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Fungi attacking springtails (Sminthuridae, Collembola) with a description of *Pandora batallata*, sp. nov. (Entomophthoraceae)

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Fungi attacking springtails, probably *Dicyrtomina* sp. (Collembola, Sminthuridae), were collected in north-western Germany. Cadavers were fixed with rhizoids onto moist pieces of dead wood lying on the soil. Fifteen specimens were carefully dissected and microscopically and genetically examined. The core data of *Pandora batallata* are as follows: The hyphal bodies measured 61.7–65.0 × 18.5 µm and contained on average 10-11 nuclei. The branched conidiophores produced mononucleate primary conidia with an average size of 20.8–21.7 × 8.9–9.6 µm. The spiny brown, usually spherical zygospores measured on average 39.6 µm excluding the spines. The long and strong cystidia had an enlarged rounded ending, which is represented by the epithet. *Pandora batallata* sp. nov. differs from related species by host, morphology (long cystidia with the spoon-like ending) and sequence differences in the LSU rDNA. Two other fungi were also found. A single dead springtail was completely filled with spherical fungal structures (diameter 9–11 µm) and a single adhering hypha. Two other dead springtails were filled with spherical structures resembling entomophthoralean resting spores, although their diameter was only 10–12 µm. Both of these fungi were not further examined but they are also considered as pathogenic to springtails.

Keywords: Insect pathogenic fungi, morphology, taxonomy, new species, LSU rDNA.

Collembolans are tiny free living apterygote insects. In spite of their ecological importance, they are not in the focus of most entomologists. Therefore, the detection of fungus infected specimens is uncommon. After a first examination of our specimens, it was clear that most were attacked by a species of Entomophthorales.

The fungus order Entomophthorales originally contained three families with arthropod-pathogenic species, the Ancylistaceae, the Entomophthoraceae and the Neozygitaceae. Later, the Neozygitaceae were placed in a separate order, the Neozygitales, based on genetic data (Humber 2012). Entomophthoraceae and Neozygitaceae contain exclusively arthropod-pathogenic species (Humber 1989, Keller & Petrini 2005).

The Entomophthoraceae consist of three subfamilies, the Entomophthoroidea (four genera including *Entomophthora*), the Erynioideae comprising six genera including *Pandora* being the most diverse and the Massosporoideae (Keller & Petrini 2005). The former two differ mainly by the conidiophores and the number of nuclei in the conidia. The Entomophthoroideae have unbranched conidiophores and multinucleate conidia, the Erynioideae have branched conidiophores and uninucleate conidia. The family Neozygitaceae consists of two genera, *Neozygites* and *Apterivorax* (Keller & Petrini 2005). The use of genetic data confirmed the correctness of this morphological classification with the exception that the genus *Massospora* became included in the subfamily Entomophthoroideae (Humber 2012, Gryganskyi et al. 2013).

There are only a few species of entomophthoroid fungi known to attack springtails, namely *Conidiobolus coronatus* and *Apterivorax sminthuri* (Steenberg et al. 1996, Keller & Steenberg 1997) and *C.* cf. *adiaeretus* (Tkaczuk et al. 2011). In this paper we describe a new species of Entomophthorales attacking a species of Dicyrtomidae, probably *Dicyrtomina* sp. Morphological as well as genetic data are presented.

Material and methods

Numerous infected springtails, probably *Dicyrtomina* sp. (Collembola, Dicyrtomidae), were col-

lected at four different locations in a forest belonging to the recreation area Hohenstein near Witten, Nordrhein-Westfalen, Germany. The collection site 1 is a floodplain forest with mainly poplars (*Populus* sp.) and alder (*Alnus* sp.) along a brook and defined by the coordinates 51.43369 N and 7.35682 E and an altitude of 118 m (Fig. 1a). The collection site 2 is a dry forest composed of an area of spruce (*Picea abies*) and an area of oak (*Quercus* sp.) and beech (*Fagus sylvatica*) and defined by the coordinates 51.43067 N and 7.36118 E and an altitude of 149 m (Fig. 1b). The collection site 3 is another floodplain forest with mainly poplars (*Populus* sp.) and defined by the coordinates 51.43093 N and 7.36360 E and an altitude of 132 m. The collection site 4 is a brook flanked with alder (*Alnus* sp.) and defined by the coordinates 51.43324 N and 7.35920 E and an altitude of 118 m. The cadavers were placed in 70 % ethanol immediately after the collection, therefore, no attempts were made to obtain secondary spores.

