



Fungal remains from late Neogene deposits at the Gray Fossil Site, Tennessee, USA

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Abstract

Interesting fungal remains were encountered during palynological investigation of the Neogene deposits at the Gray Fossil Site, Washington County, Tennessee, USA. Both *Cephalothecoidomyces neogenicus* and *Trichothyrites* cf. *padappakarensis* are new for the Neogene of North America, while remains of cephalothecoid fungus *Cephalothecoidomyces neogenicus* G. Worobiec, Neumann & E. Worobiec, fragments of mantle tissue of mycorrhizal *Cenococcum* and sporocarp of epiphyllous *Trichothyrites* cf. *padappakarensis* (Jain & Gupta) Kalgutkar & Jansonius were reported. Remains of mantle tissue of *Cenococcum* for the fossil state are reported for the first time. The presence of *Cephalothecoidomyces*, *Trichothyrites*, and other fungal remains previously reported from the Gray Fossil Site suggest warm and humid palaeoclimatic conditions in the southeast USA during the late Neogene, which is in accordance with data previously obtained from other palaeontological analyses at the Gray Fossil Site.

Key words – Cephalothecoid fungus – Epiphyllous fungus – Miocene/Pliocene – Mycorrhizal fungus – North America – palaeoecology – taxonomy

Introduction

Fungal organic remains, usually fungal spores and dispersed sporocarps, are frequently found in a routine palynological investigation (Elsik 1996). It is believed that these diverse fungal remains are often important as a palaeoecological proxy (Dilcher 1965, Lange 1976, Elsik 1978, Conran et al. 2016, Worobiec & Worobiec 2017) and for calibration in refining the divergence time estimations in the phylogenetic trees obtained using molecular clock methods (Beimforde et al. 2014, Hongsanan et al. 2016, 2017, Liu et al. 2016, 2017, Mapook et al. 2016, Phukhamsakda et al. 2016, Samarakoon et al. 2016a, b, Sánchez-Ramírez et al. 2017). Considering these potential implications, we conducted a detailed investigation on diverse fungal remains found at the Gray Fossil Site located in Washington County, Tennessee, USA (36° 23' 9.6" N, 82° 29' 52.8" W). Gray Fossil Site deposits of lacustrine origin covers an area of approximately 26,000 m² (Shunk et al. 2006). The site, initially interpreted as the fills of a palaeosinkhole within the Cambro-Ordovician Knox Group (Shunk et al. 2006), is now suggested to be a multiple sinkholes/sub-basins that could represent asynchronous events (Zobaa et al. 2011, Worobiec et al. 2013). In the organic-rich, laminated sediments are preserved a variety of abundant fossils of both animals and plants (e.g. Liu & Jacques 2010, Zobaa et al. 2011, Mead et al. 2012, Ochoa et al. 2012, 2016, Worobiec et al.

2013, Huang et al. 2014, 2015), providing a critical insight into the palaeoecology and palaeoclimate conditions in the southeast USA during the late Neogene. The animal remains at Gray Fossil Site are diverse and abundant, exemplified by bones and teeth of alligators, anurans, badgers, bats, birds, lizards, red pandas, salamanders, snakes, tapirs, turtles, wolverines, among others (Parmalee et al. 2002, Wallace & Wang 2004, Schubert & Wallace 2006, Hulbert et al. 2009, Boardman & Schubert 2011, Mead et al. 2012, Czaplewski 2017, Samuels et al. 2018), whereas the Neogene plant communities at Gray Fossil Site represent open woodlands with oaks and hickories, mixed with diverse vines and shrubs existing in a warm temperate to subtropical climate (Gong et al. 2010, Liu & Jacques 2010, Ochoa et al. 2012, Worobiec et al. 2013). On the basis of the presence of *Teleoceras* and *Plionarctos*, Gray Fossil Site deposits have been dated to a range from 7.0 to 4.5 Ma, i.e. the latest Miocene to earliest Pliocene (Wallace & Wang 2004, Shunk et al. 2006). Among the investigated fossils from Gray Fossil Site, however, fossil fungal remains were interestingly not studied in detail, although Zobaa et al. (2011) found remains of sporocarps of *Callimothallus* sp., Ochoa et al. (2012) reported a presence of fungal remains in pollen profiles (with no details) and Worobiec et al. (2013) noted the occurrence of epiphyllous fungi (Microthyriales). In our recent palynological investigation of samples collected from four test-pits within Gray Fossil Site (Worobiec et al. 2013), various fungal remains representing forms that have not previously been reported from any Neogene deposits in North America were encountered. The present investigation aims at the taxonomy and palaeoecology of three different types of these fungi, viz. isolated plates of cephalothecoid peridium, fragments of mantle tissue, and sporocarp, from the Gray Fossil Site.

