Short title: New *Hypoxylon* species from Texas

Observations on Texas Hypoxylons, including two new *Hypoxylon* species and widespread environmental isolates of the *H. croceum* complex identified by a polyphasic approach

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ABSTRACT

Two new species and a new combination of *Hypoxylon* from Texas were identified and described based on morphological, multigene phylogenetic (ITS – nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2, 28S – 5' 1,200 bp of nuc 28S rDNA, *RPB2* – partial second largest subunit of the DNA-directed RNA polymerase II, *TUB2* – partial β -tubulin) and chemotaxonomic data. *Hypoxylon olivaceopigmentum* is characterized by its pulvinate to glomerate stromata, olivaceous KOH-extractable pigments, equilateral ascospores and indehiscent perispore. *Hypoxylon texense* can be distinguished from morphologically similar species by its rust to dark brick KOH-extractable pigments and its stromatal secondary metabolites HPLC profile. *Hypoxylon hinnuleum* is proposed as the sexual morph of *Nodulisporium hinnuleum* featuring dark vinaceous glomerate stromata with dark brick KOH-extractable pigments composed of cohaerin-type azaphilones and smooth equilateral ascospores with indehiscent perispore. Based on this diagnostic characters *H. hinnuleum* forms a complex with *H. croceum* and *H. minicroceum*. More than 50 ITS sequences with high identity originating from North American and East Asian environmental isolates formed a well-supported clade with the type of *N. hinnuleum* demonstrating the widespread distribution of the species complex. In addition, updated descriptions and comprehensive illustrations with detailed information on the diagnostic feature of *H. fendleri* and *H. perforatum* are provided. The multi-locus phylogenetic reconstruction of *Hypoxylon* supported the status of the new species and broadened the knowledge about intergeneric relationships.

KEY WORDS: Ascomycota, chemotaxonomy, multigene phylogeny, taxonomy, Xylariales, three new taxa

INTRODUCTION

The systematic classification of members within the order Xylariales has evolved drastically in recent years due to the application of comprehensive multi-locus phylogenetic reconstructions, chemotaxonomy, and the recognition that taxa named from ascomata can be linked to environmental isolates. Consequently, various new families and genera have been erected and corresponding taxa re-assigned (Jaklitsch et al. 2014, 2016, Senanayake et al. 2015, Maharachchikumbura et al. 2016, Wendt et al. 2018). The Hypoxylaceae with its approximately 400 described species placed into 16 genera accounts for a vast portion of the taxonomic diversity within the order. The largest genus in the Hypoxylaceae is the type genus *Hypoxylon* with around 180 known members (Lambert et al. 2019, Wendt et al. 2018).

Hypoxylon species are encountered in all angiosperm-dominated forests, either in their saprobiotic stage or as endophytes (Kuhnert et al. 2014a, U'Ren et al. 2016). However, their diversity significantly expands towards the tropical regions of the world (Ju and Rogers 1996). In addition to their ecological importance, many species have been studied for their capacity to produce secondary metabolites (Helaly et al. 2018). The stromata, in particular, usually sequester large quantities of pigments that were traditionally extracted with a potassium hydroxide solution to reveal their characteristic species-specific color reactions. Many novel and often biologically active natural products have been discovered from the fruiting bodies and cultures of various species such as the cytotoxic hypoxyvermelhotins, the antioxidative rickenyls or the insecticidal nodulisporic acids (Bills et al. 2012, Kuhnert et al. 2014b, 2015). The biosynthesis of the latter in *H. pulicicidum* was recently elucidated emphasizing the importance of the genus as source for unique secondary metabolites (Van de Bittner et al. 2018).

