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Endophytic Micromycetes on the Leaves of the Genus *Taxodium* Richard (Cupressaceae) from the Lower Paleocene of the Amur Region

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Abstract—Traces of damage on fossil *Taxodium* leaves from the Tsagayan Formation of the Arkhara-Boguchan brown coal field (Amur Region, Russia; Paleocene) are studied using a scanning electron microscope (SEM). It is shown that the damage was caused by endophytic micromycetes. The problems of classification of microscopic damage to fossil plants are discussed.

Keywords: Taxodium, fossil plant damage, fungal endophytes, Paleocene, Amur Region

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INTRODUCTION

The first preliminary data on damage to *Taxodium* leaves from the Paleocene Arkhara-Boguchan brown coal field (Amur Region, Russia) were published by Vasilenko and Karasev (2006). Later, the morphology of these pathological structures was described, and it was suggested that they were produced by gall-forming arthropods (Vasilenko et al., 2015).

The arrival of new material and ongoing study of the morphology and anatomy of *Taxodium* shoots using SEM resulted in the new data on the structural organization of these formations, which have fundamentally changed the former interpretation of the causative agent. Our study suggests that traces of damage on the *Taxodium* leaves were caused by endophytic micromycetes. The new data indicate that classification of such microscopic damage needs to be refined, to reduce the risk of incorrect assumptions when the nature of the damaging agent is difficult to assess.

MATERIAL AND METHODS

The material studied comes from the middle subformation of the Tsagayan Formation, exposed in a quarry of the Paleocene Arkhara-Boguchan brown coal field (Amur Region, Russia) (49°18′52.3″ N, 130°12′42.7″ E). The age of these deposits is early Paleocene based on plant megafossils and palynological data (Kodrul, 2004; Markevich et al., 2004). Material with such damage was first collected by V.A. Krassilov, T.M. Kodrul, E.V. Bugdaeva, and D.V. Vasilenko, and was later supplemented by Bugdaeva (2014) and Kodrul (in 2015). The fossil plants are represented by compressions with remains of phytoleim and impressions with fragments of thin mineral incrustations. The samples were studied using a Tescan Vega XMU SEM (Low Vacuum scanning electron microscopy with gold coating or without coating). In addition, we studied the phytoleims after maceration treatment using the standard method. The photography was performed using a Nikon Coolpix 8700 digital camera and a Leica M165C stereomicroscope equipped with a Leica DFC 420C digital camera.

The collections are housed in the Geological Institute of the Russian Academy of Sciences (GIN RAS), Moscow, coll. no. 4867, and in the Borissiak Paleontological Institute of the Russian Academy of Sciences (PIN RAS), coll. no. 5142.