Materials & Methods

In the previous palynological studies (Worobiec et al. 2013) a total of 15 samples were collected from four test-pits (Bear Pit, Elephant Pit, Test Pit 2-2010, and Rhino Pit) at the Gray Fossil Site. All samples were taken from the fossiliferous laminated facies. In the current investigation, all slides from the Bear Pit (four samples), Elephant Pit (four samples), and Test Pit 2-2010 (four samples) that contain higher frequencies of fungal remains were re-examined. Depending on the abundance of palynomorphs, 4 to 8 slides from each sample were examined in detail. Samples for palynomorph analysis were prepared by means of a modification of Erdtman's acetolysis method, using HF to remove the mineral matter (Moore et al. 1991), and were rinsed in a 5 µm filter cloth. The microscope slides were made, using glycerine jelly as a mounting medium and cover-slips 24 × 24 mm. The rock samples, palynological residues, and slides are stored in the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków, Poland (Worobiec et al. 2013).

Terminology for the morphology of fungal remains (sporocarps and mantle tissue) follows Korf (1958), Ingleby et al. (1990), Wu et al. (2011). The method of measuring the size of fungal structures depends on their shape; and we used diameter measurements for regular, round or broadly elliptical structures, and length and width for quadrangular structures.

Bright field microphotography of the fossils was done using a NIKON Eclipse E400 microscope fitted with a CANON A640 digital camera.

Results

The classification of fossil and recent fungi follows Kalgutkar & Jansonius (2000), Wu et al. (2011), Hongsanan & Hyde (2017), Wijayawardene et al. (2018).

Fungi incertae sedis

Cephalothecoidomyces G. Worobiec, Neumann & E. Worobiec, Fungal Biology 121: 287 (2017)

Cephalothecoidomyces neogenicus G. Worobiec, Neumann & E. Worobiec, Fungal Biology 121: 287 (2017) Fig. 1a–b

Three isolated plates of cephalothecoid ascoma, polygonal, 62–90 µm in size, composed of radiated cells (*textura prismatica*). Cells measuring ca. 4–6 µm long and 4–6 µm wide, dark and thick-walled.

Material examined – USA, Tennessee, Gray Fossil Site, spring 2010, D. Ochoa. Bear Pit, slides 2(1), 2(2), and Elephant Pit, slide 10(6). Three specimens.

Notes – Shape and structure of the above described fungal remains are closely comparable to the isolated plates of *Cephalothecoidomyces neogenicus* described from Neogene deposits of Germany and Poland (Worobiec et al. 2017). The eminent difference between them is in the dimension of cells in that the European material is 6–14 µm long, while Gray Fossil Site one is only 4–6 µm long. Remains of *Cephalothecoidomyces* represent peculiar type of fungal sporocarps that disaggregate into plates along lines of dehiscence. They are termed cephalothecoid ascomata (Greif et al. 2004, 2009, Kirk et al. 2008). Cephalothecoid ascomata varies ranging from peridia composed of large, complex plates (e.g. *Cephalotheca*) to small, simple plates as in the extant genus *Chaetomidium* (Greif et al. 2009). *Cephalothecoidomyces neogenicus* is probably related to the extant members of family Cephalothecaceae Höhn. that comprise four genera, such as *Albertiniella* Kirschst., *Cephalotheca* Fuckel, *Cryptendoxyla* Malloch & Cain, and *Phialemonium* W. Gams & McGinnis (Lumbsch & Huhndorf 2010, Wijayawardene et al. 2012, Maharachchikumbura et al. 2016, Wijayawardene et al. 2018). From these genera, *Cephalothecoidomyces neogenicus* in respect of shape and arrangement of cells of peridial plates is most similar to the extant species of *Cephalotheca* and *Cryptendoxyla* (Worobiec et al. 2017).

