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Species delimitation in the *Podospora anserina*/ *P. pauciseta*/*P. comata* species complex (Sordariales)

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Abstract – *Podospora anserina* is a model ascomycete that has been used for over a century to study many biological phenomena including ageing, prions and sexual reproduction. Here, through the molecular and phenotypic analyses of several strains, we delimit species that are hidden behind the *P. anserina*/*P. pauciseta* and *P. comata* denomination in culture collections. Molecular analyses of several regions of the genome as well as growth characteristics show that these strains form a species complex with at least seven members. None of the traditional morphology-based characters such as ascospore and perithecium sizes or presence of setae at the neck are able to differentiate all the species, unlike the ITS barcode, mycelium growth characteristics and repartition of perithecia on the thallus. Interspecific crosses are nearly sterile and most F1 progeny is female sterile. As a result of our analyses, the taxonomy of the *P. anserina* complex is clarified by lecto- and epitypifications of the names *P. anserina*, *P. pauciseta* and *P. comata*, as well as descriptions of the new species *P. bellae-mahoneyi*, *P. pseudoanserina*, *P. pseudocomata*, *and P. pseudopauciseta.* We also report on the ability of species from this complex to form a *Cladorrhinum*-like asexual morph and to produce tiny sclerotium-like structures.

***Podospora anserina* / *Podospora pauciseta* / *Podospora comata* / *Lasiosphaeriaceae* / *Sordariales* / *Cladorrhinum*-like / sclerotium-like / microsclerotium / spermatia**

Résumé – *Podospora anserina* est un ascomycète modèle qui est utilisé depuis plus d'un siècle pour étudier de nombreux phénomènes biologiques incluant le vieillissement, les prions ou la reproduction sexuée. Nous délimitons grâce à une analyse moléculaire et phénotypique les espèces cachées derrière les dénominations *P. anserina/P. pauciseta* et *P. comata* des souches de collections. Les analyses moléculaires de plusieurs régions du génome ainsi que les caractéristiques de croissance montrent que ces espèces forment un complexe avec au moins sept membres. Aucun des caractères utilisés usuellement tels que la taille des ascospores et des périthèces, ou la présence de setae au col ne peut différencier les espèces, au contraire des analyses du code-barres ITS, des caractéristiques de croissance et de la répartition des périthèces sur le thalle. Les croisements interspécifiques sont très peu fertiles et la plupart de la descendance F1 est femelle stérile. Il en résulte une clarification par lecto- et epitypifications des noms *P. anserina*, *P. pauciseta* and *P. comata*, ainsi que la description de quatre espèces nouvelles : *P. bellae-mahoneyi*, *P. pseudoanserina*, *P. pseudocomata*, *and P. pseudopauciseta.* Nous montrons aussi la capacité de ces espèces à former un anamophe de type *Cladorrhinum* ainsi que des microsclérotes.

***Podospora anserina* / *Podospora pauciseta* / *Podospora comata* / *Lasiosphaeriaceae* / *Sordariales* / anamorphe de type *Cladorrhinum* / microsclérotes / spermaties**

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INTRODUCTION

Podospora anserina, the "friendly mold", is a model fungus that has been widely used for over a century to analyze various biological processes ranging from cell fusion, ageing, prions, sexual reproduction, differentiation and development to plant biomass degradation (Silar 2013). This species is a common inhabitant of herbivore dung and appears to have a cosmopolitan distribution (Table 1). In addition, to dung there is one report of its isolation as a plant endophyte (Matasyoh *et al.* 2011) and one strain deposited in the Westerdijk Institute Culture Collection has been retrieved from soil (Table 1, CBS 415.72). Presently, only the teleomorph is known as no asexual morph has been described. This species is pseudo-homothallic, meaning that it is formally heterothallic, *i.e.*, with two different mating types (*mat*+ and *mat*-), but it produces asci with four ascospores carrying *mat*+ and *mat*- nuclei. The heterokaryotic thalli germinating from these ascospores are thus self-fertile. Ascospores are obliquely uniseriate in the asci and measure about 20 µm × 35 µm. They have a typical cellular appendage (20 µm × 5 µm) located at the opposite of the germination pore and two non-cellular appendages at the opposite ends of the ascospore. The fruiting body produced is a perithecium with a diameter of about 0.3 mm with a well-defined neck ornamented with setae.

As noted by Atkinson in a footnote of a paper by Wolf (Wolf 1912) as early as the beginning of the 20th century, the name of this species is somewhat uncertain. Indeed, it is mostly known in scientific publications dealing with genetics, molecular biology biochemistry and genomics as *Podospora anserina* (Rahb.) Winter (Wolf 1912) or *Podospora anserina* (Ces.) Rehm (as for example in Rizet & Engelmann (1949) or in Esser (1956). Another infrequently used synonym is *Pleurage anserina* (Ces.) Kuntze (*e.g.*, Hodgkiss & Harvey 1971; Moreau & Moreau 1951). However, strains of this species are preserved under the name of *Podospora pauciseta* (Ces.) Trav. in many culture collections including that of the Westerdijk Institute, and many taxonomists use this epithet in their publications. The species appears to have been described first as *Sphaeria pauciseta* by Cesati (see Botanische Zeitung 1852 vol. 10, pp 285-288) and later a second time as *Malinvernia anserina* also by Cesati and reported by Rabenhorst (1857). Traverso validated the combination in *Podospora, P. pauciseta* (Ces.) Trav. (Traverso 1907), but some authors continued to call it *P. anserina,* as initiated by Niessl (1883) and Winter (1887). To confuse the matter, it is possible that *P. anserina* and *P. pauciseta* are two different species, a fact hinted at by Mirza and Cain (1969). Original syntype materials of both *P. pauciseta* (as *Sphaeria pauciseta* Ces. under n° 1642 in the "Klotzschii herbarium vivum mycologicum sistens fungorum per totam germaniam crescentium collectionem perfectam – Centuria XVII") and *P. anserina* (as *Malinvernia anserina* Ces. under n° 526 in the "Klotzschii herbarium vivum mycologicum sistens fungorum per totam germaniam crescentium collectionem perfectam – Editio nova Series Prima Centuria VI") are available as dried dung. However, Lundqvist (1972) stated that he could not find the fungus on these authentic types. Moreover, at the time of its first descriptions the fungus was collected directly from the wild and not cultivated in pure culture. Hence, the original types, including those of Cesati deposited in Rome's herbarium, may contain additional species and unfortunately cannot anymore be used as reference for the fungus. To complicate further the situation, a second supposedly closely-related species, *Podospora comata*, has been described (Milovtzova 1937). It differed from typical *P. anserina* by smaller spores, slender perithecia and lack of setae decorating the neck (as deduced from the drawing of Milovtzova). It is not clear whether this species is a true species or a minute form