All fifteen cadavers collected on March 10, 2020, at site 1 were carefully dissected into tiny pieces. The fungal material was mounted in lactophenol-cotton blue (LPCB) or in lactophenol-aceto-orcein (LPAO) as described by Keller (1987). All measurements were based, if not otherwise stated, on 25 structures per individual host, except cystidia, designated as one series. From each structure, usually more than one series was studied to assess variation. The number of series is given after the range of the mean values, the range of the extreme values (in brackets) and the ratio length/diameter (L/D). The microphotographs were taken with a Leica MC170 HD camera mounted on a Leica DM 1000 LED microscope.

Genomic DNA was extracted from two infected springtails collected from site 3 and 4 using a DNeasy Plant Mini Kit (Qiagen, MD, USA) following the manufacturer's protocol. The infected springtails showed the same symptoms as those used for morphological studies. PCR assays were performed on the first part of the 28S gene (LSU) and the conditions were initial denaturation for 3 min at 95 °C, followed by 33 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C and a final extension for 7 min at 72 °C. The PCR reactions were carried out in 25 µL volumes, with 200 µM dNTP, 0.2 µM of each primer nu-LSU-0018-5 (Jensen & Eilenberg 2001) and primer LSU-0805 (Kjøller & Rosendahl 2000), 1× DreamTaq™ buffer including 2.5 mM $MgCl_2$, 1.25 unit of DreamTaq™ Polymerase (Thermo Scientific, MA, USA) and 2 µl of extracted DNA. Amplicon sizes were checked by electrophoresis on a 1.5 % agarose gel in 0.5× TBE, the products were visualized with EZ-Vision (AMRESCO, OH, USA), subsequently purified using the QIAquick purification kit (Qiagen, MD, USA) and sent to Macrogen (Seoul, Korea) for sequencing in both directions.

The two sequences were edited using BioEdit version 7.2.6.2 (Hall 1999). The sequence was then, together with 22 sequences of species in the Entomophthoraceae (in particular from the Erynioideae and two *Conidiobolus* species from Ancylistaceae) retrieved from GenBank (GenBank accession numbers are found in figure 4), aligned with the ClustalW tool and trimmed manually as needed. The evolutionary distances were computed using the Maximum Composite Likelihood method. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The evolutionary history was inferred using the Neighbor-Joining method including bootstrap analysis with 500 replicates, all within MEGA version 11 (Tamura et al. 2021).

Results

Infected springtails fixed to moist but not wet pieces of dead wood lying on the soil (Fig. 1a, b) were collected between early January and end of April at day-time temperatures between 6 and 13 °C. The cadavers were either on the bark or on barkless parts, sometimes also covered with leaves. Nine cadavers showed clear symptoms of an entomophthoralean infection and were used for a detailed examination. They were fixed to the substrate with rhizoids. At a sporulating stage, the large cystidia were already visible *in situ* (Fig. 1c–f).

The PCR on the DNA extractions from the two conidia producing cadavers from sites 3 and 4 each produced an amplicon of approximate 850 base pairs. The sequencing of each amplicon produced two identical sequences of 807 unambiguous base pairs. The DNA sequence was submitted to GenBank, accession number ON176196. The evolutionary history was inferred using the Neighbor-Joining of 23 sequences and a total of 801 positions in the final dataset were used. In the phylogenetic analysis the fungus infecting the springtails cluster within the family Entomophthoraceae together with the subfamily Erynioideae which is represented by the genera *Pandora*, *Furia*, *Erynia* and *Zoophthora* supported by a boottrap value of 99 %, but it did form a separate lineage supporting a new species (Fig. 4).