Recent field work in the subtropics and tropics (e.g. Argentina, Caribbean, Thailand) continue to reveal an extensive number of undescribed *Hypoxylon* species (Kuhnert et al. 2014a, Fournier et al. 2015, Sir et al. 2015, 2016) demonstrating the potential of such areas to harbor new taxa. Much of southern Texas (USA) can be classified as subtropical, and approximately 38 % of the land remains forested (McMahan et al. 1984). Even though Texas is the second largest state in the USA, reports of Texan *Hypoxylon* species have been scarce as evidenced by a search of specimens and literature at the U.S.D.S. National Fungus Collection database. In 2007 and 2009 two extensive surveys on pyrenomycetous fungi in the Big Thicket National Preserve (southeastern Texas) were carried out by L. Vasilyeva. Subsequently, sixteen known and five new *Hypoxylon* species (following the current taxonomic concept) have been documented (Vasilyeva 2010, Vasilyeva and Stephenson 2014). However, because specimens were not cultured, illustrations were limited, and voucher specimens are not accessible in public herbaria, these findings can unfortunately not be validated. In 2016, various locations around and in Houston were explored for the diversity of *Hypoxylon* species. The resulting specimens were studied in-depth by a polyphasic approach using morphological, multigene phylogenetic (ITS, 28S, *RPB2*, *TUB2*) as well as chemotaxonomic (HPLC-DAD/MS) data. As a result, two new species, a new combination and the known taxa *H. fendleri* and *H. perforatum* are described and illustrated in detail herein.

MATERIALS AND METHODS

General

All scientific names of fungi follow the entries in Mycobank (www.mycobank.org), hence authorities and years of publications are not given. Reference specimens including the designated type material of the new species, are housed in GLM (Senckenberg Museum of Natural History Görlitz, Germany) and LIL (Fundación Miguel Lillo, San Miguel de Tucumán, Argentina) and corresponding reference cultures have been deposited with DSMZ (Braunschweig, Germany) and NRRL (ARS Culture Collection, Peoria, Illinois, USA).

Morphological characterization

Macro-morphological structures of the sexual morph were observed and measured from fresh material under a stereo microscope. Melzer's reagent was used to test the amyloid reaction of the apical apparatus and 10 % KOH to study the dehiscence of the perispore. Photos and sizes of micro-morphological structures were taken through a bright-field microscope at 400–1000 × magnification and a scanning electron microscope (FE-SEM Merlin, Zeiss, Germany). The classification of the conidiogenous structures and extraction of stromatal pigments followed the protocols of Stadler et al. (2014). Colors were assigned according to the mycological color chart (Rayner 1970). To examine conidiogenesis and to purify DNA, cultures were obtained from multi-spore isolates on yeast-malt-glucose-agar (YMG: yeast extracts 4 g/l; malt extract 10 g/l; dextrose 4 g/l) and oatmeal agar (OA) (Ju & Rogers 1996, Stadler et al. 2014).

HPLC profiling of stromatal extracts

To investigate the chemical composition of the pigments above and below the stromatal surface, pigments were extracted with acetone (Kuhnert et al. 2017a). The extracts were analyzed with an Agilent 1260 HPLC equipped with a diode array detector (DAD) and coupled to an Agilent 6120 single quadrupole mass detector using an Agilent Zorbax Eclipse Plus C18 column (5 µm, 4.6 × 150 mm). The mobile phase was composed of solvent A (acetonitrile supplemented with 0.1% formic acid) and solvent B (Millipore water supplemented with 0.1% formic acid). A linear gradient from 5 to 100% solvent A in 20 min was applied at a flow rate of 0.6 ml min⁻¹. The detection range of the compounds was set between 190 and 600 nm and mass signals were recorded in API-ES (atmospheric pressure ionization – electrospray) positive and negative ionization mode (3500 V capillary voltage).

HPLC profiling of culture extracts

Secondary metabolites produced by cultures of *H. croceum* (DSM 107926, DSM 107932) and the type strain of *N. hinnuleum* (MUCL 3621) were analyzed for comparison. Therefore, well-grown cultures of the respective strains on YMG medium (1.0% malt extract, 0.4% glucose, 0.4% yeast extract, pH 6.3) were used to inoculate 500 ml Erlenmeyer flasks with each of the following liquid media (YMG; Q6: 1.0% glycerol, 0.25% glucose, 0.5% cotton seed flour, pH 7.2; CM: 2% cornmeal, 0.4% Glucose, pH 5.5). The submerged cultures were incubated in the dark on a rotary shaker at 23 °C and 140 rpm. Fermentation was aborted after free glucose has been consumed and the mycelia was separated from the culture broth by vacuum filtration. The extraction of both parts followed a previously published protocol (Surup et al. 2014). The crude extracts were dissolved in acetone and analyzed with HPLC-MS according to Kuhnert et al. (2014b).