Table 1. Strains used in this study. The new sequences are in bold

Strain	*Previously identified as*	*Proposed new name*	*Origin*	*Substrate*	*Year*	*Ascospore size (µm)*	*Perithecium diameter*[4]	*% with setae*[5]	*GenBank numbers*			
									ITS	*Rchr3*	*Rchr4*	*Rchr6*
A	*P. anserina*	*P. anserina*	Normandie, France	dung	1937	35 ± 2 x 18 ± 1	323 ± 12	60	**MF380256**	**MF379740**	**MF379732**	**MF379769**
B	*P. anserina*	*P. anserina*	Normandie, France	dung	1941	35 ± 1 x 19 ± 1	313 ± 12	60	AY525771.1	**MF379766**	**MF379798**	**MF379796**
E	*P. anserina*	*P. anserina*	?	dung	?	36 ± 2 x 20 ± 1	371 ± 14	45	**MF380240**	**MF379744**	**MF379768**	**MF379789**
F	*P. anserina*	*P. anserina*	?	dung	?	36 ± 1 x 20 ± 1	329 ± 25	35	**MF380246**	**MF379746**	**MF379781**	**MF379799**
H	*P. anserina*	*P. anserina*	Bourgogne, France	dung	?	33 ± 1 x 20 ± 1	369 ± 10	40	**MF380244**	**MF379730**	**MF379778**	**MF379739**
I	*P. anserina*	*P. anserina*	?	dung	?	33 ± 1 x 18 ± 1	296 ± 27	55	**MF380247**	**MF379802**	**MF379735**	**MF379793**
M	*P. anserina*	*P. anserina*	Picardie, France	dung	?	35 ± 1 x 19 ± 1	311 ± 24	65	**MF380242**	**MF379708**	**MF379743**	**MF379760**
N	*P. anserina*	*P. anserina*	?	dung	?	33 ± 1 x 18 ± 1	268 ± 13	60	**MF380239**	**MF379806**	**MF379783**	**MF379787**
R	*P. anserina*	*P. anserina*	?	dung	?	33 ± 1 x 18 ± 1	337 ± 37	40	**MF380264**	**MF379697**	**MF379784**	**MF379795**
S (big S)[1]	*P. anserina*	*P. anserina*	Normandie, France	dung	?	35 ± 1 x 19 ± 1	280 ± 29	60	AY278557.1	**MF379758**	**MF379804**	**MF379755**
s (small s)	*P. anserina*	*P. anserina*	Normandie, France	dung	?	33 ± 1 x 19 ± 1	284 ± 15	50	**MF380241**	**MF379726**	**MF379754**	**MF379723**
U	*P. anserina*	*P. anserina*	Picardie, France	dung	?	35 ± 1 x 19 ± 1	351 ± 11	50	**MF380253**	**MF379786**	**MF379713**	**MF379788**
V	*P. anserina*	*P. anserina*	Picardie, France	dung	?	35 ± 1 x 19 ± 1	304 ± 18	60	**MF380236**	**MF379717**	**MF379780**	**MF379759**
W	*P. anserina*	*P. anserina*	?	dung	?	37 ± 1 x 19 ± 1	314 ± 7	50	**MF380261**	**MF379718**	**MF379775**	**MF379803**
X	*P. anserina*	*P. anserina*	Picardie, France	dung	?	37 ± 1 x 19 ± 1	285 ± 32	60	**MF380252**	**MF379701**	**MF379748**	**MF379738**
Y	*P. anserina*	*P. anserina*	?	dung	?	36 ± 1 x 19 ± 1	299 ± 12	50	**MF380237**	**MF379707**	**MF379772**	**MF379800**
Z	*P. anserina*	*P. anserina*	?	dung	?	35 ± 1 x 19 ± 1	288 ± 19	25	**MF380243**	**MF379765**	**MF379791**	**MF379733**
Pscj14	*New to this study*	*P. anserina*	Ile de France, France	dung	2003	36 ± 1 x 19 ± 1	341 ± 12	15	**MF380255**	**MF379720**	**MF379807**	**MF379704**
PSN14	*New to this study*	*P. anserina*	Picardie, France	dung	2007	36 ± 1 x 19 ±1	341 ± 17	40	**MF380249**	**MF379774**	**MF379705**	**MF379801**
PSN42	*New to this study*	*P. anserina*	Picardie, France	dung	2008	35 ± 1 x 20 ± 1	321 ± 27	29	**MF380257**	**MF379750**	**MF379805**	**MF379709**
CB1	*New to this study*	*P. anserina*	France	dung	2014	34 ± 1 x 19 ± 1	356 ± 19	5	**MF380235**	**MF379761**	**MF379729**	**MF379700**
CB2	*New to this study*	*P. anserina*	France	dung	2014	34 ± 1 x 19 ± 1	338 ± 17	20	**MF380259**	**MF379706**	**MF379724**	**MF379794**
CB3	*New to this study*	*P. anserina*	France	dung	2014	35 ± 1 x 19 ± 1	380 ± 27	12	**MF380245**	**MF379776**	**MF379741**	**MF379782**
CB4	*New to this study*	*P. anserina*	France	dung	2014	36 ± 1 x 19 ± 1	340 ± 16	54	**MF380254**	**MF379702**	**MF379767**	**MF379779**
CB5	*New to this study*	*P. anserina*	France	dung	2014	34 ± 1 x 19 ± 1	349 ± 36	28	**MF380248**	**MF379752**	**MF379773**	**MF379764**
CB6	*New to this study*	*P. anserina*	Ile de France, France	dung	2014	35 ± 2 x 19 ± 1	343 ± 14	82	**MF380250**	**MF379710**	**MF379703**	**MF379756**
CB7	*New to this study*	*P. anserina*	Ile de France, France	dung	2015	36 ± 1 x 20 ± 1	320 ± 27	66	**MF380258**	**MF379737**	**MF379728**	**MF379716**
CBS 433.50	*P. pauciseta*	*P. anserina*	Ontario	dung	1944	NA[6]	NA[6]	NA[6]	**MF380260**	**MF379753**	**MF379698**	**MF379747**
CBS 333.63	*P. pauciseta*	*P. pauciseta*	Argentina	dung	?	35 ± 1 x 19 ± 1	314 ± 20	29	GQ922516.1	**MF415406**	**MF379749**	**MF379762**
CBS 451.62	*P. pauciseta*	*P. pauciseta*	Argentina	dung	?	34 ± 1 x 18 ± 1	308 ± 62	24	GQ922517.1	**MF415407**	**MF379742**	**MF379797**
CBS 237.71	*P. comata*	*P. pauciseta*	Israel	dung	1970	32 ± 1 x 19 ± 1	247 ± 24	1	**MF380263**	**MF415408**	**MF379722**	**MF379792**
CBS 112042	*P. pauciseta*	*P. bellae-mahoneyi*	Western Australia	dung	2001	39 ± 1 x 23 ± 1[2]	397 ± 31	50	DQ166956.1	**MF379763**	**MF379736**	**MF379719**
CBS 411.78	*P. comata*	*P pseudopauciseta*	Venezuela	dung	1972	32 ± 1 x 19 ± 1	372 ± 18	30	**MF415409**	**MF379731**	**MF379770**	**MF379790**
CBS 253.71	*P. pauciseta*	*P. pseudoanserina*	Central Africac	dung	?	34 ± 1 x 19 ± 1	253 ± 71	38	GQ922515.1	**MF379745**	**MF379777**	**MF379715**
CBS 124.78	*P. pauciseta*	*P. pseudoanserina*	India	dung	1977	33 ± 1 x 19 ± 1	326 ± 18	27	**MF380251**	**MF379711**	**MF379785**	**MF379712**
T	*P. comata*	*P. comata*	France	dung	?	32 ± 1 x 17 ± 1[3]	228 ± 16	65	AF443849.1	**MF379757**	**MF379734**	**MF379725**
CBS 415.72	*P. pauciseta*	*P. pseudocomata*	Pakistan	soil	?	33 ± 1 x 19 ± 1	277 ± 17	13	**MF380238**	**MF379771**	**MF379751**	**MF379721**

[1] Sequenced strain (Espagne *et al.* 2008; Grognet *et al.* 2014); deposited as CBS141519 for the *mat+* and CBS141520 for the *mat-* homokaryons and as n° 6597 in the collection of the Museum National d'Histoire Naturelle de Paris for the *mat+/mat-* heterokaryon. – [2] Ascospore height and width are significantly larger than most of the other strains. – [3] Ascospore width is significantly smaller than most of the other strains. – [4] Average diameter of the largest mature perithecia when cultivated on M2 medium with 4 g/L of dextrin as carbon source. – [5] Percentage of perithecia with clearly differentiated setae when cultivated on medium with wood shavings as carbon source. – [6] Strain CBS 433.50 has lost sexual reproduction by loss of the *mat-* nuclei.

of *P. anserina* (Krug & Khan 1989). Note that the long awaited revision of the species related to *P. anserina* is unlikely to improve the matter. The genus *Podospora* is polyphyletic (Miller & Huhndorf 2005) and belongs to the *Lasiosphaericeae*, a paraphyletic family from which stem the mostly monophyletic *Chaetomiaceae* and *Sordariaceae* (Cai *et al.* 2006; Kruys *et al.* 2015; Madrid *et al.* 2011; Miller & Huhndorf 2005). Although, the type species of the genus is *Podospora fimiseda*, an eight-spored species (Niessl 1883) and a close relative in molecular phylogeny to *Podospora pauciseta* (Kruys *et al.* 2015), a formal taxonomic revision of the family will likely result in a change of genus name for most species, including possibly that of *P. anserina*, *P. pauciseta* and *P. comata*. Here, as a start, we have undertaken the analysis of most available strains of *P. pauciseta, P. anserina* and *P. comata* present in culture collections and show that they belong to a species complex with not three, but at least seven members.

MATERIALS AND METHODS

Strain sampling

Strains A to Z (Table 1), including S (big S), s (small s) and T, were from the collection of Georges Rizet and have been used for many early studies on *P. anserina* (see for example Bernet 1965 and Marcou 1961). Both *mat*+ and *mat*- isolates were already available for all these strains. They were crossed and fresh *mat*+ and *mat*- homokaryotic progeny for each strain was selected for phenotypic analysis. The S (= big S) strain is the reference strain for which the genomes of the *mat*+ (Espagne *et al.* 2008) and *mat*- (Grognet *et al.* 2014) nuclei have been sequenced. The origin of some of these strains (date and location of sampling) is unfortunately not known. Moreover, they were kept in the lab for over 60 years and some may have acquired mutation(s), possibly accounting for some of the deviation seen for a few characters. Nonetheless, most of them look exactly like the newer isolates, indicating that phenotypically-relevant mutations have not accumulated at high level. The newer strains, PSN14, Pscj14, PSN42 and CB1 to CB7, were obtained from dung sampled from various French regions and at different dates (Table 1). Ascospores ejected from perithecia present on the dung were collected and germinated. Thalli produced from these ascospores were immediately stocked at –80°C to prevent any mutation accumulation. These self-fertile thalli were incubated for the production of perithecia. Homokaryotic *mat*+ and *mat*- ascospores, recognized by their small sizes, were collected and selected for phenotypic analysis. The remaining strains were purchased from the Westerdijk Institute. Like for the previous strains, homokaryotic progeny for all Westerdijk Institute strains were used for phenotypic analysis. Note that for some strain of the Westerdijk Institute Culture Collection, "*incolore*" mutations that frequently occur in *P. anserina* (Rizet 1939) were detected. Only progenies devoid of this mutation were selected for analysis.

Phenotypic analysis

Standard culture conditions, media and genetic methods for *P. anserina* have been described (Rizet & Engelmann 1949; Silar 2013) and can be accessed at http://podospora.i2bc.paris-saclay.fr. M0 minimal base medium has the following

composition KH2PO4 0.25 g/L, K2HPO4 0.3 g/L, MgSO4/7H2O 0.25 g/L, Urea 0.5 g/L, Thiamine 0.05 mg/L, Biotine 0.25 µg/L, Citric Acid 2.5 mg/L, ZnSO4 2.5 mg/L, CuSO4 0.5 mg/L, MnSO4 125 µg/L, Boric Acid 25 µg/L, Sodium Molybdate 25 µg/L, Iron Alum 25 µg/L, Agar 12,5 g/L. It can be supplemented with Dextrins at 4 g/L or 6 g/L to yield the M2 minimal medium, crystalline cellulose (cat n° CC41 from Whatman) at 5 g/L to yield M4 medium or 1 g of *Guiboursia deumeusi* wood shavings. Importantly, when cultivated on these media, all strains yielded in 7 to 10 days matured perithecia typical of the species. These looked alike perithecia obtained on sterile horse dung, a more natural growth medium for the fungus. Germination medium was ammonium acetate 4.4 g/L, bactopeptone 15 g/L and Agar 13 g/L. Perithecium formation was assayed as in (Tangthirasunun *et al.* 2016). Ascospore and perithecium sizes were the average + SD of 10 different samples. Hyphal Interference was assayed as in (Silar 2005). Crippled Growth as in (Haedens *et al.* 2005). Microsclerotia developed on plates with wood shavings and the *Cladorrhinum*-like anamorphic structures were obtained by incubating homokaryotic strains on M2 medium overlaid with a cellophane sheet for three weeks at 18°C.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted as described by Lecellier & Silar (1994). The ITS locus was amplified using primers ITS1 and ITS4 primers (White *et al.* 1990). The amplified three intergenic loci were (1) a region between *Pa_3_1380* and *Pa_3_1390* on chromosome 3 (Rchr3) using primers 1380F (acgcgcacatacacagg) 1380R (tggatgccattgggctat), (2) a region on chromosome 4 between Pa_4_7610 and Pa_4_7620 (or Rchr4) with primers 7610F (tcctggtgagctgtatgtaggctggacacg) and 7610-2R (accaggcagtagagtgaaaaggtcgaaggc) and (3) a region on chromosome 6 between Pa_6_5500 and Pa_6_5510 (or Rchr6) with primers 5500F (agatgggttcttgtcatgagagggctggttt) and 5500R (tgggcttgatatcgtgctatattggcggcc). The sequencing was performed by Genewiz UK (Takeley, UK), with primers ITS1, ITS4, 1380F, 7610F and 5500F. Sequences were manually assembled.