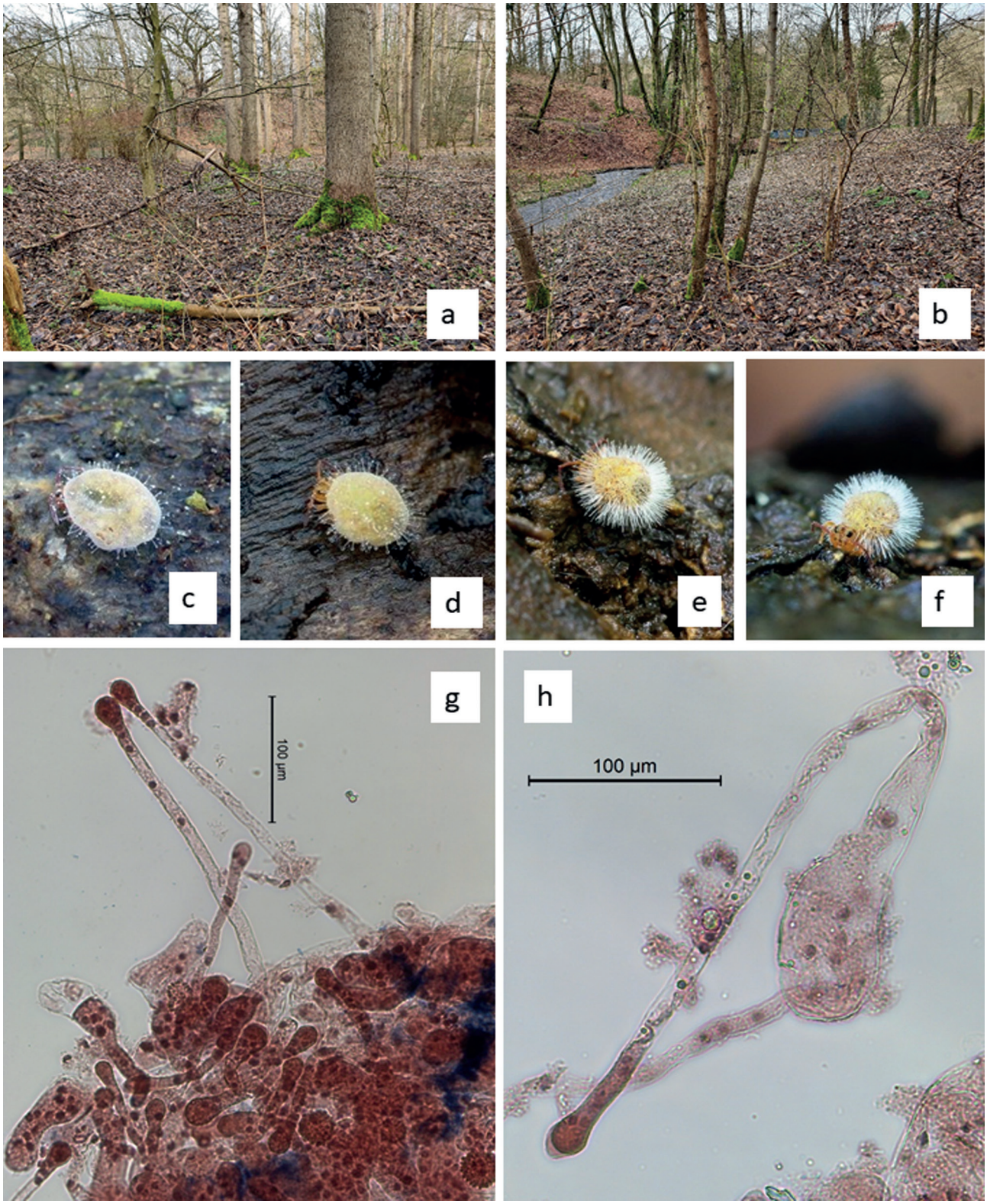

Fig. 1. **a.** Collection site 1. **b.** Collection site 2. **c–f.** Infected springtails at different stages of sporulation. Note the powerful cystidia in figures e and f (natural size of the springtails about 1.5–2 mm). **g.** Hyphal bodies and two fully developed cystidia. **h.** Single cystidium with large basal cell and enlarged ending.