Occurrence – Up to now *Cephalothecoidomyces neogenicus* is known only from two Neogene localities in Europe, viz. Adendorf, Germany and Mizerna-Nowa, Poland (Worobiec et al. 2017). Fossil remains to some degree resembling genus *Cephalothecoidomyces* were reported as other fossil-taxa from Oligocene to Holocene deposits (see Worobiec et al. 2017).

Ascomycota

Gloniaceae (Corda) Boehm, Schoch & Spatafora, Mycological Research 113 (4): 468 (2009)

Cenococcum Moug. & Fr., Systema Mycologicum 3: 65 (1829)

Cenococcum cf. *geophilum* Fr., Systema Mycologicum 3: 66 (1829)

Fig. 1c–e

Several fragments of mantle tissue, up to ca. 200 µm in size, composed of more or less rectangular cells (*textura prismatica*). Net synenchyma plectenchymatous, cells with thick walls, up to 17 µm long, and 7.5–10 µm wide. Cells arranged in more or less regular stellate aggregations.

Material examined – USA, Tennessee, Gray Fossil Site, spring 2010, D. Ochoa. Bear Pit, slides 7(3), 19(2), 19(3), 19(4), and Elephant Pit, slides 10(2), 10(3). Thirteen specimens.

Notes – Cellular structure of the above described fungal remains, namely stellate arrangement of cells, is rather characteristic of mantle tissue (synenchyma), of one of the most frequently found extant mycorrhizal fungus *Cenococcum geophilum* Fr. (Ingleby et al. 1990, Agerer 1999, LoBuglio 1999, Agerer 2006). Somewhat similar structure of mantle tissue occurs in some extant species of genus *Tomentella* Pers. ex Pat. (Basidiomycota) (Jakucs & Erős-Honti 2008). However, the cell arrangement in *Tomentella* is different from that in *Cenococcum* (see Jakucs & Erős-Honti 2008, Fig. 1g). LoBuglio (1999) considered the characteristic cell arrangement of mantle tissue of *Cenococcum* as “stellate or cephalothecoid”. Moreover, the cephalothecoid arrangement of cells of peridium is typical for the genus *Elaphomyces* Nees, which is considered as the sexual stage (teleomorph) of *Cenococcum geophilum* (LoBuglio et al. 1996). The cephalothecoid structure of fungal peridia is found in many extant often unrelated fungal genera (Worobiec et al. 2017), and rather similar cephalothecoid peridial wall structure to mantle tissue of *Cenococcum geophilum* could be found in some species of *Chaetomidium* (Zopf) Saccardo (Greif et al. 2009), rarely also from the genus *Chaetomium* Kunze (Doveri 2011, 2013, 2014). Rather similar cephalothecoid peridium to the mantle tissue of *Cenococcum geophilum* Fr. has fossil-species *Adendorfia miocenica* G. Worobiec, Neumann & E. Worobiec, described from Miocene locality of Adendorf, Germany (Worobiec et al. 2017). Taking this into account, we reconsidered the determination of