Molecular phylogenetic analyses

DNA extraction of fresh cultures and amplification of the ITS (nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2), 28S (5' 1,200 bp of nuc 28S rDNA), *RPB2* (partial second largest subunit of the DNA-directed RNA polymerase II) and *TUB2* (partial β -tubulin) loci was performed as described by Wendt et al. (2018). Sequences were generated for eleven Hypoxylaceae strains from Texas (nine *Hypoxylon* strains, two *Annulohypoxylon* strains) and the ex-type strain of *Nodulisporium hinnuleum* (MUCL 3621) representing seven species. In addition, corresponding sequences of the genome-sequenced strain *Nodulisporium* sp. (no. 65-12-7-1) were provided by Dr. Dan Hu (Wang et al. 2018). Furthermore, the rare species *H. lienhwacheense* is included for the first time in multigene phylogenetic analyses due to the availability of a full genome sequence (unpublished data). The new sequences of each locus were aligned with 49 corresponding Hypoxylaceae sequences and a *Xylaria hypoxylon* sequence as outgroup by using 100 iterations of MUSCLE alignment software (Edgar 2004) (for sequence details see TABLE 1). For the phylogenetic assessment of sequence data the general procedure of Lambert et al. (2019) was followed. Alignment data generated by a fast Fourier transformation approach (MAFFT) was processed via Geneious® 7.1.9 (<http://www.geneious.com>) and subjected to a curation of phylogenetic informative characters via the Castresana Lab Gblocks Server (Talavera and Castresana 2007). Further processing, concatenation and phylogenetic inference as well as substitution model selection was conducted using the PhyML Online Server (<http://www.atgc-montpellier.fr/phyml/>) with the PhyML 3.0 algorithm for phylogenetic relationship inference and preliminary determination of the best fitting substitution model via smart model selection as previously described by Lambert et al. (2019). 1,000 bootstrap (BS) replicates were executed and the support values were mapped onto the best-scoring tree.

RESULTS

Molecular phylogeny.–

Amplification products of the ITS, 28S, *TUB2* and *RPB2* could be obtained for all new strains with the exception of the ITS locus of one *H. fendleri* isolate (DSM 107923). The size of the ITS fragments ranged from 574 to 894 bp and spans the complete ITS1, 5.8S rDNA and ITS2 region. The lengths of the 28S (721 – 1,368 bp), *TUB2* (1,027 – 1,415 bp) and *RPB2* fragments (892 – 1,054 bp) depended in most case on the sequence quality. *Annulohypoxylon truncatum* (DSM 107925) carries a 323 bp long insert around the 5' 800 bp position in the 28S region, which does not occur in other related *Annulohypoxylon* species including the very close relative *A. annulatum*. Similarly, all isolates of *H. texense* contain a 172 bp insertion around the 5' 1,100 bp position of the 28S locus, which is unique among the known 28S sequences within the Hypoxylaceae. In the course of a genome comparison project targeting representatives of the latter family, *H. lienhwacheense* was fully sequenced (genomic data are not yet publicly available). Hence, the complete sequences of the *TUB2* (MK287550) and *RPB2* (MK287563) gene could be extracted and used for improved alignments. The *TUB2* gene had a length of 1,927 bp and the *RPB2* gene a length of 3,909 bp. The previously published *RPB2* sequence of the epitype of *H. fragiforme* (KM186296) was identified as mistake (most likely belongs to *H. investiens*) and re-sequenced in the course of this study (MK887342).

Alignments of the individual loci were used for single-locus ML phylogenetic reconstructions (SUPPLEMENTARY FILE 1 - 4). The 28S, *TUB2* and *RPB2* calculations contained 63 sequences derived from 62 Hypoxylaceae strains (including 30 *Hypoxylon* species, seven *Annulohypoxylon* species, four *Daldinia* species, three *Jackrogersella* species, three *Pyrenopolyporus* species, two *Hypomontagnella* species, two *Nodulisporium* isolates and one species of *Entonaema*, *Rhopalostroma* and *Thamnomycetes*) and one *Xylaria hypoxylon* strain as outgroup. For the ITS phylogeny 58 additional sequences with high similarity ($\geq 97\%$) to *H. hinnuleum* were retrieved from GenBank and used for