***Pandora batallata* S. Keller, sp. nov.** – Figs. 1–3e.
MycoBank no.: MB 843636

Description. – Rhizoids monohyphal with a diameter ranging from 8–14 µm (n = 10). Endings enlarged, rounded, foot-like or with short root-like branches (Fig. 2a, b). – Hyphal bodies irregularly short rod-shaped, usually straight or slightly bent, 61.7–65 × 18.5 µm with a length/diameter ratio of 3.34–3.51 (2 series) (Fig. 3a) and containing 6–16 (average 10.6) nuclei (n = 25) with a diameter of 5.7–5.9 (5–7) µm (two series) (Tab. 1). – Conidiophores densely packed, hyaline and branched (Fig. 2c–e). – Primary conidia mononucleate, bitunicate, subcylindrical to slightly pyriform with rounded apex, papilla flat to slightly rounded, often asymmetrical (Fig. 2f), 20.8–21.7 (19–24) µm × 8.9–9.6 (8–11) µm with a length/dia-

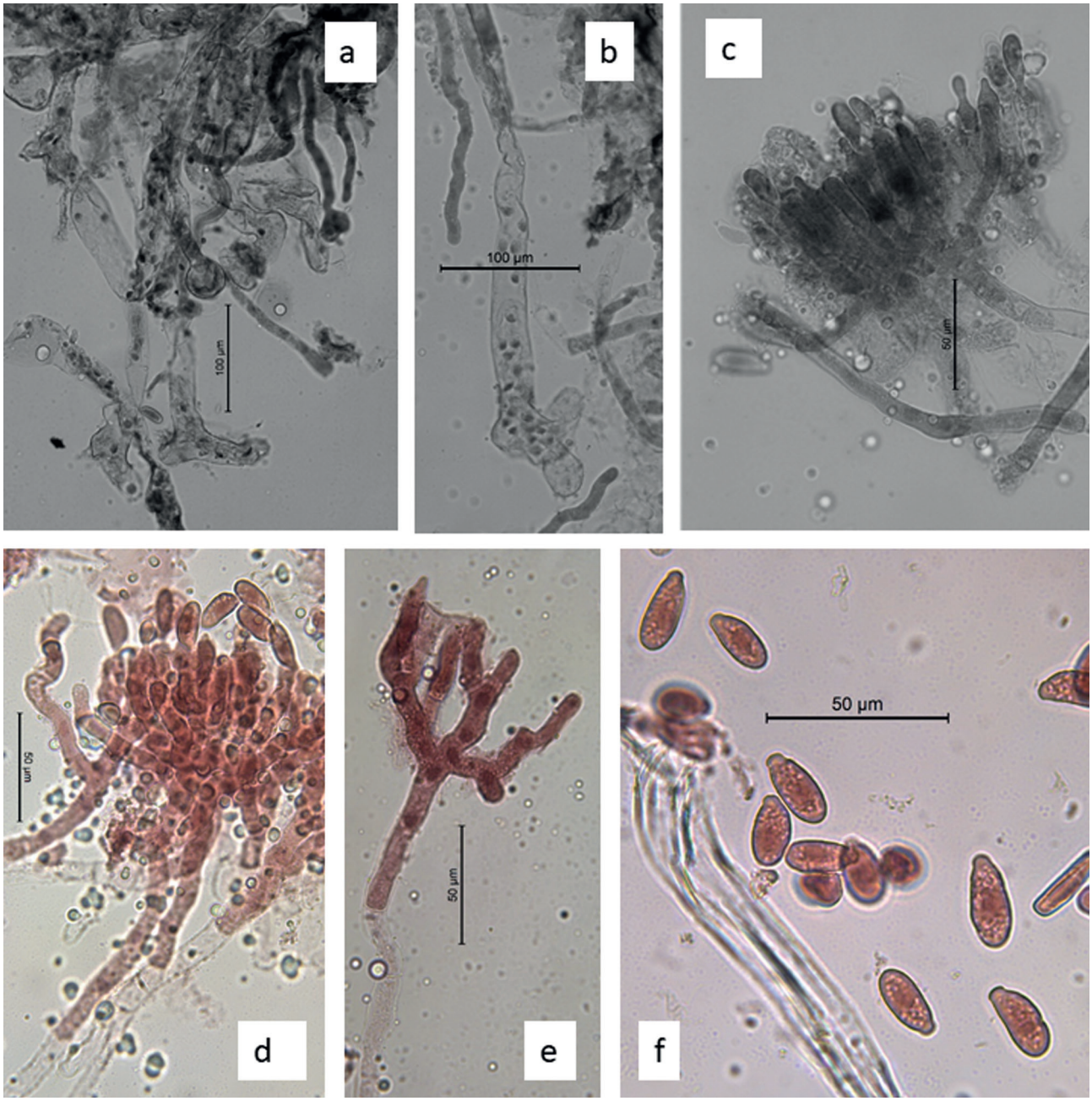

Fig. 2. a. Two rhizoids with foot-like endings. **b.** Rhizoid with branched ending and nuclei. **c.** Bundle of conidiophores. **d.** Conidiophores with developing conidia. **e.** Single branched conidiophore. **f.** Primary mononucleate conidia.

Tab. 1. Dimensions of the fungal structures in µm (PC = primary conidia, mRS = mature resting spores, Cyst. term. = diameter of the terminal part of cystidia, Cyst. glob. = diameter of the terminal globular enlargement of the cystidia, HB = hyphal bodies, Nuclei HB = diameter of nuclei in hyphal bodies, Nuclei cHB = diameter of nuclei in conjugating hyphal bodies, s.d. = standard deviation), based on 25 measurements each.

Structure and slide number	Length (L) (s.d.) min-max	Diameter (D), (s.d.), min-max	Ratio L/D	Stain
PC 33	20.8 (1.23) 19-24	9.2 (0.51) 8-9	2.27	LPCB