remains of *Adendorfia miocenica* and found that some of these remains with strongly elongated cells could in fact represent the mantle tissue of *Cenococcum*. On the other hand, remains of *Adendorfia miocenica* from Germany were accompanied by numerous fragments of trichomes similar to that covering the peridia of extant genera *Chaetomidium* and *Chaetomium*. In some cases, these trichomes were also preserved attached to small fragments of the peridium from Adendorf, Germany. *Chaetomidium* and *Chaetomium* are considered to be closely related to *Adendorfia* (Worobiec et al. 2017). As trichomes of this type were completely absent in the Gray Fossil Site, the presence of *Adendorfia* should be excluded and further discussed if these fungal remains seem to really represent the mantle tissue of *Cenococcum geophilum*. Presence in the fossil state of mantle tissue of *Cenococcum* is related to its resistance to the decomposition (Bird & McCleneghan 2005). Nevertheless, as the deposits of the Gray Fossil Site lie rather shallow beneath the recent soil layer, it could not be excluded that remains of *Cenococcum geophilum* could be remnants of subfossil or even recent mycorrhizas from roots penetrating the soils. Kołaczek et al. (2013) pointed to possible contamination of mire deposits with spores of mycorrhizal fungi by roots penetrating the peat and discussed resulting problems with the interpretation of diagrams of non-pollen palynomorphs including spores of mycorrhizal fungi. On the other hand, the state of preservation, similar in all studied fungal remains, points rather to fossil origin of the studied *Cenococcum* remains.

Occurrence – To date remains of mantle tissue of *Cenococcum* have not been reported from the fossil state. Mudie & Lelièvre (2013) reported “fragment of seriate fungal fruit body” from late Holocene deposits of Maligomisch, Nova Scotia, Canada that probably represents the fragment of mantle tissue of *Cenococcum*. Fossil sclerotia of *Cenococcum*, sometimes in high quantities, were reported from numerous localities of Pliocene to Quaternary age, particularly from archaeological sites (Shay & Kapinga 1998, Bennike et al. 2002, Fischer & Butzmann 2006). From the early Eocene amber of the Tadkeshwar Lignite Mine, Gujarat, India, Beimforde et al. (2011) described fragments of ectomycorrhizas of *Eomelanomyces cenococcoides* Beimforde, Dörfelt & A. R. Schmidt. These authors considered preserved hyphae with iris diaphragms at the septa that extend outward from mantle as similar to the recent genus *Cenococcum*. However, in the opinion of Beimforde et al. (op. cit.), *Eomelanomyces cenococcoides* differs from *Cenococcum* in the high variability in the branching of the ectomycorrhizal systems and by the regular formation of microsclerotia. It should be added that preserved fragments of *Eomelanomyces cenococcoides* show no characteristic for *Cenococcum* stellate arrangement of cells of mantle tissue.

Microthyriaceae Sacc., Sylloge Fungorum 2: 658 (1883) vel Trichothyriaceae Theiss., Beihefte zum Botanischen Centralblatt 32: 3 (1914)

Trichothyrites Rosend. emend. Smith, Palaeontology, 23(1): 209 (1980)

Synonyms:

Notothyrites Cookson, Proceedings of the Linnean Society of New South Wales 72: 208 (1947)

Sphaerialites Venkatach. & R.K. Kar, Palaeobotanist 17: 181 (1969)

Trichothyrites* cf. *padappakarensis (Jain & Gupta) Kalgutkar & Jansonius, American Association of Stratigraphic Palynologist Foundation Contributions Series 39: 303-304 (2000) Fig. 1f–i

One whole sporocarp and two fragments of other sporocarps, 63–127 µm in diameter. Scutellum composed of radiating rows of quadrilateral (*textura prismatica*) nonporate cells, up to 7 µm long and 2.5–5.0 µm wide. Radial cell walls usually straight, tangential cell walls usually rounded. In one specimen [Bear Pit 7(6)] are preserved both upper and lower wall of scutellum, their cells have more or less the same shape and dimensions. Ostiole central, roundish, 12.5–20.0 µm in diameter. Collar distinct, composed of three rows of small, isodiametric cells, with very thick and dark walls, ca. 3–4 µm in diameter.

Material examined – USA, Tennessee, Gray Fossil Site, spring 2010, D. Ochoa. Bear Pit, slide 7(6), Elephant Pit, slide 3(3), and Test Pit 2-2010, slide 2(5). Three specimens.