RESULTS

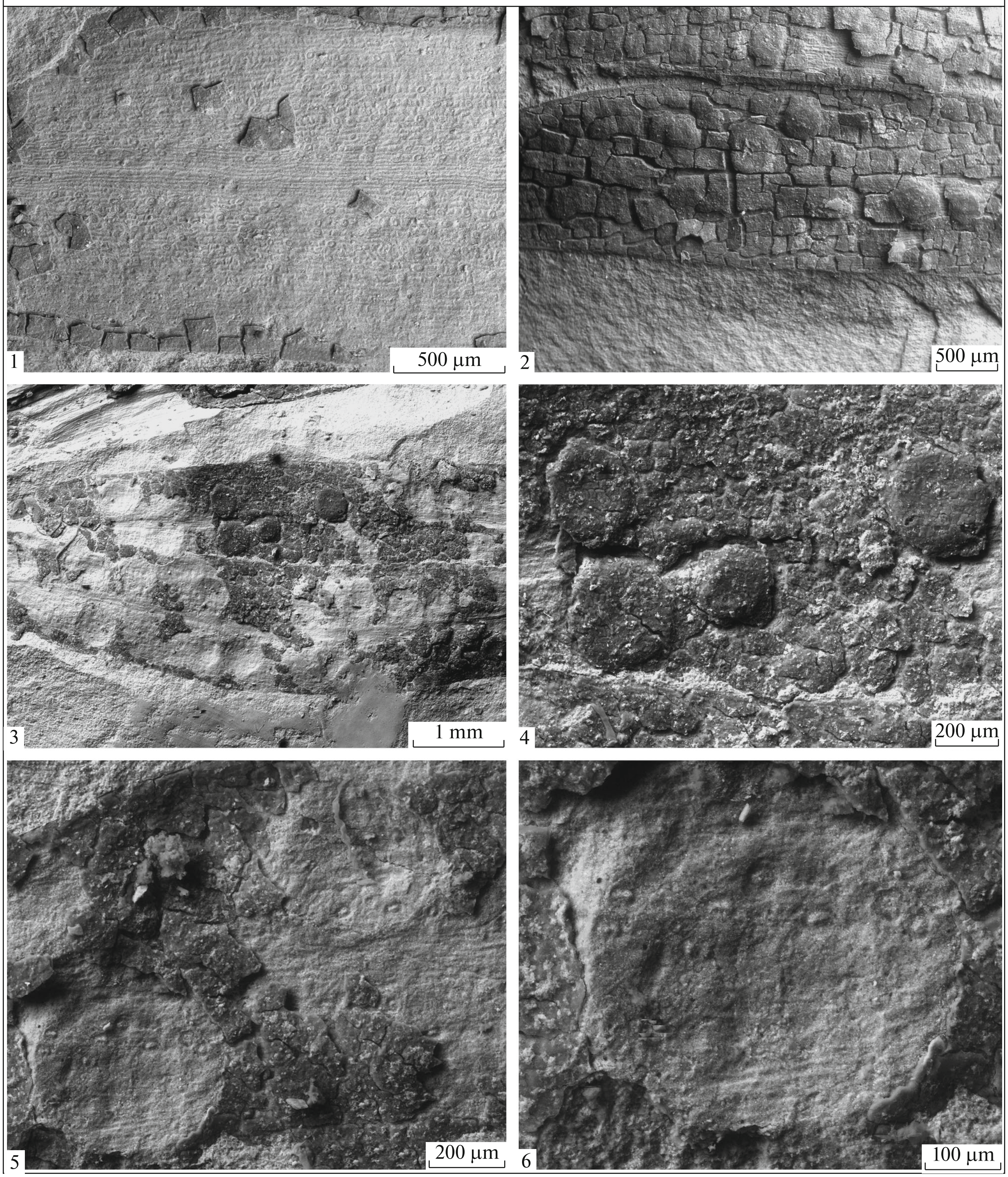
We examined over 130 conifer shoots, which were attributed to a new species of the genus *Taxodium*, based on an assemblage of characteristic features (the new species will be described in a separate paper).The

Fig. 1. General view of the shoots of *Taxodium* sp., damaged by micromycetes (a, b) and some damaged leaves (c, d): (a) specimen GIN no. 4867-AB1-264; (b) specimen GIN no. 4867-AB1-601; (c, d) specimen PIN no. 5142/2.

shoots have a straight axis and linear-lanceolate or oblong-oval leaves, alternating or nearly opposite (Figs. 1a, 1b). The longest leaves are located in the middle of the shoot, and their length decreases toward the shoot base and apex. The leaves are amphistomatic. The stomata are amphicyclic or incompletely amphicyclic, with four to six subsidiary cells, oriented longitudinally, transversely, or obliquely, and are arranged in two stomatal bands on each side of the leaf (Pl. 15, fig. 1). Each stomatal band has three to eight stomata across its width (Pl. 15, fig. 1).