PC 34	21.0 (1.29) 19-24	9.2 (0.68) 8-11	2.28	LPAO
PC 35	21.0 (1.09) 20-24	8.9 (0.77) 8-11	2.36	LPCB
PC 40	21.7 (1.27) 19-24	9.6 (0.56) 8-11	2.25	LPCB
mRS 44		39.6 (2.44) 35-42		LPCB
Cyst. term.		13.3 (2.17) 11-18		LPCB
Cyst. glob.		21.3 (0.96) 17-31		LPCB
HB 37	61.7 (7.3) 52-80	18.5 (1.95) 15-22	3.34	LPAO
HB 38	65.0 (10.8) 42-90	18.5 (1.41) 15-22	3.51	LPCB
Nuclei HB 38		5.7 (0.32) 5-6		LPAO
Nuclei HB 39		5.9 (0.40) 5-7		LPAO
Nuclei cHB 41		5.9 (0.63) 5-7		LPAO

meter ratio of 2.25–2.36 (four series) (Tab. 1). – Secondary conidia rare, produced laterally of the primary ones and 14–18 × 9–11 µm (n = 4). – Cystidia powerful reaching lengths of more than 500 µm including the large base, usually towering 200–400 µm above the conidial layer and containing numerous nuclei; diameter decreasing from the base to the top to end with a typical spoon-like or globular enlargement (Figs. 1g, h, 3a); diameter of this enlargement 21.3 (17–31) µm, diameter before the enlargement 13.3 (11–18) µm (one series each) (Tab. 1). – Resting spores. Zygospores produced by conjugation of two equally looking hyphal bodies (Fig. 3b, c). At an early stage of zygospore formation, the hyphal bodies containing 6–16 nuclei with an average of 9.1–9.2 nuclei (two series). Nuclei of the same diameter as those in conidiogenous hyphal bodies (Tab. 1). Resting spores brown, spherical to slightly subspherical with a spiny episporium; spherical ones 39.6 (35–42) µm excluding the spines (one series, Tab. 1, Fig. 3d, e); spines 2–4 µm long.

Etymology. – The name *batallata* (Latin *„batallum”,* clapper of bell) refers to the clapper-shaped cystidia.