Notes – Sporocarps with diameter exceeding 100 μm , structure of collar and scutellum cell size and arrangement are rather similar to the fossil-species *Trichothyrites padappakarensis* described from upper Miocene deposits of Padappakkara, Western Ghats, South India (Jain & Gupta 1970). However, differences among the discussed three specimens from the Gray Fossil Site prevent unequivocal assignment of these fungal remains to *Trichothyrites padappakarensis*. Similar sporocarps (ascocarps) with roundish, central ostiole are usually found among extant members of the families Microthyriaceae and Trichothyriaceae (Wu et al. 2011, Hongsanan & Hyde 2017). Among the recent representatives of Trichothyriaceae, two species of genus *Lichenopeltella* Höhn., *L. pinophylla* (Höhn.) P.M. Kirk & Minter and *L. nigroannulata* (J. Webster) P.M. Kirk & Minter, are the most similar to *T. cf. padappakarensis* from the Gray Fossil locality. The state of preservation of *Trichothyrites* remains from Gray Fossil Site (e.g. absence of asci and ascospores) prevents from detailed comparison with mentioned recent species of *Lichenopeltella*.

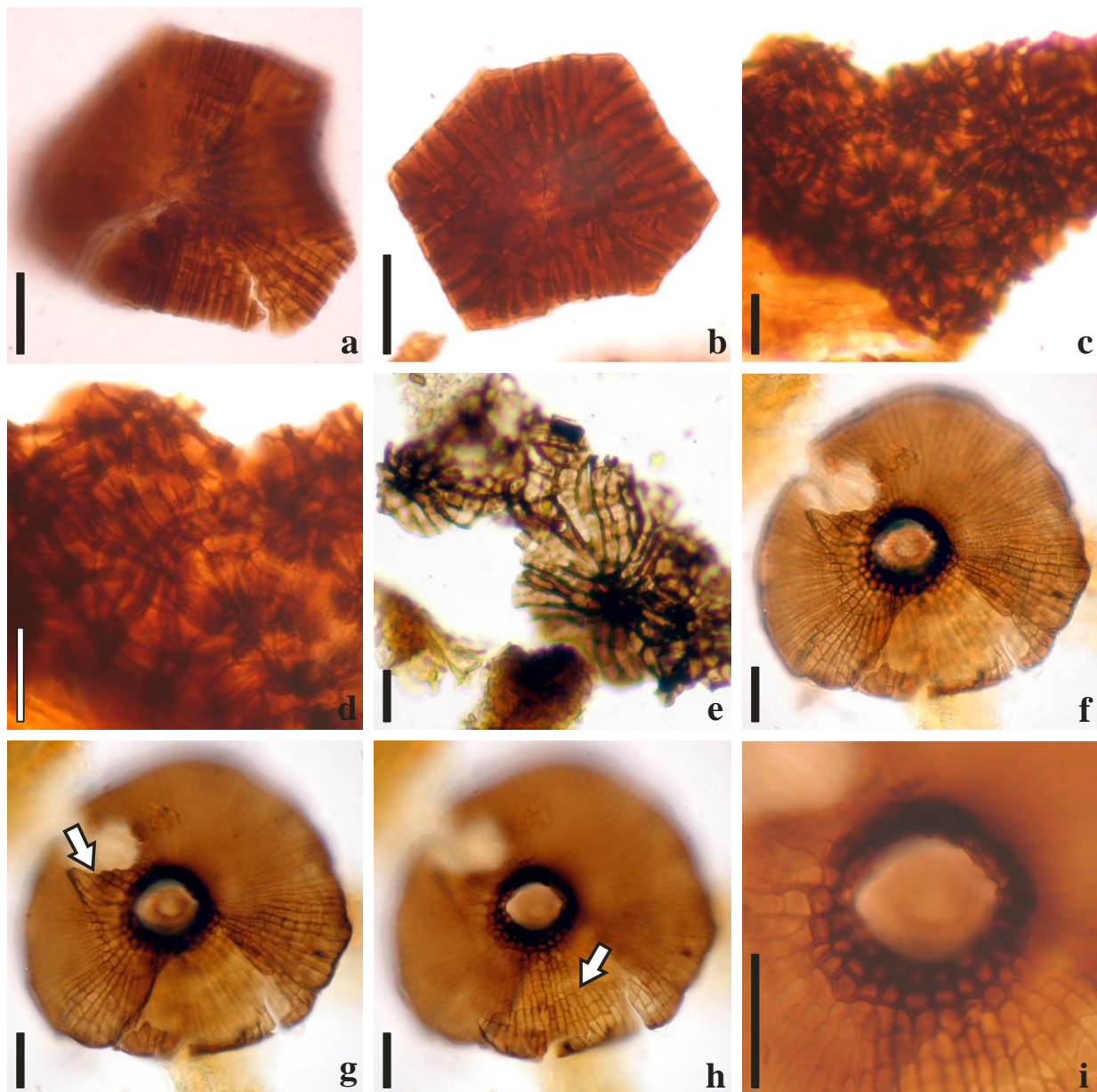


Figure 1 – Fungal remains from the Gray Fossil Site. a, b *Cephalothecoidomyces neogenicus*, isolated plates of cephalothecoid peridium. c–e *Cenococcum cf. geophilum*, fragments of mantle tissue with characteristic stellate-like arrangement of cells. f–i *Trichothyrites cf. padappakarensis*, f–sporocarp, g–sporocarp note upper layer of scutellum (arrow), h–sporocarp, note lower layer of scutellum (arrow), i–detail of ostiolae. Scale bars: 20 μm .