Of all the shoots studied, 10 specimens have leaves with damage readily visible under a binocular microscope as rounded or oval tubercles slightly raised over the leaf surface and occurring near the stomatal bands (Pl. 15, figs. 2–4; Figs. 1c, 1d). The diameter of these tubercles varies from 130 to 570 μm, more commonly 300 μm. The tubercles are semispherical in shape, sometimes cup-like, weakly concave centrally, numerous on the leaf or less commonly solitary (Pl. 15, figs. 2–6; Pl. 16, figs. 1, 2; Fig. 1d). The leaf epidermis is not damaged on the surface of the tubercles; stomata are visible (Pl. 15, figs. 5, 6). The preparation for the SEM study shows a tear in the cuticle, allowing the fruiting body of micromycete (conidiomata), developed inside the leaf, to be studied. The fruiting body wall is formed of tangled hyphae. The shape of the fruiting body is discoid, cup-like. There is a stoma-like pore, diameter ca. 30 μm, oriented outwards (Pl. 16, fig. 3). The fruiting body when separated from the leaf tissue by maceration, shows accumulation of spores (Pl. 16, figs. 4, 6), as well as isolated hyphae (Pl. 16, fig. 5). Spores (conidia) are oval to rounded, with a maximum diameter 8–10 μm. The hyphae are 3–4 μm in cross-section.