the respective calculation. The curated alignments of the individual gene loci showed 396 (1260 original positions, OP), 1076 (3023 OP), 819 (3943) and 1034 (2417) phylogenetic informative characters for the ITS, 28S, *RPB2* and *TUB2* region, respectively. In all single-locus trees the backbone was not statistically supported. The majority of main and subclades in the ITS as well as 28S inferences were unsupported and the number of nucleotide substitutions per site were in average lower between species as compared to trees inferred from gene-coding trees. In contrast, the *TUB2* tree showed the highest bootstrap support for the subclade architecture. The ITS sequences of the closest BLAST hits for *H. hinnuleum* clustered together with the type strain of *N. hinnuleum* into one well supported clade (84% BS support) with very low nucleotide substitution rate per site (< 0.05). We refrained from an in-depth evaluation of the single-loci trees as Wendt et al. (2018) and Daranagama et al. (2018) already demonstrated that multigene phylogenies generated from concatenated alignments deliver a much more robust picture of the inter- and infrageneric relationships. Therefore, they have been accepted as state-of-the-art molecular analyses to deduce the phylogenetic position and relationships of new species within the Hypoxylaceae.

For the multigene phylogenetic reconstruction (FIG. 1) the concatenated informative characters of the individual DNA loci of 63 fungal strains were used. The Tamura and Nei 1993 model (TN93+I +G), using proportion of invariant sites and distribution of rates at variable sites modeled on a discrete gamma distribution with four rate classes, was estimated to be the best-fit likelihood model of evolution for ML. The best multigene phylogenetic tree calculated showed a ML score of $\ln L = -39763.56902$. The supported backbone of the ML tree (53 % BS support) shows the polyphyletic origin of *Hypoxylon* and *Daldinia*. All other genera are monophyletic. The new species *H. olivaceopigmentum* clusters together with *H. hinnuleum*, *H. investiens*, *H. lateripigmentum* and *H. pulicidum* with 93 % BS support. *Hypoxylon texense* forms a clade with *H. rubiginosum*, *H. cercidicola* and *H. petriniae* (67 % BS support). The Texan *H. fendleri* strains group together with a representative of the same species from French Guiana (100 % BS support) and the morphologically related *H. crocopeplum*. The sequences of *H. perforatum* collected in Texas are phylogenetically closely related to the French *H. perforatum* (100 % BS support), *H. musceum* and *H. pilgerianum*. *Hypoxylon hinnuleum* is revealed as the closest relative of the type of *Nodulisporium hinnuleum* and the Chinese *Nodulisporium* sp. with maximum BS support. The multigene tree includes for the first time *H. lienhwacheense*, which forms an unsupported long branch basal to the *Daldinia* clade inside of a 100 % BS supported major clade including members of *Pyrenopolypous*. Identical to previous studies, the position of *H. griseobrunneum* and *H. trugodes* within the family could not be resolved (Wendt et al. 2018). The remaining clades are in accordance with the results of Wendt et al. (2018). Due to new RPB2 sequence of *H. fragiforme*, the position of this species has changed and now clusters correctly with its sister taxon *H. howeianum*.

TAXONOMY

Based on morphological, chemotaxonomic and phylogenetic data it was concluded that two specimens of *Hypoxylon* species represent new taxa and one species represents the so far unknown sexual morph of *Nodulisporium hinnuleum* for which a new combination is made. All *Hypoxylon* species collected during the survey in Southeast Texas are depicted and described in detail in the following including *H. fendleri* and *H. perforatum*.

Hypoxylon olivaceopigmentum Kuhnert & Sir, sp. nov.

FIGS. 2-3

MB 829104

Typification: USA. TEXAS: Harris Co., Houston, Memorial Park, Houston Arboretum & Nature Center, on a trail between Willow Oak Trail and Arboretum Lake, on dead hardwood, 30 Oct 2016, E. Kuhnert EKMP16002 (**holotype**, GLM-F116104; **isotype**, LIL). Ex-type culture DSM 107924, NRRL 66878. GenBank: ITS = MK287530; 28S = MK287542, *RPB2* = MK287555, *TUB2* = MK287568.

Etymology: In reference to its olivaceous KOH-extractable pigments.

Diagnosis: Differs from *Hypoxylon kanchanapisekii* by having olivaceous extractable pigments, larger perithecia and wider ascospores. Differs from *Hypoxylon croceum* by having vinaceous stromatal surface, olivaceous extractable pigments, smaller perithecia and wider ascospores.