Occurrence – To date fossil-species *Trichothyrites padappakarensis* has not been found in the Cenozoic deposits of USA. Sporocarps of fossil-genus *Trichothyrites* were reported from Pleistocene deposits of Springfield, Minnesota (as *T. pleistocaenicus* Rosendahl, Rosendahl 1943) and Pleistocene deposits of Lee County, Iowa (as *Trichothyrites* sp., Wilson 1952). Up to now 14 fossil-species of *Trichothyrites* have been described worldwide from Cenozoic localities (Kalgutkar & Jansonius 2000). Worobiec et al. (2013) reported remains of Microthyriales from the Gray Fossil Site, but these authors did not specify the taxonomical position of these fungi.

Discussion

Remains of sporocarps, morphologically similar to the extant cephalothecoid fungi from family Cephalothecaceae (*Cephalothecoidomyces neogenicus*) and epiphyllous member of Microthyriaceae vel Trichothyriaceae (*Trichothyrites* cf. *padappakarensis*) along with fragments of mantle tissue (synenchyma) of mycorrhizal fungus that are almost identical to the extant *Cenococcum geophilum*, were found in the Neogene deposits of the Gray Fossil Site, USA. Both *Cephalothecoidomyces neogenicus* and *Trichothyrites* cf. *padappakarensis* are new for the fossil mycota of North America, while the mantle tissue remains of *Cenococcum* in the fossil state are reported for the first time. The only fossil record of the genus *Cenococcum* from North America concerns its sclerotia found in Quaternary deposits, particularly from archaeological sites (e.g. Shay & Kapinga 1997). As it was mentioned earlier, sporocarps of fossil representatives of *Trichothyrites* were earlier reported from Pleistocene localities of USA (Rosendahl 1943, Wilson 1952).

