. Neighbour-joining analysis of an ITS alignment of 535 sites. Genetic distances were computed according to the Kimura-2-parameter model. Bootstrap values from 1000 replicates are indicated above the branches.

## DISCUSSION

*Petriella setifera* was originally found on horse dung in Silesia in Poland (Schmidt 1912). In 1957 the fungus was found by A. L. Shigo on a wilted oak tree in West Virginia, USA (Barrone et al. 1961) and in 1962 in soil in Tokyo, Japan (Udagawa 1963). The fungus is known also from pepper, seed of mustard, wood, skin lesion of a dolphin (*Tursiops*) and human tissues (Becker and Lenz 1976; Ioachimescu-Dimulescu 1978; Shah and Jain 1993; Issakainen et al. 1999; Freire et al. 2000).

*Petriella setifera* is a pleomorphic fungus in the family Microascaceae, with more than one type of sporulation. Schmidt (1912) recorded its teleomorph. Barron et al. (1961) observed a *Sporotrichum* anamorph and a *Graphium* anamorph in the early stage of fungus incubation. The *Graphium* anamorph did not reappear and was not described by Barron et al. (1961), who did not doubt of its existence, however, particularly since all other species of *Petriella*, e.g. *P. sordida* (Zukal) Barron and Gilman, *P. gutullata* Barron and Cain, *P. lindforsii* Curzi and *P. boulangeri* Curzi, have a *Graphium* conidial stage. Udagawa (1963) observed a distinct *Graphium* anamorph formed by a Japanese isolate of *P. setifera*.

Chlamydo-spores produced by *P. setifera* are described here for the first time. Chlamydo-spores, 'the asexual spores originating by modification of a hyphal segments', represent in *P. setifera* the third type of asexual sporulation. Chlamydo-spores were observed previously only in *P. boulangeri*, in which they are 30 x 12 µm, many-celled, yellow-brown, and often form next to perithecia (Curzi 1930). Considering that the production of chlamydo-spores within *Microascaceae* is rather common, the failure to observe them in *P. setifera* in the past was presumably due to the rarity of the fungus and inconsistency of its asexual sporulation. *Petriella setifera* was observed to cause advanced post-emergence rot of mustard seedlings (Shah and Jain 1993). As the causative agent of soil-borne disease, the fungus is likely to depend on chlamydo-spores for its survival in soil. All anamorphs of the isolate of *P. setifera* studied, as well as the teleomorph, formed simultaneously, quickly and easily *in vitro* after a few days of incubation on SNA or PDA. Occurrence of anamorphs in *P. setifera* can be however erratic and dependent on substrate conditions (Barron et al. 1961).

Considering the resemblance of *P. setifera* to *Ceratocystis* and *Ophiostoma*, particularly when its *Graphium* and *Sporotrichum* anamorphs are concern (Wingfield et al. 1993) and its nutritional preferences for an oak (after occurrence of the fungus on the declined oak in USA and Poland) the pathogenicity of *P. setifera* to oak cannot be excluded.

*Oidiodendron setiferum* appears to be extremely rare in nature. This is the third record of its appearance after those of Udagawa and Toyazaki (1987), who found it in house dust, and Pagano and Zucconi (1994), who found it in soil. So far the fungus was most associated with the soil habitat. The isolation of the fungus by Pagano and Zucconi (1994) was possible only with bait consisting of fallen leaves of *Rubus procerus* P. J. Müller. Our isolate of *O. setiferum*, however, grew and sporulated well directly on the surface of sapwood of Scots pine timber after incubation in the dark, at 25° and 95% humidity, for 3 months. The fungus was subcultured from the timber and kept *in vitro*. The fungus produces setiform hairs surrounding the fertile heads. Hairs were formed both on wood and in agar media.

The hairs of all three isolates of known *O. setiferum* differ in details. The hairs of our isolate were mono- or biseriate, abundantly branched, dark brown, septate, smooth or finally roughened, recurved, up to 80–100 µm long, and resembled those described by Udagawa and Toyazaki (1987) more closely than those described by Pagano and Zucconi (1994), which were rather straight and only once or twice branched. The main difference is, however, in the shape and character of the hairs tips. The hairs of our isolate terminated with hyaline, swollen, often dichotomously branched and sterile apices. Those of the Udagawa and Toyazaki (1987) isolate tapered gradually to pointed tips. The hairs of the Pagano and Zucconi (1994) isolate had the fertile apices. The second characteristic which distinguishes our isolate from those described previously is the production of numerous coils formed by delicate hyaline hypha in the submerged mycelium. These have not been reported previously.

The molecular studies confirmed the taxonomy of the both fungi studied, and so the newly observed characteristics contribute significant diagnostic and behavioural information on both species.

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### Morfologia i analiza sekwencji rDNA grzybów *Petriella setifera* i *Oidiodendron setiferum* wyizolowanych z pędów chorych dębów

#### Streszczenie

U polskich izolatów *Petriella setifera* i *Oidiodendron setiferum* wyizolowanych z pędów dębu bezszypułkowego (*Quercus petraea*) z objawami zamierania koron oraz drewna sosny zwyczajnej (*Pinus sylvestris*), stwierdzono obecność nowych cech morfologicznych. Po raz pierwszy zaobserwowano i opisano (i) chlamydospory tworzone przez *P. setifera*, (ii) hialinowe, nabrziałe, cienkościenne, sterylne końcówki na bezpłodnych setach otaczających płodne rozgałęzienia konidioforów oraz liczne pętle zbudowane z cienkich strzępek tworzone przez *O. setiferum*. Zaprezentowano pierwsze zdjęcia anamorf *P. setifera*, które uzupełniają oryginalny opis grzyba z 1912 roku. Stanowisko taksonomiczne obu grzybów potwierdziły badania sekwencji rDNA.