Distinguishing characters. – *Pandora batallata* can be distinguished unequivocally from all other species within the Entomophthoraceae by its host and by sequence differences in the LSU rDNA. The long cystidia with the spoon-like ending are another distinguishing character. Spiny resting spores are rare in the *Erynia*/*Furia*/*Pandora* group. They are present in comparable shape and dimension in *Erynia echinacea* (Ben-Ze'ev & Kenneth) Remaudière & Hennebert (1980) attacking aphids and in *Pandora echinospora* (Thaxter) Humber (1989) attacking flies.

Holotypus. – Deposited at the herbarium ZT, ZT Myc 58425.

Other fungi

Three dead springtails were infected by other fungi. The body of one of the springtails was filled with globular structures most of them with adhering hyphae. The globules had an average diameter of 9.7±0.57 (9–11) µm. Other structures were absent (Fig. 3f). The body of the two other springtails was completely filled with resting spore-like structures. They were spherical with a diameter of 11.2–11.5± 0.40–0.43 (10–12.5) µm (2 series). The content was granular, some spores had a dominant vacuole (Fig. 3g). The spore wall was about 1 µm thick.

Discussion

Reports of fungi as pathogens of collembolans are extremely rare. Steenberg et al. (1996) mentioned *Conidiobolus coronatus* and another entomophthoralean fungus attacking the lucerne flea *Sminthuri viridis* in Denmark, the latter was subsequently described by Keller & Steenberg (1997) as *Neozygites sminthuri* (now placed in the genus *Apterivorax*, Neozygitaceae). This species was recently reported from Norway as pathogen of a species of the family Isotomidae (Andreasen et al. 2021). In a