The presence of the fungal remains studied could be used for the reconstruction of Neogene paleoenvironment and palaeoclimate of the Gray Fossil Site. *Cephalothecoidomyces neogenicus* was most probably growing on decaying wood similarly as extant members of Cephalothecaceae, which are typically found as saprobes on rotten wood or on other fungi (Cannon & Kirk 2007). *Cenococcum* formed mycorrhizal associations with both woody and herbaceous plants similarly as extant *Cenococcum geophilum* (Trappe 1962, LoBuglio 1999, Obase et al. 2016). Extant members of family Cephalothecaceae, most probably related to fossil *Cephalothecoidomyces* are found in temperate areas of the Northern Hemisphere (Cannon & Kirk 2007). *Trichothyrites* cf. *padappakarensis*, epiphyllous fungus closely related to the extant members of families Microthyriaceae and Trichothyriaceae is a better climatic proxy than *Cephalothecoidomyces*. Modern microthyriaceous fungi show the highest abundance and taxonomic diversity in warm and humid regions of the Earth (Reynolds & Gilbert 2005, Thaug 2006, Hofmann 2010, Hosagoudar et al. 2011, Piepenbring et al. 2011) since high precipitation and air humidity are crucial factors for their growth (Selkirk 1975, Johnson & Sutton 2000, Limaye et al. 2007). Considering this, some researchers correlate the presence of epiphyllous, microthyriaceous fungi in a fossil state with a humid and warm, even tropical climate (Dilcher 1965, Lange 1976, Elsik 1978, Kalgutkar & Jansonius 2000, Tripathi 2009, Conran et al. 2016). Some extant microthyriaceous fungi, however, do not require favourable thermal conditions and are found even in polar areas with wet climate (Lind 1928, Dennis 1968, Holm & Holm 1984). On the other hand, at the Gray Fossil Site were found remains of sporodochia of fossil-genus *Callimothallus* Dilcher (Zobaa et al. 2011). The presence of fungal sporodochia of *Callimothallus* is a good proxy of climatic conditions in the past (Worobiec & Worobiec 2017) and points to warm and humid climates in the southeast USA during the late Neogene. To sum up, on the basis of the presence of fungal remains of *Callimothallus*, *Cephalothecoidomyces*, and *Trichothyrites*, we can infer a warm temperate and humid climate of the period of sedimentation of deposits of the Gray Fossil Site.

The fungal remains from the Gray Fossil Sites were accompanied by moderately diversified palynoflora with a strong domination of angiosperm pollen, mainly from two genera (viz., *Quercus* and *Carya*). These trees were the main components of an oak-hickory forest, with a small admixture of other plants, such as *Ulmus*, *Juglans*, *Pinus*, *Vitis*, among others. Pollen grains of herbs (Cyperaceae, Poaceae and Asteraceae) were encountered regularly, but they were not abundant (Ochoa et al. 2012, Worobiec et al. 2013). In addition, microfossils of freshwater algae were present in all Gray Fossil Site samples. Their compositions vary among the pits, nevertheless

in all pits peridinioid dinoflagellate cysts and resting cells of the Zygnemataceae green algae (most probably *Spirogyra* and *Zygnema*) were the most frequent. Those resting cells point to the presence of shallow sinkhole pond water, permitting easy warming and possible periodical drying up (Worobiec et al. 2013). Warmer and drier climatic conditions were previously deduced using plant fossils identified from the Gray Fossil Site, as well as from the common occurrence of charcoals all over the fossiliferous laminated layers, contributed by forest fires (Jiang & Liu 2008, Liu & Zavada 2009). On the other hand, the presence of epiphyllous fungal sporocarps in samples from all the pits and the palaeoclimatic reconstruction of the Gray Fossil Site fossil flora (Liu & Zavada 2009) likely indicate a rather high mean annual precipitation (over 1000 mm) in the late Neogene. It would be most probable that the higher precipitation during the wettest months made possible the development of the microthyriaceous epiphyllous fungi, whereas during the driest months the water bodies in the palaeosinkhole might have dried up and forest fires could then have occurred (Worobiec et al. 2013).

Fossil fungi are important also for the calibration of the divergence time estimations in the phylogenetic trees (e.g. Sánchez-Ramírez et al. 2017). This is especially true in case of *Cephalothecoidomyces neogenicus*. The presence of this fossil-species at the Gray Fossil Site is the first fossil record of cephalothecoid fungi in the area of North America. Previous results on investigation of fossil cephalothecoid fungi revealed their presence in the fossil state in the Neogene of Europe and, on the basis of presumably remains, in Africa, Asia and South America, mostly in Neogene deposits (Worobiec et al. 2017). Thus, the fossil record of cephalothecoid fungi now covers almost all the continents (with exception of Australia and Antarctica) and confirms presence of cephalothecoid fungi at least from Neogene period. Mycorrhizal *Cenococcum* from the Gray Fossil Site could constitute the oldest record of this genus in the fossil state and first record of mantle tissue of *Cenococcum* in Neogene deposits. It provides an important premise of the presence of ectomycorrhizas in the fossil state (Sánchez-Ramírez et al. 2017) and to date back mycorrhizal associations of *Cenococcum* to the Neogene period.

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