Plate 15

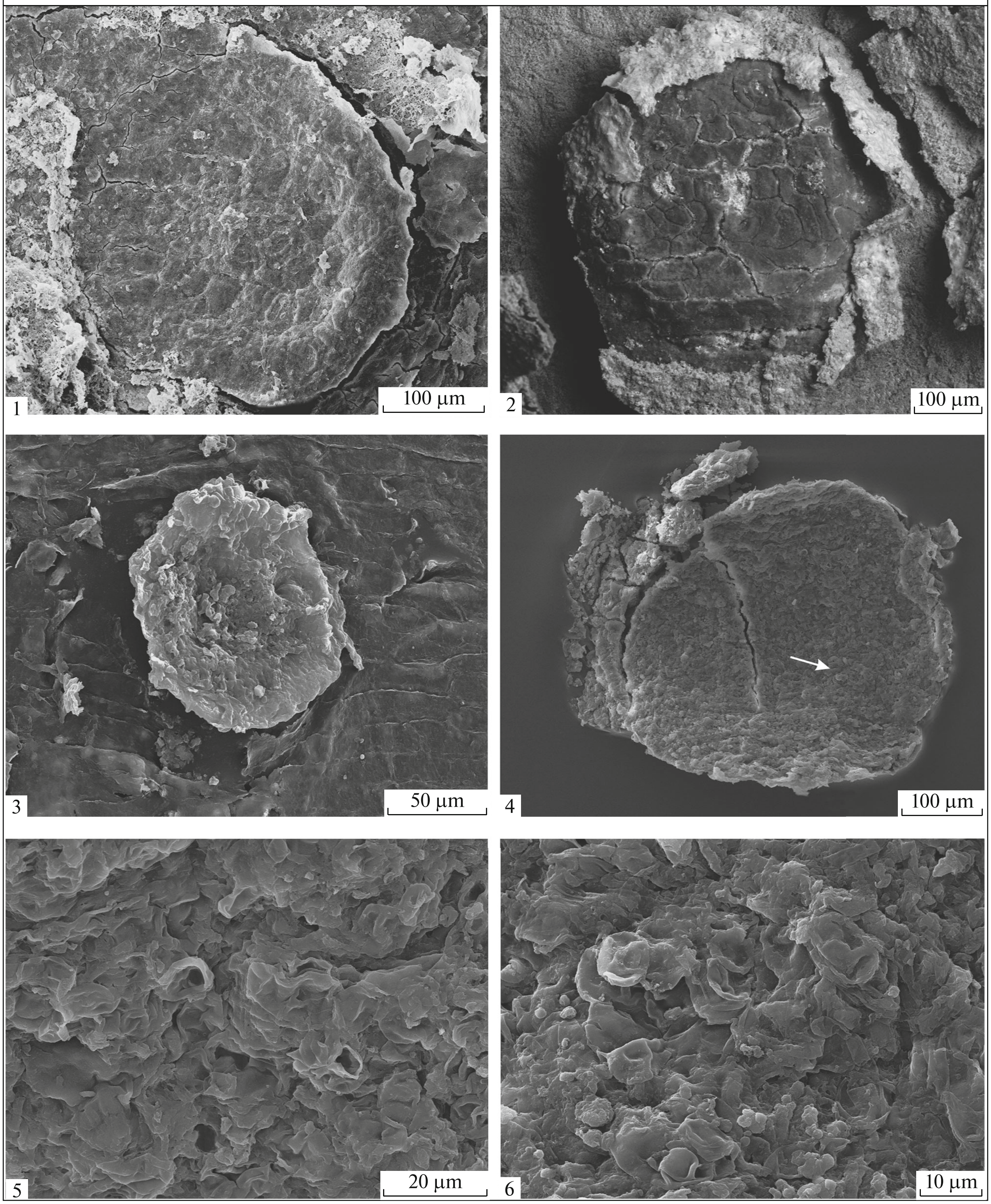


Explanation of Plate 15

Fig. 1. Epidermal structure of the leaves of *Taxodium* sp., specimen GIN no. 4867-AB1-264, incrustation, SEM.

Figs. 2–6. Leaves of Taxodium sp., damaged by micromycetes, specimen PIN no. 5142/2, SEM; (5, 6) incrustation showing preserved epidermis with stomata covering the picnidium.

Plate 16



DISCUSSION

Despite the presence of various plant remains collected from the Tsagayan Formation, leaf damage represented by microscopic tubercles confined to the stomatal rows was only discovered on the leaves of the genus *Taxodium*. The SEM study of the anatomical structure of these leaves showed that the contaminants causing the leaf damage are produced by micromycete condiomata developing beneath the leaf cuticle. The leaf epidermis does not undergo any pathological changes (at least until the time when the spores are released). The incrustations distinctly mark the preserved stomata. Thus, no swelling growth on the external and subsurface tissues, which is usually observed in plants affected by gall producers, is observed. An excessive outgrowth of plant tissue in response to a pathogen is essential to identify the gall nature of damage. Both small and relatively large galls emerge on plants infested, mainly, by various arthropods. The plants act as a habitat and a food source for an invasive gall-forming agent throughout its entire life cycle. The plant tissue grows in response to the pathogen's invasion to form a dense sheath surrounding the affected area, to prevent the invasion from spreading over larger areas. Note that the damage examined on the *Taxodium* leaves lacks the typical characters of galls.

The occurrence of the pathological structures within the stomatal bands suggests that the endophytic fungi most likely invaded via open stomatal apertures. The shape of the fruiting body and the presence of a stoma-like pore facing the surface of the epidermis, suggest that these fruiting bodies belong to Ascomycota fungi. Both ascomata (perithecia or ascolocular stromata) and pycnidial conidiomata have these characters. The absence of asci in the fruiting bodies, and the size of these structures, suggest that these fruiting bodies most likely represent pycnidia. The presence of spores (apparently conidia) in the fruiting bodies agrees with the suggestion that these structures are pycnidial conidiomata which supports the fungal nature of the invasive agent that caused damage on the leaves of *Taxodium*. Based on the observed characters, we consider that these microscopic fungal remains could most likely be assigned to the anamorphic Ascomycota fungi. The presence of the fruiting bodies (conidiomata) allow these micromycetes to be assigned to pycnidial Coelomycetes (in traditional taxonomy – order Sphaeropsidales of the phylum Deuteromycota). The state of preservation of the material, in particular the absence of data on the interior of the conidiomata, and the structure and coloration of the conidia, does not allow a more detailed characterization of these fossils or determination of their taxonomic assignment. Note that the identification even of extant anamorphic fungi is not limited to their external morphology, but requires detailed cytological examination (in particular of conidiogenesis), and often study in culture to enable a relationship to be made between the anamorph and teleomorph and physiological-biochemical characters of the fungi to be determined, as well as molecular analysis. The position of the mycelium and pycnidia in the leaf tissues and the absence of any obvious negative effect of the micromycetes on the host cells allows its assignment to the ecological group of endophyte fungi.