Phylogenetic analyses

DNA sequences were compared using Mafft with the default parameters (Katoh & Standley, 2014) and transferred to Jalview for visualization (Waterhouse *et al.*, 2009). Alignments were used to construct phylogenetic trees using the maximum likelihood method (PhyML software) with the GTR model (Guindon & Gascuel, 2003; Guindon *et al.*, 2005). The optimized trees were transferred to the iToL server for visualization (Letunic & Bork, 2007). Bootstrap values are expressed as percentages of 100 replicates.

RESULTS AND DISCUSSION

Molecular phylogeny

To delimit potential species, we sequenced four genomic regions (Table 1): (1) the nucITS rRNA (ITS1-5.8S-ITS2) located on chromosome3, (2) an intergenic

region (called Rchr3) also situated on chromosome 3 and closely linked to a locus under balanced selection, the Het-s/het-S incompatibility locus (Debets *et al.* 2012), (3) an intergenic region situated on chromosome 4 (Rchr4) and an intergenic region on chromosome 6 (Rchr6). The last three regions were chosen because, being intergenic, they likely accumulated more differences than the elongation factor 1-α or tubulin genes used frequently for phylogenetic analysis. Moreover, Rchr3 being associated with a locus under balanced selection should exhibit more polymorphisms than Rchr4 and Rchr6.

All three intergenic regions differentiated the same seven sets (Fig. 1). Moreover, these sets were identical to those defined by the ITS sequence (Fig. 2). We find no evidence of recombination between the four regions between the species sets, suggesting that the seven sets represented in fact seven species. The largest set contained all strains from the Rizet collection except strain T, all the strains newly collected from France (*i.e.*, Pscj14, PSN14, PSN42 and CB1 to CB7) and strain CBS 433.50. They all had the same ITS, Rchr4 and Rchr6. They could only be differentiated by Rchr3, which clustered them into two subgroups (Fig. 2). As expected, this clustering reflected the one obtained when the Het-s/Het-S region was analyzed (Debets *et al.* 2012), with the S (big S) strain belonging to one subgroup and the s (= small s) strain to the other. The six additional sets were (1) strain T, (2) strain CBS 112042, (3) strain CBS 411.78, (4) strains CBS 237.71, CBS 451.62 and CBS 333.63, (5) strain CBS 415.72 and (6) strains CBS 253.71 and CBS 124.78. As expected when combined, phylogeny with the four sequences gave seven statistically-well-supported sets (Fig. 3). Although individual branches for each set are statistically well supported, the branching order is poorly defined by the present data (Fig. 2 and 3). Most strains are equally distant from each other, except groups (5) and (6) that seem to be more closely related to each other than to the other strains (Fig. 3 and Table 2). Note that the percentage of similarity of the sequences between the sets was ten times greater than those within sets (Table 2) in the range of 1-3%, which are typical of differences between species rather than those of different strains from the same species. Fertility of interspecific crosses was very low (few hundreds of ascospores obtained per Petri plate, instead of the hundreds of thousands obtained in most intraspecific crosses). F1 progeny from interspecific crosses often had poor growth and most of them were female sterile. This showed that the seven sets were in fact seven species, a conclusion supported by morphological and cultural properties of the strains (see below). Names for these species are indicated in Figs 1, 2, 3, 5 and 7 as well as in Table 1 and 2.

Table 2. Percentage of nucleotide sequence identity between the indicated species

	P. anserina	*P. pauciseta*	*P. comata*	*P. bellae-mahoneyi*	*P. pseudoanserina*	*P. pseudopauciseta*	*P. pseudocomata*
P. anserina	< 0.1%[a]						
P. pauciseta	1.8%	< 0.1%					
P. comata	2.2%	2.4%	NA				
P. bellae-mahoneyi	2.7%	3.2%	3.1%	NA			
P. pseudoanserina	2.0%	2.3%	2.6%	3.1%	0%		
P. pseudopauciseta	2.3%	2.5%	2.8%	3.4%	0.7%	NA	
P. pseudocomata	1.7%	2.1%	2.1%	2.7%	2.1%	2.3%	NA

[a] This number is obtained when the Rchr3 region is omitted; when this region under balanced selection is included, the percentage of identity reached 0.3%.

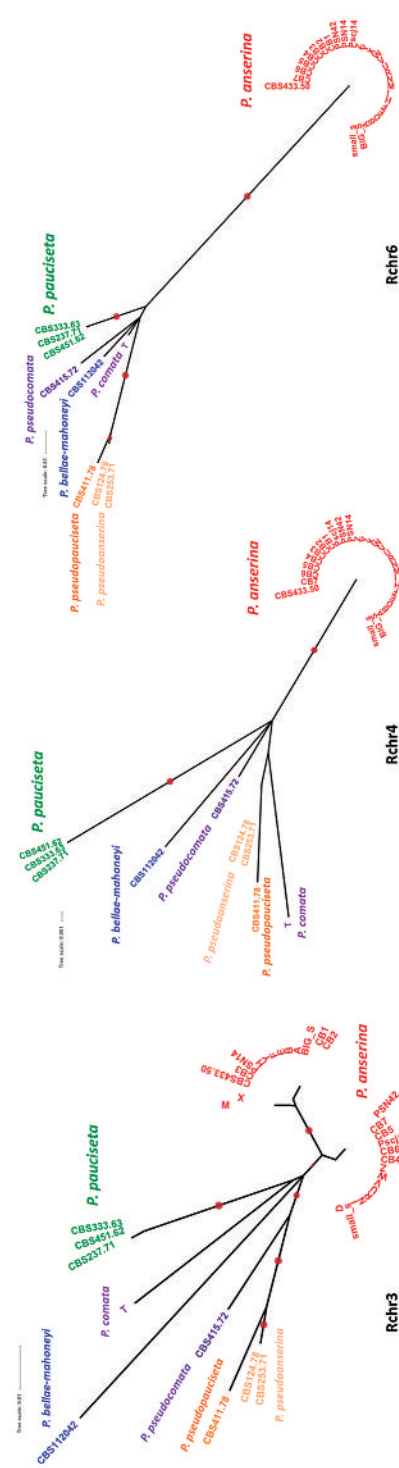

Fig. 1. **Phylogenetic trees constructed using Rchr3, Rchr4 and Rchr6.** The trees were constructed by maximum likelihood with PhyML. Bootstrap supports (> 80%; 100 replicas) are indicated by the dots.

Fig. 2. **Comparison of the ITS1 & ITS2 regions.** The 5.8S is masked; no difference was found in the 5.8S gene.

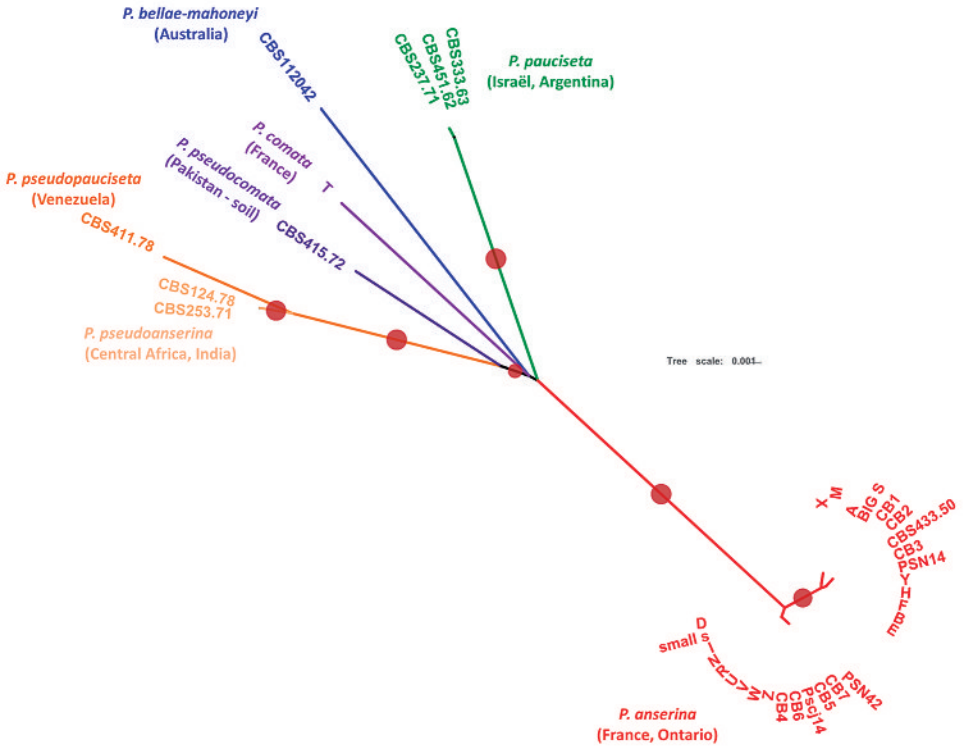

Fig. 3. **Species tree inferred from the concatenated dataset (ITS + Rchr3 + Rchr4 +Rchr6).** Bootstrap support values (> 80%; 100 replicas) are indicated by the dots. Known regions where strains from the relevant species were collected are indicated.

Taxonomy

Podospora pauciseta Traverso, Flora Italica Cryptogama Pars I: Fungi Pyrenomycetae, p. 431 (1907) Fig. 7B

= *Sphaeria pauciseta* Ces., Botanische Zeitung vol. 10, p. 285 (1852)

Lectotypification: ITALY, Vercelli, on pig dung, Cesati, Rabenh. Herb. Viv. Mycol., Cent. XVII, 1642 (HAL, **lectotypus hic designatus**, Mycobank: MBT#249597).