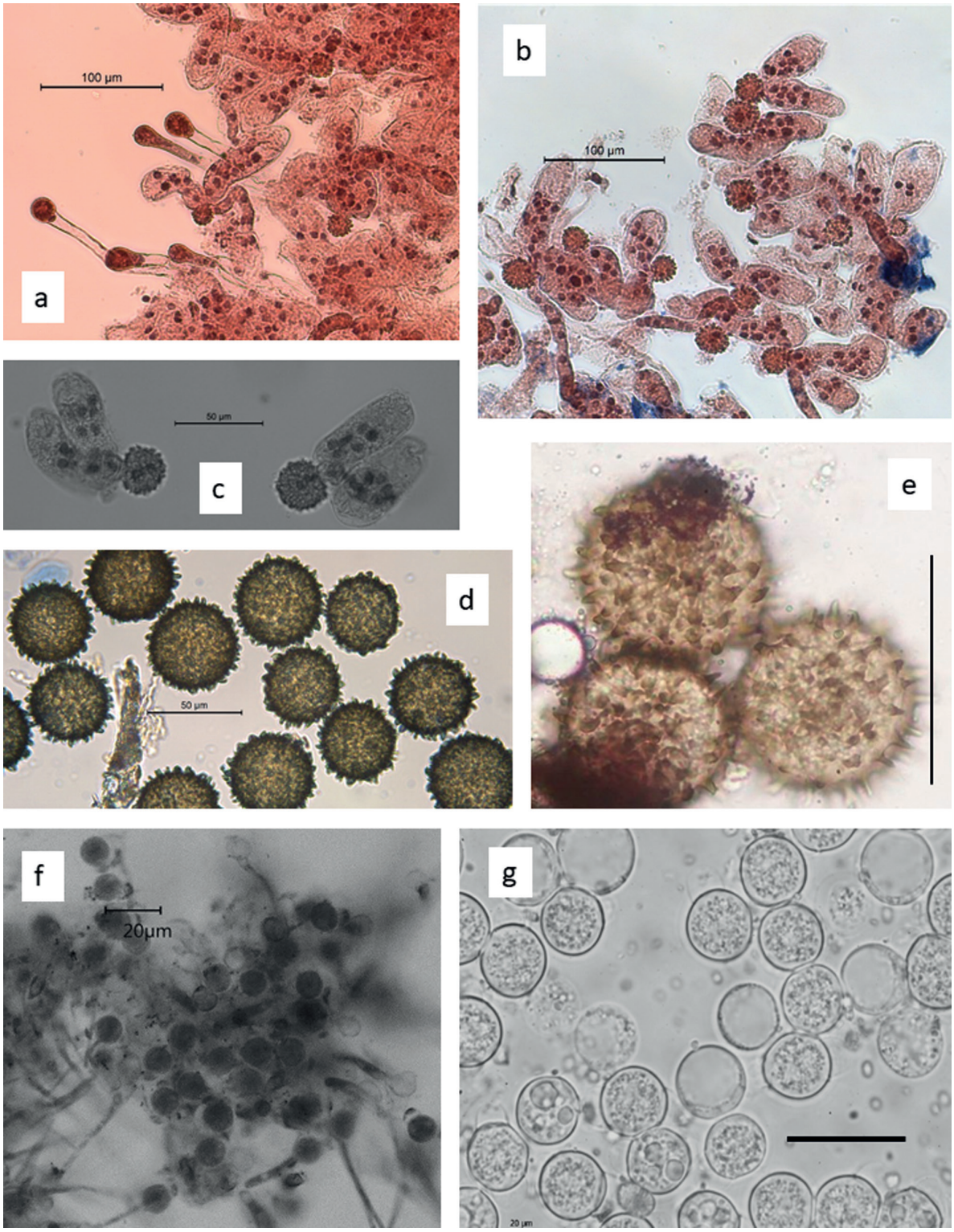

Fig. 3. a. Resting spore forming hyphal bodies together with the endings of five cystidia. **b.** Early stages of zygospore formation with nuclei. **c.** Detail of zygospore formation showing the two conjugating hyphal bodies with the young zygospores, which contains two nuclei at this stage of development. **d.** Mature zygospores. **e.** Detail of zygospores showing the spiny episporium (bar represents 50 µm). **f.** Unidentified fungus showing globular structures with adhering hyphae. **g.** *Tarichium*-like fungus found in two dead springtails (bar represents 20 µm).