Extant microscopic fungi with pycnidial sporogenesis affect various organs of plants causing an array of disease symptoms: leaf streak and spot disease, necrosis of branches and trunks, fruit and seed rot. It is noteworthy that there is little evidence of disease of *Taxodium* (irrespective of the pathogen). In contrast, many text-books on plant disease prevention note a relatively high resistance of *Taxodium* to various kinds of disease (*Ecosystems of the world*, 2005; Zhukov et al., 2013; etc.). Considering the high host-specificity of microscopic fungi affecting extant conifers (Zhukov and Gordienko, 2003; Isikov and Konoplya, 2004, etc.), it can be suggested which micromycetes are characteristic contaminants of modern *Taxodium*. Possible agents affecting *Taxodium* include microscopic fungi of the genera *Pestalotia* de Notaris and *Pestalotiopsis* Steyaert (anamorphic fungi of the order Ascomycota, families Amphispyariaceae and Pestalotiopsidaceae, respectively), differing in the teleomorphs and the number of septa in the conidia. The microscopic fungus *Pestalotiopsis funerea* (Desm.) Steyaert (Syn. *Pestalotia funerea* Desm), a facultative parasite of drought-weakened, sun-damaged, or cold-affected plants, affects the leaves of *Taxodium* (Pirone, 1978). The micromycetes on the leaves of the fossil *Taxodium* that we described differ from *P. funerea* by the shape of the pycnidia and spores. Another species described as *Pestalotiopsis microspora* (Speg.) G.C. Zhao et N. Li (Li et al., 1996), affects bark and vascular elements rather than leaves. The fungus *Stereum taxodi* Lentz et H.H. McKay from the order Basidiomycota (Burns and Honkala, 1990), which has large fruiting bodies, is also an agent affecting *Taxo-*

Explanation of Plate 16

Figs. 1–4. Fruiting body of micromycete, SEM: (1) specimen PIN no. 5142/1, globose fruiting body under the cuticle; (1) specimen GIN no. 4867-AB1-602; tears in the cuticle show incrustation of the leaf epidermis with stomata; (3) specimen GIN no. 4867-AB1-606, cuticle after maceration; the tear in the cuticle shows a fruiting body with a slit-like pore, view from the side facing the leaf surface; (4) specimen PIN no. 5142/1, fruiting body, view from the side facing inside the leaf blade. The arrow points to the rounded stomata.

Figs. 5 and 6. The surface of the micromycete fruiting body, specimen PIN no. 5142/1, SEM photograph, (5) hyphae forming the surface of a dense fructification showing cross-sections; (6) spore on the surface of a fruiting body.

dium wood. Representatives of three classes of fungi (Dothideomycetes, Eurotiomycetes and Sordariomycetes) from the phylum Ascomycota were discovered on *Taxodium* shoots affected by the gall midges *Taxodiomyia cupressi* Schweinitz and *T. cupressiananassa* (Osten Sacken), Diptera: Cecidomyiidae (Washburn and Van Bael, 2017). The taxonomic diversity of micromycetes inside and outside galls is significantly different, related to the role of insects in the transition and further growth of fungi.

Arthropods are also recorded among the agents affecting *Taxodium*. Apart from gall midges causing formation of large galls on shoots (Chen and Appleby, 1984; Gagné and Hibbard, 2008), there are records of leaf mining by the lepidopteran larvae *Coleotechnites apicitripunctella* (Clemens) (Hartman et al., 2000).