Epitypification: Mycobank: MBT#121083: ISRAEL, Northern Region, between Moledet and Jubla, isolated from Antelope dung, Apr. 1970, *J.C. Krug* (PC0735081 **epitypus hic designatus**; duplicate deposited at HAL sub nr. 3208 F,; ex-epitype culture: CBS 237.71).

Notes: The three strains available for this species produce perithecia all over the culture on M2 medium and on a ring similar to that of *P. anserina* on M4. They fructify abundantly on wood shavings. Their ITS sequence differs at one position from the *P. anserina* reference ITS: ITS2 – C insertion after nucl. n° 11.

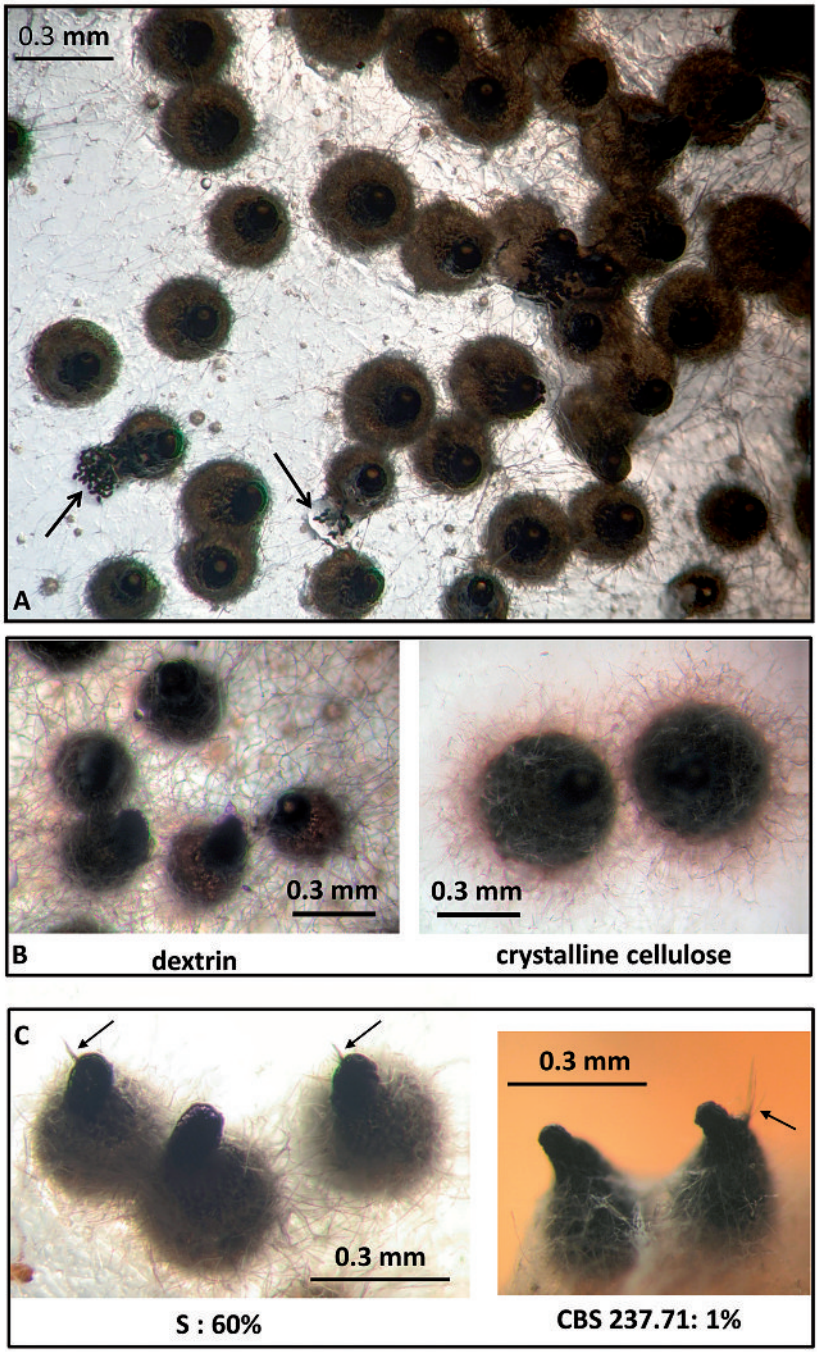

Fig. 4. (**A**) Variation of the size of the perithecia. Arrows point towards ascospores that originated from small perithecia, showing that these were matured. (**B**) Perithecia of strain CBS 411.78 on two different media containing either dextrin or crystalline cellulose as carbon source. (**C**) Strain CBS 237.71 has very low percentage (1%) of perithecia with setae (arrows), while strain S has 60%.

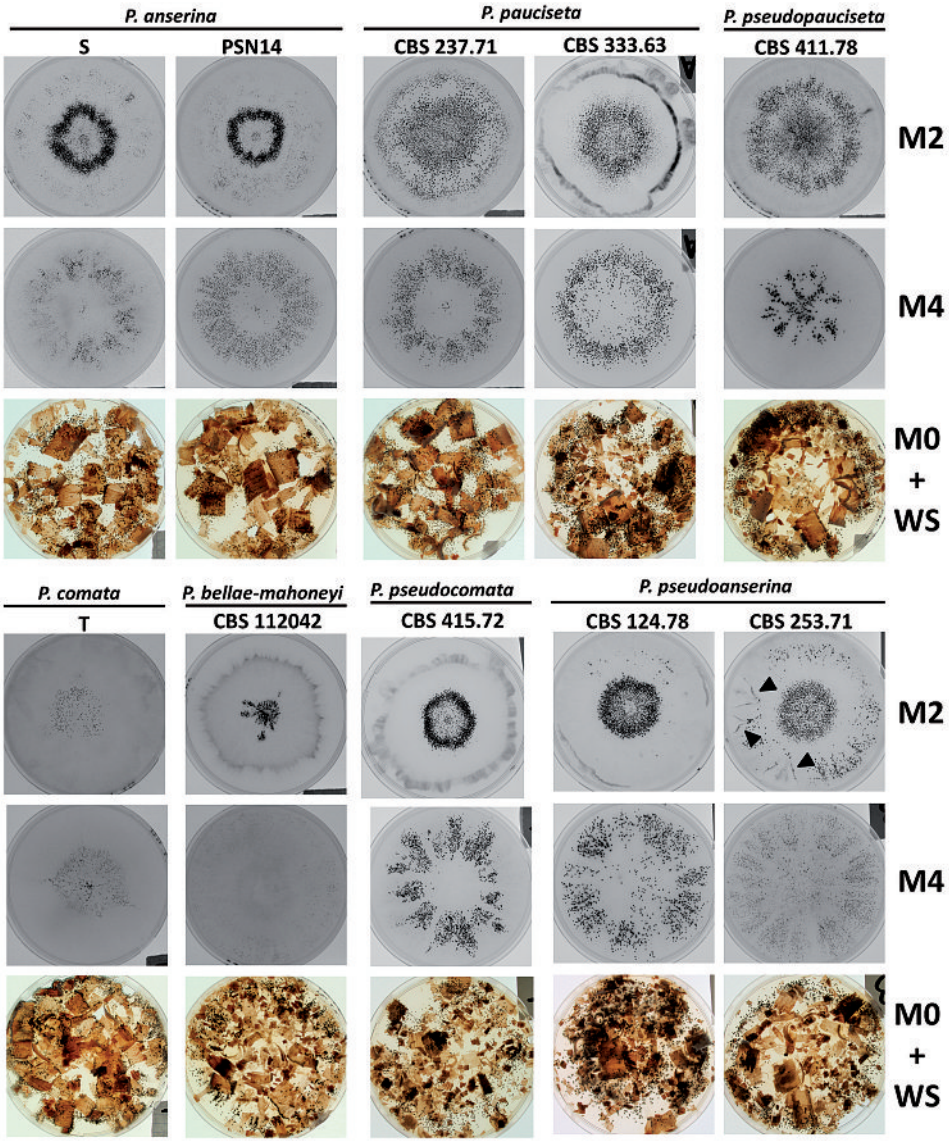

Fig. 5. **Perithecium differentiation patterns on various carbon sources.** Only representative strains are illustrated. M0 + WS= M0 supplemented with wood shavings as carbon source. Arrowheads on the M2 plate of CBS 253.71 point towards sectors of altered growth.