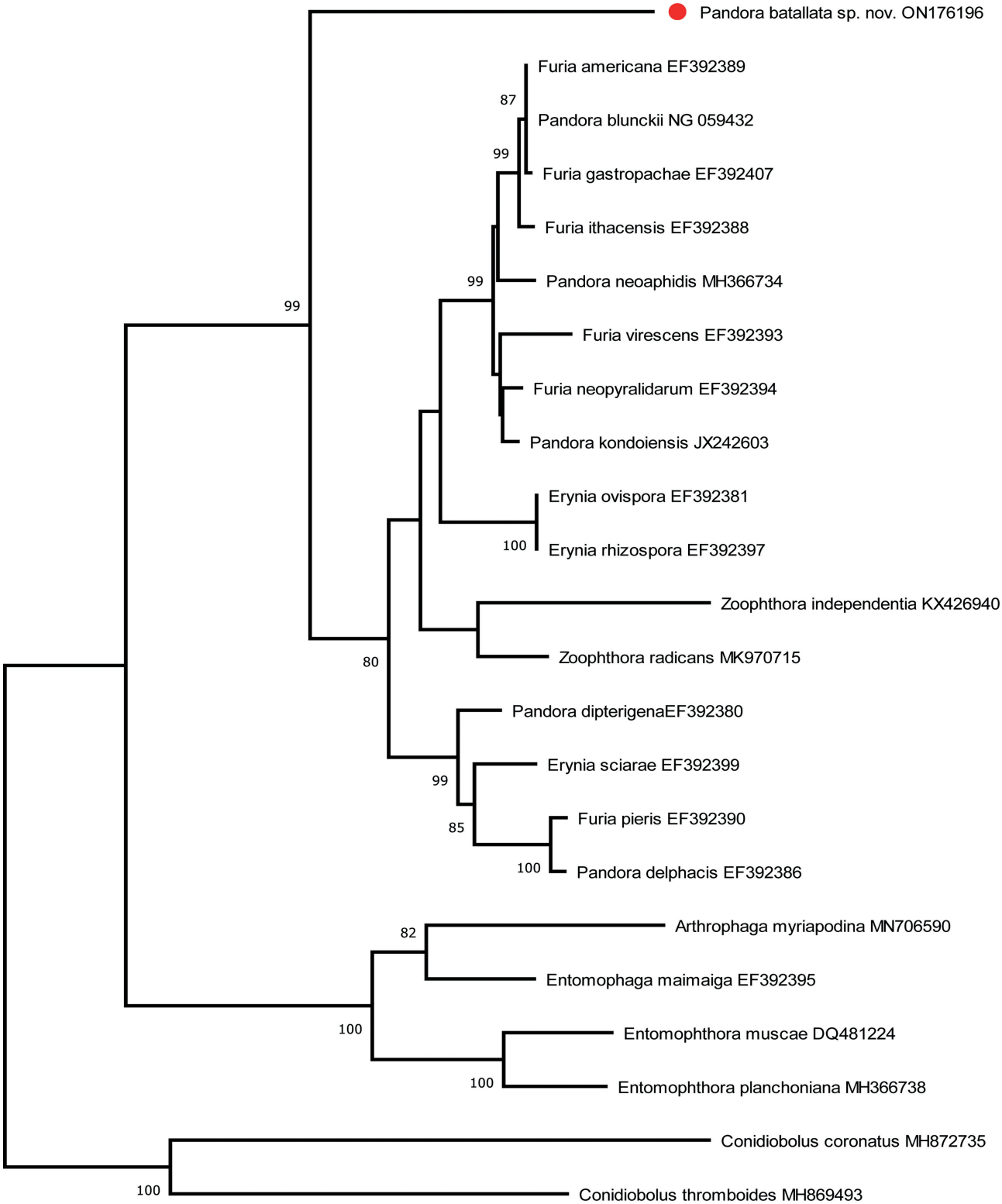

Fig. 4. *Pandora batallata*, sp. nov., (red dot) clustering within the Entomophthoraceae and within the subfamiliy Erynioideae. Evolutionary history inferred using the Neighbor-Joining method, bootstrap values above 80 % written next to the branches. GenBank number of the sequences given for each species included. *Conidiobolus thromboides* transfered to *Neoconidiobolus* (Nie et al. 2020).

large study on fungal pathogens, Dromph et al. (2001) found the same species together with three related but not formally described species. They all caused only little mortality. During studies on the diversity of arthropod-pathogenic fungi in Austria and Poland an unidentified Collembola was found in Austria infected with *Conidiobolus* cf. *adiaeretus* (Tkaczuk et al. 2011). Further, Bałazy (1993) reported *Podura* sp. floating on the water surface infected with *Zoophthora (Erynia) curvispora,* a finding which we consider as doubtful.

We consider all three fungi currently found as pathogens of springtails. These findings indicate that more fungi pathogenic to these insects can be expected as demonstrated by Dromph et al. (2001). *Pandora batallata* is the first reported species within the family Entomophthoraceae to attack a Collembola. We have placed the species in the genus *Pandora* since it shows a good accordance with the morphological features of this genus. However, some doubts about this placement are justified based on the results of the LSU sequence. *Pandora batallata* did cluster together with the Erynioideae including the *Pandora/Erynia/Furia* and the *Zoophthora* clusters but as a basal branch (Fig. 4). However, the taxonomic resolution of the genera within Erynioideae is still contradictory (Gryganskyi et al. 2012). In-depth studies including multigene or genome analysis might shed light over the most important morphological and biopathological taxonomic characters within Erynioideae and may demonstrate if the placement in *Pandora* is correct or if a new genus should be erected for this species.

It is possible that the resting spore like structures observed in two other springtails (Fig. 3g) also belong to the Entomophthorales. They resemble some species of *Tarichium* described from mites by Bałazy (1993). These species have resting spores distinctly smaller than any other resting spores of classified Entomophthorales. DNA sequencing of the resting spores might in the future elucidate if there is a link between the currently described species or other entomophthoralean species (Scorsetti et al. 2012, Thomsen & Jensen 2002), but for now we leave it open whether the resting spores are correctly placed in the family Entomophthoraceae or should be placed in another taxon.

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