Evidently, the kind of leaf damage cause by microscopic endophyte pycnidial fungi that we described from the Paleocene *Taxodium*, is not characteristic of extant species. Species of the other two genera of the subfamily Taxodioideae—*Cryptomeria* Hayata and *Glyptostrobus* Endlicher—are also relatively resistant to fungal damage. Among possible fungal agent damaging leaves of these genera, only the genus *Pestalotia* has been recorded (Isikov and Konoplya, 2004; Zhukov et al., 2013). It should be noted that, according to molecular data, leaves of the *Taxodium* contain considerable amounts of micromycetes, among which anamorphic ascomycetes of the genera *Phyllosticta* Pers. and *Diaporthe* Nitschke have been recorded (Washburn and Van Bael, 2017). Both conidiomata of *Phyllosticta,* and those of the anamorphic stage of the species of the genus *Diaporthe* (species of the genus *Phomopsis* (Sacc.) Sacc.) are characterized by the morphology (shape and arrangement of pycnidia and unicellular conidia), similar to pycnidia discovered on the leaves of *Taxodium* from the lower Paleocene deposits. Note that pycnidial micromycetes commonly affect some extant conifers, e.g., *Pinus* L., *Larix* Miller, *Abies* Miller, *Cupressus* L. (Semenkova and Sokolova, 2003; Kuzmichev et al., 2004; Zhukov et al., 2013, etc.). Thus, by analyzing modern interpretations of associations between *Taxodium* and the micromycetes that have historically affected this taxon, we propose a possible change in these associations in the modern biota, the reasons and stages of which require further study.

Pycnidia of the extant agents causing fungal diseases of plants can be observed with the naked eye on the leaves and shoots as small black dots, tubercles or streaks. It is not possible to assign them to any taxonomic group solely based on morphology (Zhukov and Gordienko, 2003; *Ecosystems of the world*, 2005; etc.). The macromorphology of such fungal damage is almost identical to that of galls caused, for instance, by small mites. To confirm the identity of the agent, it is necessary to find out its microstructural organization (in the case of fungal disease, its reproductive type, interior structure, sporogenesis, and spore morphology), by studying micromycetes collected from the extant plant using microscopic technologies. It is even more difficult to recognize microscopic galls and damage caused by endophytic micromycetes in fossil plants because the type of preservation of the fossil does not always permit microstructural examination. Such damage are usually interpreted as arthropod-plant interactions. This interpretation is supported by the vast diversity of forms of damage caused by arthropods, and by the fact that arthropod damage is typically more scattered, not occurring in such concentrations as typical micromycete damage. Most often microscopic formations on fossil plants (1 mm or less in diameter) are interpreted as galls (Labandeira et al., 2007 (DTs 32, 80, 116, 125, 144, etc.); Donovan et al., 2016 (text-fig. 1b), etc.). It is commonly suggested that such galls are produced by insects, but galls can be also initiated by small mites. Some morphologically very similar structures are interpreted as traces of stabbing or sucking agents (e.g., Labandeira et al., 2007; DTs 46, 47, 48, 132, etc.).

The fungal cause of the microscopic damage to *Taxodium* leaves, originally identified as galls based on their general morphology, was only established after microscopic examination. It seems obvious that no confident interpretation of the origin of such damage in plants is possible without studying its microstructure. The same applies to other types of damage, the nature of the agents causing which is not obvious due to the similarity of their macromorphology (e.g., rounded holes rimmed by callus tissue could be produced by insect feeding or tissue destruction after mining). Apparently a new approach is needed to identify types of damage of fossil plants, which are only represented by imprints not permitting microstructural examination of damage traces. It appears that a more correct approach is to assign such damage traces using O.S. Vyalov's morphological classification, which was later supplemented by other authors (Vyalov, 1975; Vasilenko, 2005, 2006, 2007, 2008; Aristov et al., 2013, etc.). The application of this classification to microscopic damage similar to those described above is not yet developed, and needs to be improved. At present we emphasize that, based only on external morphology, without anatomical study, it is impossible to confidently establish the nature of the initiation agent of micro-damage to fossil plants in the shape of tubercles, dots, or streaks. Traditional interpretation of the damage traces as having been produced by insects can be erroneous, and can lead to incorrect evaluations of the taxonomic diversity of insects identified by damage on plants, and of co-evolutionary relationship in the paleobiotas of the past.

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