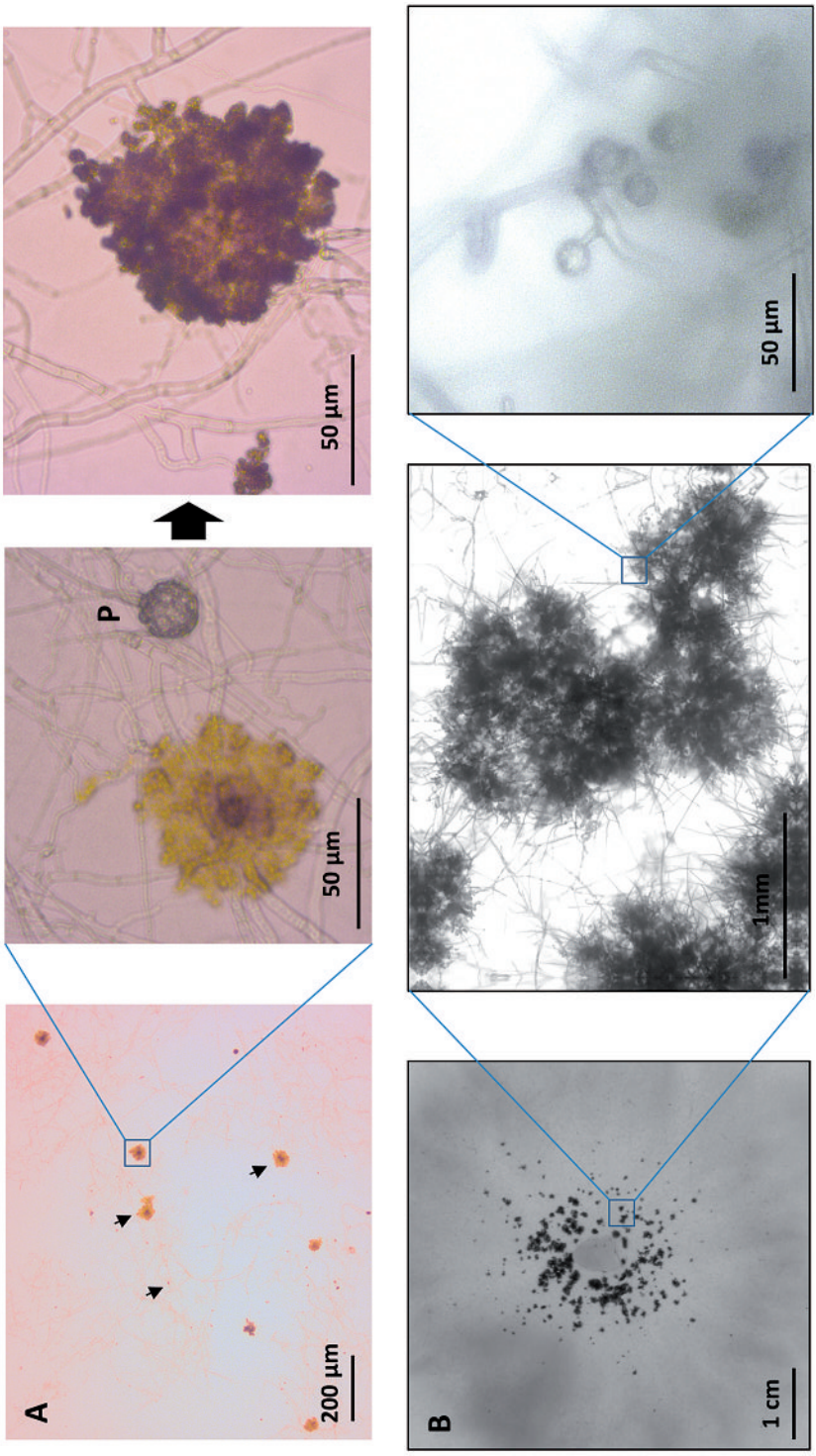

Fig. 6. **Microsclerotia (A) and *Cladorrhinum* anamorphs (B).** P: protoperithecium.

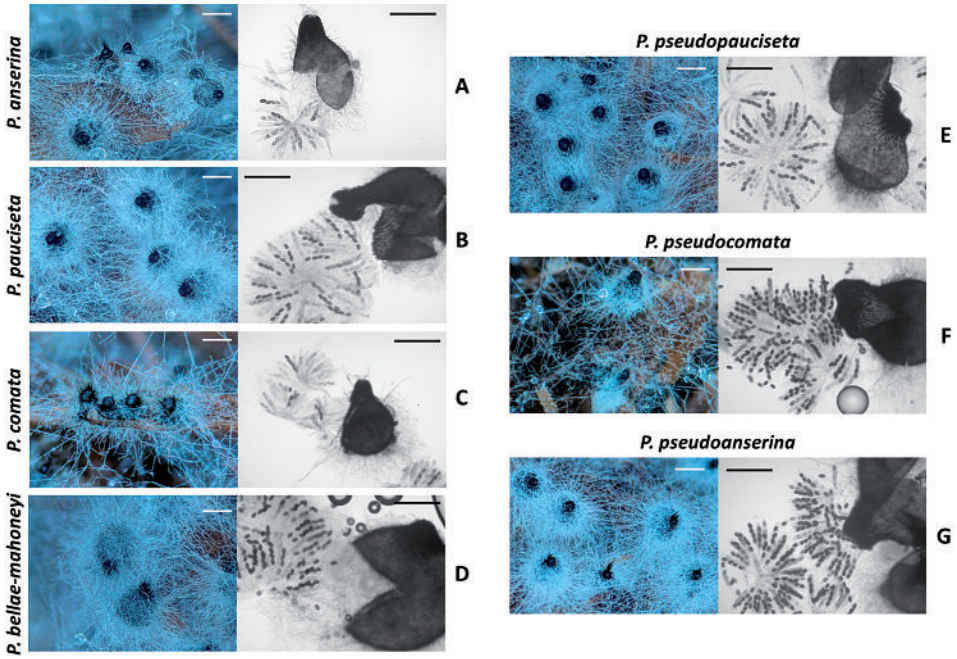

Fig. 7. For each species on the left are the perithecia obtained on sterile dung and on the right squash mount of perithecia obtained on M2 showing perithecial wall, neck and centrum with asci. Left panel, white bar = 0.3 mm, right panel black bar = 0.25 mm.

Podospora anserina Niessl Ueber die Theilung der Gattung *Sordaria*. *Hedwigia* 22: 156. (1883)

= *Malinvernia anserina* Ces., Rabenhorst, Erklärung der Taf. XV. Hedwigia 1: 116 – pl. 115 fig. 114 (1857)

Lectotypification: ITALY, Vercelli, on goose dung, Cesati, Rabenh., Herb. Viv. Mycol., Ed. Nov., Cent. VI, 526 (HAL, **Lectotypus hic designatus**, MBT#183160).

Epitypification: FRANCE. Normandie, isolated from dung. Date of collection unknown but around 1940 (PC0735082 & HAL 3205 F; **Epitypus hic designatus**, Mycobank: MBT#100818); ex-epitype culture: Strain S deposited at the Museum National d'Histoire Naturelle, Paris n° 6597; Fig. 7A).

Notes: Many strains are available for this species. Diagnostic features are the presence of a ring of perithecia on M2 for *mat+/mat-* heterokaryotic cultures and the ability to undergo Crippled Growth. Perithecia form a larger and more diffuse ring on M4 medium and are produced all over wood shavings. Sequence of its ITS, the first sequenced, is a defining feature and is used as reference for all the other strains of the species complex. This species seems to be prevalent in Western Europe.

Podospora comata Milovtz., Trav. Inst. Bot. Kharkov 2: 20 (1937) Fig. 7C

Lectotypification (Mycobank MBT#261400): UKRAINE, on horse dung, Trav. Inst. Bot. Kharkov vol. 2: 20 fig. 2 (1937), **lectotypus hic designatus**.

Epitypification (Mycobank MBT#261400): FRANCE. Isolated from dung. (PC0735083, **epitypus hic designatus**, duplicate deposited at HAL sub nr. 3207 F; ex-epitype culture: Strain T deposited at the Mycotheque du Museum National d'Histoire Naturelle de Paris, n° 6598).

Notes: Type material of *P. comata* could not be traced and is not preserved in the herbarium of the Kharkov National University (Dr. A. Akulov, pers. comm.). Therefore, the original illustration published in Milovtzova (1937), which is part of the original material (Art. 9.3), is designated as lectotype (Art. 9.2), and the current application of *P. comata* is clarified by epitypification. The only strain available for this redefined species was collected in France. It has smaller ascospores (32 + 1 × 17 + 1 μm) and perithecia on M2 (diam. = 228 + 16 μm) than strains of the other species. This strain requires at least 6g/L of dextrin in M2 for abundant pigmentation and fructification. It is poorly able to utilize the crystalline cellulose present in the M4 medium. It is however very fertile on wood shavings. Its ITS sequence differs at three positions from the *P. anserina* reference ITS: ITS1 – nucl. n° 12 G to A, ITS1 – nucl. n° 26 G to A and ITS2 – CC insertion after nucl. n° 141.

Podospora bellae-mahoneyi C. Boucher, TS Nguyen & P Silar, ***sp. nov.*** Fig. 7D

Mycobank: MB821832.

Etymology. Refers to Ann Bell and David Mahoney who isolated and deposited the strain.

Type: Western Australia. Isolated from kangaroo dung, Feb. 2001, leg. A. Bell & D.P. Mahoney (PC0735079 **holotypus**; HAL 3206 F isotypus; ex-type culture: CBS 112042).

Description: Perithecium diameter 397 + 31 mm, pyriform, membranous, semitransparent, pale brown, covered with numerous hyphoid hairs. Neck blackish, coriaceous, often with a tuft of dark, rigid, agglutinated hairs, sometimes with a few scattered hairs too. Asci 4-spored, clavate-lageniform. Spores spoon-shaped in the early stages. Mature spores obliquely uniseriate: spore head 38 + 1 × 23 + 1 mm, ellipsoidal sometimes slightly asymmetrical, flattened at the base, smooth, thick-walled, with a central germ pore. Presence of a primary appendage (pedicel) 20-22 × 5-6 mm (exceptionally longer), cylindrical, slightly tapering towards the apex. Upper secondary appendage (cauda) lash-shaped, not covering the germ pore; lower cauda solid, filiform, ephemeral, arising from the pedicel apex and with three additional short, quite coiled appendages at the pedicel base, near the septum.

Notes: The only strain available for this species makes bigger spores (39 + 1 × 23 + 1 μm) and perithecia on M2 (diam. = 397 + 31 μm) than all the strains from the other species. It produces few perithecia on M2 and none on M4. It produces many perithecia on wood shavings. Its ITS sequence differs at one position from the *P. anserina* reference ITS: ITS1 – A insertion after nucl. n° 146.

Podospora pseudoanserina C. Boucher, TS Nguyen & P Silar ***sp. nov.*** Fig. 7G

Mycobank: MB821835.

Etymology: Refers to its relatedness to *P. anserina*.

Type: Central Africa. Isolated from dung of *Cobus defassa*, leg, *R. Cailleux* (PC0735075 **holotypus**; HAL 3209 F isotypus; ex-type culture: CBS 253.71).

Description: Perithecium diameter 200-400 mm, pyriform, membranous, semitransparent, pale brown, covered with numerous hyphoid hairs. Neck blackish, coriaceous, often with a tuft of dark, rigid, agglutinated hairs, sometimes with a few scattered hairs too. Asci 4-spored, clavate-lageniform. Spores spoon-shaped in the early stages. Mature spores obliquely uniseriate: spore head 34 + 1 × 19 + 1 mm,

ellipsoidal sometimes slightly asymmetrical, flattened at the base, smooth, thick-walled, with a central germ pore. Presence of a primary appendage (pedicel), 20-22 × 5-6 mm (exceptionally longer), cylindrical, slightly tapering towards the apex. Upper secondary appendage (cauda) lash-shaped, not covering the germ pore; lower cauda solid, filiform, ephemeral, arising from the pedicel apex and with three additional short, quite coiled appendages at the pedicel base, near the septum.