Morphology: Stromata glomerate to pulvinate or effused-pulvinate; coalescent, sometimes growing on top of old stromata, 0.5–12 mm long × 0.5–10 mm broad × 0.4–0.5 mm thick; with inconspicuous perithecial mounds; surface Brown Vinaceous (84) becoming black; pruinose; orange-brown granules immediately beneath the surface and between perithecia; with KOH extractable pigment Olivaceous (48); tissue below the perithecial layer inconspicuous, black. Perithecia pyriform to spherical, 0.2–0.25 mm high × 0.2–0.22 mm diam. Ostioles conical papillate. Asci 8-spored, cylindrical, the spore-bearing parts 65–79 µm long × 9–11 µm wide, stipes 25–45 µm long, with amyloid apical apparatus, discoid, 0.5–0.7 µm high × 1.5–2 µm broad. Paraphyses filiform, copious. Ascospores brown, nearly equilateral, with broadly rounded ends, (9.3–)9.7–11.9(–15.7) × (4.5–)5.0–6.6(–7.2) µm (N= 60, Me= 10.7 × 5.9 µm), with straight germ slit 1/2 to 2/3 of spore-length on the more convex side; perispore indehiscent in 10% KOH, smooth; episporium smooth. Conidiogenous structure on artificial medium with virgariella-like branching patterns (FIG. 3E), rarely with periconiella-like branching patterns (FIG. 3D). Conidiophores hyaline, smooth to roughened. Conidiogenous cells hyaline, smooth to roughened, (17.6–)21.0–32.4(–38.7) × (1.3–)1.6–2.2(–2.3) µm. Conidia ellipsoid, hyaline, smooth to slightly roughened, 3.5–4.3(–4.9) × (1.8–) 2.0–2.7(–2.8) µm (Me= 3.9 × 2.3 µm).

Culture: Colonies on OA covering a 6 cm Petri dish after 3 weeks, at first whitish becoming white and Isabelline (65), velvety to felty, azonate with entire margin; reverse, Apricot (42), Umber (9). Sporulating regions scattered over entire surface of colony.

Secondary metabolites: Stromatal acetone extracts contain BNT and an unknown cohaerin type compound with m/z = 498 (FIG. 14).

Notes: This new species can be differentiated from other *Hypoxylon* species by the following combination of characters: 1) glomerate to pulvinate stromata, 2) pale Olivaceous extractable pigments, 3) nearly equilateral ascospores with straight 1/2 to 2/3 spore-length germ-slit and 4) indehiscent perispore. In general, some of the diagnostic features are similar to *H. kanchanapisekii*, *H. croceum* and *H. hinnuleum*. *Hypoxylon kanchanapisekii* differs by having stromata with less than 20 perithecia, Brown Vinaceous or Umber extractable pigments and different shape and size of the perithecia (spherical 0.1–0.2 vs pyriform to spherical 0.2–0.25 × 0.2–0.22 mm). In addition, this species was only found on monocotyledon hosts. *Hypoxylon croceum* and *H. hinnuleum* can be separated from *H. olivaceopigmentum* in principal by their dark stromatal surface color; extractable pigments, and dimensions of the perithecia and ascospores. Some stromata of *H. olivaceopigmentum* were found to grow on older stromata. This rare phenomenon was so far only reported for some specimens of *H. griseobrunneum* and *H. spengazzinianum* (Kuhnert et al. 2014a, Sir et al. 2016).

Despite the strong statistical support of the phylogenetic relationships within the *H. olivaceopigmentum* clade, this new taxon can easily be distinguished from *H. investiens*, *H. lateripigmentum* and *H. pulicidum*. The latter group features effused stromata and rather small