Notes: The two strains available for this species produce perithecia with very different size. Moreover, sizes differ on M2 and M4 but in opposite directions for both strains. Diagnostic feature of both strains appears to be the production of perithecia as a disk of 2 cm of diameter on M2. Their ITS sequence differs at one position from the *P. anserina* reference ITS: ITS2 – nucl. n° 144 C to A.

Podospora pseudocomata C. Boucher, TS Nguyen & P Silar, ***sp. nov.*** Fig. 7F

Mycobank: MB821833.

Etymology: refers to its relatedness to *P. comata*.

Type: PAKISTAN. Lahore, Jamrud-Landi Kotal Road, isolated from soil, leg. *S. Ahmed* (PC0735078 **holotypus**; HAL 3210 F isotypus; ex-type culture: CBS 415.72).

Description: Perithecium diameter 277 + 17 mm, pyriform, membranous, semitransparent, pale brown, covered with numerous hyphoid hairs. Neck blackish, coriaceous, often with a tuft of dark, rigid, agglutinated hairs, sometimes with a few scattered hairs too. Asci 4-spored, clavate-lageniform. Spores spoon-shaped in the early stages. Mature spores obliquely uniseriate: spore head 33 + 1 × 19 + 1 mm, ellipsoidal sometimes slightly asymmetrical, flattened at the base, smooth, thick-walled, with a central germ pore. Presence of a primary appendage (pedicel) 20-22 × 5-6 mm (exceptionally longer), cylindrical, slightly tapering towards the apex. Upper secondary appendage (cauda) lash-shaped, not covering the germ pore; lower cauda solid, filiform, ephemeral, arising from the pedicel apex and with three additional short, quite coiled appendages at the pedicel base, near the septum.

The only strain available for this species was isolated from soil. This strain undergoes senescence rapidly on M2 and produces abundant and large perithecia on M4. Its ITS sequence differs at one position from the *P. anserina* reference ITS: ITS2 – C insertion after nucl. n° 141. Point of insertion is the same as CC insertion in *P. comata*.

Podospora pseudopauciseta C. Boucher, TS Nguyen & P Silar, ***sp. nov.*** Fig. 7E

Mycobank: MB821834.

Etymology: refers to its relatedness to *P. pauciseta*.

Type: VENEZUELA. Edo Anzoategui, West of Barcelona, isolated from cow dung, July 1972, *J.C. Krug*. (PC0735080 **holotypus**; HAL 3211 F isotypus; ex-type culture: CBS 411.78)

Diagnosis: differs from other *Podospora* in its ability to produce perithecia ten times more voluminous on M4 than on M2. Its ITS sequence differs at two positions from the *P. anserina* reference ITS: ITS1 – nucl. n° 109 C to A and ITS2 – nucl. n° 144 C to A. Change in ITS2 – nucl. n° 144 C to A is also present in *P. pseudoanserina*.

Description: Perithecium diameter 372 + 18 mm, pyriform, membranous, semitransparent, pale brown, covered with numerous hyphoid hairs. Neck blackish, coriaceous, often with a tuft of dark, rigid, agglutinated hairs, sometimes with a few scattered hairs too. Asci 4-spored, clavate-lageniform. Spores spoon-shaped in the early stages. Mature spores obliquely uniseriate: spore head 32 + 1 × 19 + 1 mm,

ellipsoidal sometimes slightly asymmetrical, flattened at the base, smooth, thick-walled, with a central germ pore. Presence of a primary appendage (pedicel) 20-22 × 5-6 mm (exceptionally longer), cylindrical, slightly tapering towards the apex. Upper secondary appendage (cauda) lash-shaped, not covering the germ pore; lower cauda solid, filiform, ephemeral, arising from the pedicel apex and with three additional short, quite coiled appendages at the pedicel base, near the septum.

Notes: The only available strain for this species was isolated from dung from Venezuela. It produced perithecia all over the thallus on M2 in a manner similar to *P. pauciseta*. Diagnostic feature exhibited by the strain is its ability to produce perithecia ten times more voluminous on M4 than on M2. Its ITS sequence differs at two positions from the *P. anserina* reference ITS: ITS1 – nucl. n° 109 C to A and ITS2 – nucl. n° 144 C to A. Change in ITS2 – nucl. n° 144 C to A is also present in *P. pseudoanserina*.

Ascospore, perithecium size and setae are not able to differentiate species

Measure of ascospore sizes showed that only two strains statistically differed from all the others ($p < 5\%$). CBS 112042 had significantly larger ascospores (both in length and width) and strain T had significantly narrower ascospores (Table 1). However, the differences were small. All the other strains had ascospores of about the same size, including the two strains labelled as *P. comata* in the Westerdijk Institute Culture Collection (CBS 411.78 and CBS 237.71). Regarding the size of the perithecium, we observed variation in their diameter even for mature perithecia (Fig. 4A). Moreover, for some strains, perithecium diameter depended greatly upon the medium they were cultivated on. For example, strain CBS 411.78 produced perithecia nearly ten times more voluminous when fed with crystalline cellulose than when fed with dextrins (Fig. 4B). Interestingly, strains from *P. pseudoanserina* (group 6) behaved in opposite direction with respected to their perithecium size: CBS 124.78 produced larger perithecia on crystalline cellulose than on dextrin (413 + 48 vs 326 + 18), while CBS 253.71 produced smaller ones (185 + 32 vs 253 + 71). Note that neck size depends upon lighting, because perithecium necks orient towards light and perithecia grown under low light radiating from the side have longer necks than those illuminated from above with an intense light. Perithecium height cannot thus be used for differentiating strains. Consequently, Table 1 gives only the diameter of the largest mature perithecia when cultivated on M2 medium with 4g/L of dextrin as carbon source. While strain T and CBS 112042 had perithecia with the smallest and largest diameters, respectively, these were not statistically different from those of strains having similar sized perithecia (*e.g.*, strain N for small perithecia and strain CB3 for large ones).

Presence of setae at the neck is also often used to differentiate *P. comata* from *P. pauciseta/anserina*. However, analysis of setae in the various strains showed that they were not present on all perithecia (Fig. 4C). Moreover, the proportion of perithecia with setae as well as the length of the setae varied with the medium. For example for strain S, setae were short and not clearly visible on M2 medium in which dextrin was present at 4 g/L; they decorated 60% of the perithecia (n = 100). On M2 medium with 6 g/L, setae were more readily observed, but only in 25% of the perithecia (n = 100). On medium with wood shavings as carbon source, they were clearly observed and present in 55% of the perithecia (n = 100) (Fig. 4C) and on medium with crystalline cellulose, they were shorter and present in 30% of the perithecia (n = 100). Finally, on horse dung, a natural substrate of the fungus, they were easily seen and present in 60% of the perithecia (n = 100). Table 1 gives the

proportion of setae when the strains were grown on wood shaving medium, as setae were the most clearly counted on this medium (Fig. 4C). Percentage of setae was very low in CBS 237.71 (1%), identified as a *P. comata* strain in the Westerdijk Institute Culture Collection (now a *P. pauciseta* strain), but also in CB1 a newly-isolated strain of *P. anserina*. All the other strains had setae on more than 15% of their perithecia, typically in around 50% of them (Table 1). Note that variation in setae was previously reported (Griffiths 1901).

Overall, these data showed that the characters traditionally used to differentiate *P. comata* from *P. pauciseta/anserina* were not reliable in differentiating most species of the complex. Nevertheless, based on the small sizes of its ascospore and perithecia, strain T appears to be the closest to the one described by Milovtzova. At the other end, strain CBS 112042 has larger spore and perithecia than all the other strains. These morphological differences confirmed the fact that these two strains belong to two different species having specific intergenic and ITS sequences different from all the other strains.

Perithecium repartition pattern in different culture conditions can discriminate between species

While morphological attributes of ascospores and perithecia were often poor discriminators for the species, we notice that perithecium formation patterns delimitated the various species fairly well, especially when different carbon sources were assayed (Fig. 5). This confirmed that the seven species defined by sequence analysis were *bona fide* species. On the M2 minimal medium containing 4 g/L of dextrin as carbon source, all the strains of *P. anserina* formed a ring of small perithecia with an inner diameter of about 1 cm and a width of about 1 cm, with little strain-dependent variations. Some additional perithecia appeared later following a larger and more diffuse ring. On M4 that contained 5.5 g/L of crystalline cellulose, all *P. anserina* strains formed larger rings of small perithecia with inner diameters of about 2 cm and widths of about 2 cm. On wood shaving medium, they formed small perithecia on most of the wood shavings. None of the other strains had a similar pattern. On M2, strains of *P. pauciseta* formed perithecia on most of the thallus as did strains of *P. pseudopauciseta*. Yet strains of *P. pauciseta* also formed a ring on M4 similar to *P. anserina*, while *P. pseudopauciseta* produced fewer, but much enlarged perithecia (Fig. 4), at the center of the plate. On wood shavings both strains produced more perithecia than *P. anserina*. *P. comata* produced few small perithecia at the center of the plate on M2 containing 4 g/L of dextrin, while it was much more fertile on M2 with 6 g/L of dextrin (*i.e.*, it produced more and larger perithecia; data not shown). Differences on perithecium production between 4 g/L and 6 g/L of dextrin were not as pronounced in the other species as in *P. comata*. Fertility of *P. comata* on M4 was also poor, but important on wood shaving, where perithecia concentrated at the periphery of the plates. *P. bellae-mahoneyi* produced few perithecia at the center of M2 plates, none on M4 and a medium amount of large perithecia mostly at the periphery of the plate on wood shavings. *P. pseudocomata* differs in having many perithecia on M2 mostly along a ring similar to that of *P. anserina*. It formed large perithecia on M4 and smaller ones on wood shaving. The two strains of *P. pseudoanserina* both formed a disk of perithecia on M2, a more diffuse ring on M4 and numerous ones on wood shavings. However, because CBS 124.78 produced fewer but larger perithecia than CBS 253.71, especially on M4, the figures for these two strains appeared different.

Species from the complex can form microsclerotia and *cladorrhinum* like-anamorphs

All strains from the *P. anserina* species complex were able to produce microsclerotium-like structures (Fig. 6, Table 3) when cultivated with wood shavings as carbon source. These structures measured up to about 50 µm in diameter and started as an orange material deposited at discrete region of the mycelium. Upon aging the material turned blackish (Fig. 6).

One-celled uni-nucleated "conidia" have been reported for *P. anserina* as early as 1916 (Satina 1916). Because they do not germinate readily on all tested media, they were thought to serve only as male gametes during fertilization and thus renamed spermatia (Dodge 1936). They are carried by small peg-like hyphae called phialides (if one thinks that they are actual conidia) or spermogonia (if one sees them only as male gametes) that look like a typical *cladorrhinum* anamorph. Although accumulation of phialides/spermogonia into branched conidiophores has been described (Dodge 1936), the available descriptions never mention them as visible to the naked eye. Interestingly, at low temperature (18°C) in the dark on M2 overlaid with cellophane, most strains from the *P. anserina* species complex were able to form anamorphic structures visible with the naked eye (Fig. 6, Table 3). However, production of these structures was rather unreliable even when the strains were tested in standardized conditions, making it a poor discriminating criterion to characterize the species. Table 3 lists the species for which we were able to see clearly some anamorphic structures. Lack of detection in our experiments does not preclude the formation of *Cladorrhinum*-like anamorphs by the strains when additional experiments will be carried out. These were composed of numerous branched hyphae carrying phialides interspersed with long sterile hyphae, as described for *Bahupaathra samala* (Subramanian & Lodha 1964), syn. *Cladorrhinum samala* (Mouchacca & Gams 1993). The phialides thus observed carry several

Table 3. Main phenotypic features of the various species of the *Podospora anserina* species complex

	P. anserina	*P. pauciseta*	*P. comata*	*P. bellae-mahoneyi*	*P. pseudo-anserina*	*P. pseudo-comata*	*P. pseudo-paucistea*
***Cladorrhinum*-like anamorph**	+[1]	CBS 333.63:– CBS 237.71:+ CBS 451.62:–	+	+	–	+	+
Microsclerotia	yes	yes	yes	yes	yes	yes	yes
Appressorium-like	yes	yes	yes	yes	yes	yes	yes
Hyphal Interference (oxidative burst)	++	CBS 333.63:++ CBS 237.71:++ CBS 451.62:–	–	+	CBS 253.71: + CBS 124.78: ++	++	+
Hyphal Interference (killing)	efficient	inefficient	inefficient	inefficient	inefficient	inefficient	inefficient
Crippled Growth	yes	no	no	no	no	no	no
Other sector-like phenomenon	no	no	yes	no	CBS 253.71: yes CBS 124.78: no	no	yes
Senescence	yes	yes	yes	yes	yes	yes	yes

[1] *Cladorrhinum*-like anamorphs were detected for all strains except for Psjc14.

dozens of uninucleated cells of 3 to 5 µm in some mucilage, tentatively suggesting that they are actual conidia used for dispersal. Low level of germination of spermatia in the range of one out of 10^3 to 10^2 has been described to occur on medium with sorbose and yeast extract (Esser & Prillinger 1972). Attempts to reproduce these data with typical spermatia obtained on M2 medium or with the uninucleated cells from the *Cladorrhinum*-like morph have so far failed; only about one out of 10^6 typically germinates. Media tested for their germination included sorbose + yeast extract, M2, potato dextrose, V8 and malt + yeast extract media. A 30 minutes heat shock at 65°C does not improve germination rate. The low percentage of germination of these cells may be due to the fact that we have yet to find the proper trigger.

Additional features common to all species and features unique to some species

Strain S (Big S) of *P. anserina* is known to exhibit several biological features that can easily be evaluated. We thus assayed whether these were conserved in the other strains. Firstly, strain S is able to form appressorium-like structures to penetrate cellophane (Brun *et al.* 2009). All the strains investigated here were also able to form these structures in a few days (Table 3). It is known that their production is controlled by the same pathways as the ones involved in germination of the ascospores (Lambou *et al.* 2008). Accordingly, all the strains had the same pattern for ascospore germination. Their ascospores germinated with nearly 100% efficiency on G medium and sterile dung, but failed to germinated with good efficiency on M2 medium (*i.e.*, typical less than 1% germination was observed), unless a heat shock (65°C for 30 min.) was applied in which case germination occurred for about 80% of the ascospores. As previously described (Geydan *et al.* 2012; Marcou 1961), all strains of all species underwent senescence (Table 3).

Strain S is able to present a hyphal interference defense mechanism when encountering other filamentous fungi (Silar 2005). This phenomenon is associated with an oxidative burst at the contact point with the contestant and may result in the hyphal death of the contestant upon contact with the S strain hyphae. Both the burst and the cell death can be assayed by colorimetric tests and are not correlated (*i.e.*, mutant strains with the highest burst may not be the best killers (Silar 2005). We evaluated whether the other strains exhibited such phenomenon when confronted with *Penicillium chrysogenum*, a species readily killed by the S strain (Table 3). All *P. anserina* strains were efficient at generating a burst and good at killing *P. chrysogenum*. On the contrary, strains from the other species were not efficient at killing, although some of them generated an important oxidative burst (Table 3).

P. anserina strain S is known to produce sectors of "Crippled Growth" when cultivated on M2 medium supplemented with 5g/L of yeast extract (M2 + YE; Haedens *et al.* 2005; Silar *et al.* 1999). Crippled Growth can readily be assayed by inoculating explants taken from stationary phase culture onto fresh M2 + YE medium; thalli initiated from such explants exhibit large sectors of Crippled Growth (Haedens *et al.* 2005; Silar *et al.* 1999). All strains of *P. anserina* presented this cell degeneration when explants were taken from stationary phase (Table 3). On the contrary, none of the strains for the six other species were able to undergo Crippled Growth (Table 3), indicating that this cell degeneration is specific to *P. anserina*. However, we noticed that strains T, CBS 411.78 and CBS 253.71 (visible for this strain on the M2 plates of Fig. 5) also produced sectors of abnormal growth,

although of a different morphology than Crippled Growth and appearing with different modalities since they were not induced by passage into stationary phase. These sectors had thus a genetic or epigenetic basis that was different from that of Crippled Growth.

CONCLUSION

Overall, the data showed that strains identified as *P. pauciseta/P. anserina* or *P. comata* belong to a species complex that contains at least seven *bona fide* species sharing many features. All these species are pseudo-homothallics that produce similarly-shaped perithecia, asci and ascospores after seven days of incubation on M2 medium or dung, and ten days on M4 and wood shavings media. Descriptions for the morphology of these structures are available in (Lundqvist 1972) under the name *P. pauciseta* and in (Doveri 2004) under the name *P. anserina*. They are illustrated for the seven species in Fig. 7. All strains produce on M2 a mycelium growing at about 7 mm/d and that accumulates a green pigment at the center of the thallus. All strains senesce after 10 to 20 cm of growth on M2. Modalities of ascospore germination are conserved for all strains. They can however be distinguished by the sequence of their Rchr3, Rchr4 and Rchr6 regions as well as their ITS, some mycelium characteristics (Table 3) and their perithecium formation patterns on various media (Fig. 5). Two species also differ from all the others by the size of their ascospores and fruiting bodies. We thus redefine three species and propose four new ones (Figs 2, 3, 4, 7 and in Table 1). We propose to retain *P. pauciseta* for two strains known under this name in the Westerdijk Institute Culture Collection: CBS 451.62 and CBS 333.63, as well as for CBS 237.71 that is referenced as a *P. comata* in the Westerdijk Institute Culture Collection. We attribute the name *P. anserina* for the strains that has been used in most studies under this name, including strain S used for genome sequencing. We propose to keep *P. comata* for strain T of the G. Rizet collection, as this strain seems to be the closest to the one described by Milovtzova, based on its ascospore and perithecium size. Moreover, most sequences for *P. comata* present in Genbank, including the ITS region, were obtained from this strain. Additionally, strain T has been used under the name of *P. comata* in a few scientific publications (*e.g.*, (Belcour *et al.* 1997; Deleu *et al.* 1993; Koll *et al.* 1996). New species are *P. bellae-mahoneyi* (CBS 110242), *P. pseudoanserina* (CBS 124.78 and CBS 253.71), *P. pseudocomata* (CBS 411.78) and *P. pseudopauciseta* (CBS 415.72).

Podospora pauciseta/anserina is a widespread and frequent coprophilous fungus that has been reported from all continents and climates. Like its *Sordariales* relatives in the genera *Neurospora* (*e.g.*, *N. crassa*) and *Sordaria* (*e.g.*, *S. macrospora* and *S. fimicola*), it has been used for over a century in many genetical and biochemical studies thanks to the ease in obtaining its complete lifecycle under artificial conditions. Yet, little is known about its genetic diversity. Importantly, *P. comata*, a second closely related species has been described and several strains have been deposited under this name in culture collections. Phenotypic analyses of *P. comata*, *P. anserina* and *P. pauciseta* strains showed that the characters traditionally used to differentiate these species, *i.e.*, ascospore and perithecium sizes as well as presence of erect setae at the base of the neck, were often not reliable. Importantly, molecular analyses of four regions of the genome located on three different chromosomes

showed that seven species could be recognized. Further phenotypic analyses confirmed the molecular data and were able to differentiate the seven species mostly based on their growth and fertility behavior on various media differing by their carbon sources. Finally, fertility of interspecific crosses was very low. Key diagnostic feature is the sequence of the ITS region, which is specific for each species. Regrettably, strains deposited under different names in the culture collection may belong to the same species and strains deposited under the same name may belong to different ones (Table 1).

Although specimens of the morpho-species have been described from all continents, our results tentatively suggest that the seven *bona fide* species may present some geographical structure (Table 3, Fig. 3). Indeed, all strains of *P. anserina* now redefined have been obtained from Western Europe with one exception from Ontario. Another strain (CBS 455.64) likely belonging to this species (as deduced from its ITS sequence), present in the Westerdijk Institute Culture Collection but not analyzed here, has been isolated from Switzerland. All the strains from the other species come from different geographic locations, except strain T which has also been found in France. Unfortunately, the sample size from the six other species is too small to draw any definitive conclusions. Moreover, transport of domesticated animals by human to various regions of the world as well as natural migration may obscure geographical structure. Only analyses of large samples should be able to decide on the matter. However, it is noteworthy that the different species show different abilities to exploit various carbon sources. This may be related to the differences in the floras and faunas present in the different regions of the world. It is thus possible that each species is better suited for particular dung produced in relation to the major floras and faunas present in the different parts of the world.