ascospores (7–9.5 μm), while *H. olivaceopigmentum* possesses pulvinate stromata and larger ascospores (9.5–12 μm) (Kuhnert et al. 2014a). *Hypoxylon hinnuleum*, which is also part of the cluster, forms pulvinate stromata as well but with much darker tones. In contrast, the rather equilateral ascospores with indehiscent perispore can be found in all species of the clade except *H. lateripigmentum* which shows perispore dehiscence in KOH. Moreover, all group members contained BNT in their stromatal crude extracts as a major constituent besides multiple other metabolites. *Hypoxylon investiens* is known to produce daldinone A in addition (Stadler and Fournier 2006), which lacks in related taxa. In respect to the additional secondary metabolites found in the stromata of *H. olivaceopigmentum*, *H. hinnuleum*, *H. lateripigmentum* and *H. pulicicidum*, each species shows a unique chemical pattern that is useful as a discriminative character. The unknown metabolites of *H. pulicicidum* and *H. lateripigmentum* were speculated to belong to the azaphilone family (Bills et al. 2012, Kuhnert et al. 2014a). This assumption is supported by a currently ongoing study, which focuses on the isolation of the chemical entities. First results indicate a close relationship to minutellin type azaphilones (unpublished).

Due to the unique combination of morphological characters, the unique chemical composition of the stromata and the distinct position of the species in the phylogenetic reconstructions we see the erection of *H. olivaceopigmentum* as justified, despite the fact that the species concept is based on a single collection, which however, contains ample amounts of stromata.

Hypoxylon texense Kuhnert & Sir, sp. nov.

FIGS. 4-5

MB 829105

Typification: USA. TEXAS: San Jacinto Co., Sam Houston National Forest, Big Creek Scenic Area, Big Creek Trail, on dead hardwood, 5 Sep 2016, *E. Kuhnert* EKSHN16004 (**holotype**, GLM-F116099; **isotype**, LIL). Ex-type culture DSM 107933, NRRL 66876. GenBank: ITS = MK287536; 28S = MK287548, *RPB2* = MK287561, *TUB2* = MK287574.

Etymology: In reference to the state of Texas where the holotype was found.

Diagnosis: Differs from *Hypoxylon canariense*, *Hypoxylon fendleri*, *Hypoxylon lusitanicum*, *Hypoxylon retpela* and *Hypoxylon urriesii* by having Rust (39) to Dark Brick (86) KOH-extractable pigments. Differs from *Hypoxylon rubiginosum* and *Hypoxylon petriniae* by having orange to reddish-orange stromatal granules, and ascospores with straight to sigmoid germ slit.

Morphology: Stromata effused to effused-pulvinate; 2–60 mm long $\times$ 0.8–20 mm broad $\times$ 0.6–1.1 mm thick; plane or wrinkled to reticulate with inconspicuous perithecial mounds; surface Livid Vinaceous (83), Brown Vinaceous (84), pruinose; yellowish-orange to reddish-orange granules immediately beneath the surface and between perithecia; with KOH extractable pigment Rust (39) to Dark Brick (86); tissue below the perithecial layer inconspicuous, black. Perithecia obovoid, 0.3–0.6 mm high $\times$ 0.28–0.5 diam. Ostioles punctiform, sometime fringed with a white layer. Asci 8-spored, cylindrical, the spore-bearing parts 63–80 μm long $\times$ 5–9.5 μm wide, stipes 34–75 μm long, with amyloid apical apparatus, discoid, 0.7–1.2 μm high $\times$ 1.8–2.8 μm broad. Paraphyses filiform, copious. Ascospores brown, ellipsoid-inequilateral, with narrow rounded ends, (8.7–)9.1–10.8(–11.5) $\times$ (4.0–)4.5–5.4(–5.7) μm (N= 60, Me= 10.0 $\times$ 4.9 μm) with straight or slightly sigmoid germ slit spore-length in the convex side; perispore dehiscent in 10% KOH, inconspicuous to conspicuous transversal striations; epispore smooth.

Conidiogenous structure on natural substrate and artificial medium with virgariella-like to nodulisporium-like branching patterns (FIG. 5C-F). Conidiophores hyaline to pale yellow-brown, smooth. Conidiogenous cells hyaline to pale yellow, smooth, $8.2\text{--}24.9 \times 1.8\text{--}3.7 \mu\text{m}$. Conidia ellipsoid, hyaline, smooth, $3.7\text{--}5.9 \times 2.3\text{--}3.3 \mu\text{m}$ (Me= $4.6 \times 2.8 \mu\text{m}$).

Culture: Colonies on OA covering a 6 cm Petri dish after 3 weeks, at first whitish becoming whitish with Luteous (12) and Isabelline (65) areas, velvety to felty, with rays at the center to the margin, azonate with irregular margin, reverse, pale Umber (9), Isabeline (65). Sporulating regions scattered over entire surface of colony.