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REFERENCES

BELCOUR L., ROSSIGNOL M., KOLL F., SELLEM C. H. & OLDANI C., 1997 — Plasticity of the mitochondrial genome in *Podospora*. Polymorphism for 15 optional sequences: group-I, group-II introns, intronic ORFs and an intergenic region. *Current Genetics* 31: 308-317.

BERNET J., 1965 — Mode d'action des gènes de "barrage" et relation entre l'incompatiblité cellulaire et l'incompatibilité sexuelle chez *Podospora anserina*. *Annales des Sciences Naturelles, Botaniques Paris* 6: 611-768.

BRUN S., MALAGNAC F., BIDARD F., LALUCQUE H. & SILAR P., 2009 — Functions and regulation of the Nox family in the filamentous fungus *Podospora anserina*: a new role in cellulose degradation. *Molecular Microbiology* 74: 480-496.

CAI L., JEEWON R. & HYDE K. D., 2006 — Molecular systematics of *Zopfiella* and allied genera: evidence from multi-gene sequence analyses. *Mycological Research* 110: 359-368.

DEBETS A. J. M., DALSTRA H. J. P., SLAKHORST M., KOOPMANSCHAP B., HOEKSTRA R. F. & SAUPE S. J., 2012 — High natural prevalence of a fungal prion. *Proceedings of the National Academy of Sciences U.S.A.* 109: 10432-10437.

DELEU C., CLAVÉ C. & BÉGUERET J., 1993 — A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*. *Genetics* 135: 45-52.

DODGE B. O., 1936 — Spermatia and nuclear migration in *Pleurage anserina*. *Mycologia* 28: 284-291.

DOVERI F., 2004 — *Fungi Fimicoli Italici*. Associazione Micologia Bresadola, Trento.

ESPAGNE E., LESPINET O., MALAGNAC F., DA SILVA C., JAILLON O., PORCEL B. M., COULOUX A., AURY J. M., SEGURENS B., POULAIN J., ANTHOUARD V., GROSSETETE S., KHALILI H., COPPIN E., DEQUARD-CHABLAT M., PICARD M.,

CONTAMINE V., ARNAISE S., BOURDAIS A., BERTEAUX-LECELLIER V., GAUTHERET D., DE VRIES R. P., BATTAGLIA E., COUTINHO P. M., DANCHIN E. G., HENRISSAT B., KHOURY R. E., SAINSARD-CHANET A., BOIVIN A., PINAN-LUCARRE B., SELLEM C. H., DEBUCHY R., WINCKER P., WEISSENBACH J. & SILAR P., 2008 — The genome sequence of the model ascomycete fungus *Podospora anserina*. *Genome Biology* 9: R77.

ESSER K., 1956 — Gen-Mutaten von *Podospora anserina* (Ces.) Rehm mit männlicher Verhalten. *Naturwissenschaften* 43: 284.

ESSER K. & PRILLINGER H., 1972 — A new technique to use spermatia for the production of mutants in *Podospora*. *Mutation Research* 16: 417-419.

GEYDAN T. D., DEBETS A. J., VERKLEY G. J. & VAN DIEPENINGEN A. D., 2012 — Correlated evolution of senescence and ephemeral substrate use in the *Sordariomycetes*. *Molecular Ecology* 21: 2816-2828.

GRIFFITHS D., 1901 — North american *Sordariaceae*., pp. 59-61, 127 – Pl. 125, Figs 124-126 in *Memoirs of the Torrey Botanical Club*.

GROGNET P., BIDARD F., KUCHLY C., TONG L. C., COPPIN E., BENKHALI J. A., COULOUX A., WINCKER P., DEBUCHY R. & SILAR P., 2014 — Maintaining Two Mating Types: Structure of the Mating Type Locus and Its Role in Heterokaryosis in *Podospora anserina*. *Genetics* 197: 421-432.

HAEDENS V., MALAGNAC F. & SILAR P., 2005 — Genetic control of an epigenetic cell degeneration syndrome in *Podospora anserina*. *Fungal Genetics & Biology* 42: 564-577.

HODGKISS I. J. & HARVEY R., 1971 — Factors affecting fruiting of *Pleurage anserina* in culture. *Transactions of the British Mycological Society* 57: 533-536.

KOLL F., BOULAY J., BELCOUR L. & D'AUBENTON-CARAFA Y., 1996 — Contribution of ultra-short invasive elements to the evolution of the mitochondrial genome in the genus *Podospora*. *Nucleic Acids Research* 24: 1734-1741.

KRUG J. C. & KHAN R. S., 1989 — New records and new species of *Podospora* from East Africa. *Canadian Journal of Botany* 67: 1174-1182.

KRUYS A., HUHNDORF S. M. & MILLER A. N., 2015 — Coprophilous contributions to the phylogeny of *Lasiosphaeriaceae* and allied taxa within *Sordariales* (*Ascomycota*, *Fungi*). *Fungal Diversity* 70: 101-113.

LAMBOU K., MALAGNAC F., BARBISAN C., THARREAU D., LEBRUN M. H. & SILAR P., 2008 — The crucial role during ascospore germination of the Pls1 tetraspanin in *Podospora anserina* provides an example of the convergent evolution of morphogenetic processes in fungal plant pathogens and saprobes. *Eukaryotic Cell* 7: 1809-1818.

LECELLIER G. & SILAR P., 1994 — Rapid methods for nucleic acids extraction from Petri dish grown mycelia. Current Genetics 25: 122-123.

LUNDQVIST N., 1972 — 5. *Podospora pauciseta*, pp. 147-152 in *Nordic Sordariaceae S. Lat. Acta Universitatis Upsaliensis*, Uppsala.

MADRID H., CANO J., GENÉ J. & GUARRO J., 2011 — Two new species of *Cladorrhinum*. *Mycologia* 103: 795-805.

MARCOU D., 1961 — Notion de longévité et nature cytoplasmique du déterminant de la sénescence chez quelques champignons. *Annales des Sciences Naturelles, Botaniques Paris Sér*. 12, 2: 653-764.

MATASYOH J. C., DITTRICH B., SCHUEFFLER A. & LAATSCH H., 2011 — Larvicidal activity of metabolites from the endophytic *Podospora sp.* against the malaria vector *Anopheles gambiae*. *Parasitology Research* 108: 561-566.

MILLER A. N. & HUHNDORF S. M., 2005 — Multi-gene phylogenies indicate ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology in the *Sordariales* (*Ascomycota*, *Fungi*). *Molecular Phylogenetics and Evolution* 35: 60-75.

MILOVTZOVA M., 1937 — Data on the microflora of Ukraine (in Ukrainian). *Travaux de l'Institut de Botanique, Kharkov* 2: 17-22.

MIRZA J. H. & CAIN R. F., 1969 — Revision of the genus *Podospora*. *Canadian Journal of Botany* 47: 1999-2048.

MOREAU F. & MOREAU M., 1951 — Observations cytologiques sur les Ascomycètes du genre *Pleurage*. *Revue de Mycologie* 16: 198.

MOUCHACCA J. & GAMS W., 1993 — The hyphomycete genus *Cladorrhinum* and its teleomorph connections. *Mycotaxon* 48: 415-440.

NIESSL, 1883 — Ueber die Theilung der Gattung *Sordaria*. *Hedwigia* 22: 153-156.

RABENHORST L., 1857 — Erklärung der Taf. XV. *Hedwigia* 1: 116 – pl. 115 fig.114.

RIZET G., 1939 — De l'hérédité du caractère absence de pigment dans le mycélium d'un ascomycète du genre *Podospora*. *Comptes-Rendus de l'Académie des Sciences, Paris* 209: 771-774.
RIZET G. & ENGELMANN C., 1949 — Contribution à l'étude génétique d'un Ascomycète tétrasporé : *Podospora anserina* (Ces.) Rehm. *Revue de Cytologie et Biologie Végétale* 11: 201-304.
SATINA S., 1916 — Studies in the development of certain species of the *Sordariaceae*. *Bulletin de la Société impériale des naturalistes de Moscou, nouvelle série.* 30: 106-142 Pl. 101-102.
SILAR P., 2005 — Peroxide accumulation and cell death in filamentous fungi induced by contact with a contestant. *Mycolological Research* 109: 137-149.
SILAR P., 2013 — *Podospora anserina*: from laboratory to biotechnology, pp. 283-309 in *Genomics of Soil – and Plant-Associated Fungi*, edited by Benjamin A. Horwitz, P. K. M., Mala Mukherjee, Christian P. Kubicek. Springer, Heidelberg New York Dordrecht London.
SILAR P., HAEDENS V., ROSSIGNOL M. & LALUCQUE H., 1999 — Propagation of a novel cytoplasmic, infectious and deleterious determinant is controlled by translational accuracy in *Podospora anserina*. *Genetics* 151: 87-95.
SUBRAMANIAN C. V. & LODHA B. C., 1964 — Four new coprophilous hyphomycetes. *Antonie van Leeuwenhoek* 30: 317-330.
TANGTHIRASUNUN N., NAVARRO D., GARAJOVA S., CHEVRET D., CHAN HO TONG L., GAUTIER V., HYDE K. D., SILAR P. & BERRIN J. G., 2016 — Inactivation of *Podospora anserina* cellobiose dehydrogenases modifies its cellulose degradation mechanism. *Applied & Environmental Microbiology* 83: e02716-02716.
TRAVERSO J. B., 1907 — *Flora Italica Cryptogama Pars I: Fungi Pyrenomycetae*.
WHITE T. J., LEE S. & TAYLOR J., 1990 — Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315-322 in *PCR Protocols: a Guide to Methods and Applications*, edited by Innis, M., D. Gelfand, J. Sninsky & T. White. Academic Press, New York.
WINTER G., 1887 — CLXI. *Podospora*, pp. 169-177 in *Dr. L. Rabenhorst's Kyrogamen-Flora von Deutschland, Oesterreich und der Schweiz Zweite auflage*.
WOLF F. A., 1912 — Spore formation in *Podospora anserina* (*Rahb.) Winter. Annals of Mycology* 10: 60-64.