Secondary metabolites: Stromatal extracts contain rubiginosin A and mitorubrinol acetate as major metabolites accompanied by further unknown compounds of the rubiginosin type (FIG. 14).

Other specimens examined: USA. TEXAS: Hardin Co., Saratoga, Big Thicket National Preserve, Lance Rosier Unit, on dead hardwood, 12 Nov 2016, *E. Kuhnert EKB16001* (GLM-F116100, LIL; culture DSM 107928, NRRL 66882) and *EKB16003* (GLM-F116101, LIL; culture DSM 107929, NRRL 66883).

Notes: *Hypoxylon texense* is characterized by having stromata with vinaceous shades, yellowish orange to reddish orange granules, Dark Brick to Rust extractable pigments, ascospores with straight or slightly sigmoid germ slits and anamorph with virgariella-like to nodulisporium-like branching patterns. The diagnostic characteristics of this taxon resemble several species, which are compiled in TABLE 2. In general, the color of the KOH-extractable pigments, the color of the stromatal granules and germ slit morphology is useful to set *H. texense* apart from these species.

Hypoxylon texense is part of a morphological and chemical rather homogeneous phylogenetic clade comprised of *H. cercidicola*, *H. petriniae* and *H. rubiginosum*. All species usually form effused brown-vinaceous stromata with orange to brown KOH-extractable pigments and inequilateral ascospores with inconspicuous to conspicuous ornamentation. Their stromatal extracts are composed of azaphilones of the mitorubrin- and rubiginosin-type with rubiginosin A usually prevailing (Stadler et al. 2004). Each species exhibits a different HPLC profile depending on the composition of the minor metabolites, which can be used as discriminating character. The phylogenetic distance of *H. texense* supports the status as a new species.

***Hypoxylon hinnuleum* (G. Sm.) Kuhnert & Sir comb. nov.**

FIGS. 6-7

MB 830754

Basionym: *Nodulisporium hinnuleum* G. Sm., Trans. Br. Mycol. Soc. 45(3): 390 (1962).

Typification: USA. KANSAS: Manhattan, Kansas State University, isolated from *Sorghum bicolor* seeds, Apr 1958, S. M. Pady S. 168 (ATCC 36255 as *Nodulisporium hinnuleum*), [duplicates in IMI 083003, LSHB BB373, CBS 286.62, MUCL 3621 (studied), GenBank Acc. No: ITS – MK287537, 28S – MK287549, TUB2 – MK287575, RPB2 – MK287562].

Morphology: Stromata glomerate to pulvinate or effused-pulvinate, coalescent, rarely perithecioid; 0.8–7 mm long $\times$ 0.5–6 mm broad $\times$ 0.4–0.6 mm thick; with inconspicuous to conspicuous perithecial mounds; surface Dark Vinaceous (82) to Sepia (63), becoming black; orange brown granules immediately beneath the surface and between perithecia; with KOH extractable pigments Dark Brick (86); tissue below the perithecial layer inconspicuous, brown to black, 0.1–0.3

mm thick. Perithecia piriform to rarely obovoid, 0.25–0.33 mm high $\times$ 0.22–0.3 mm diam. Ostioles with prominent conical black papillae. Asci 8-spored, cylindrical, the spore-bearing parts 50–63 μ m long $\times$ 4.3–6.6 μ m wide, stipes 30–48 μ m long, with amyloid apical apparatus, discoid, 0.5–0.7(–0.9) μ m high $\times$ 1.4–1.6(–2.2) μ m broad. Paraphyses filiform, copious. Ascospores brown, ellipsoid nearly equilateral, rarely pyriform or oblong ovoid, with narrowly rounded, less frequently broadly rounded ends, (7.2–)7.6–9.5(–11.7) $\times$ (2.8–)3.7–4.5(–4.8) μ m (N= 60, Me= 8.6 $\times$ 4.1 μ m), with faint straight 2/3 spore-length germ-slit; perispore indehiscent in 10% KOH, smooth; episporium smooth. Conidiogenous structure on natural substrate and artificial medium with virgariella-like, nodulisporium-like and periconiella-like branching patterns (FIG. 7C–H). Conidiophores hyaline, smooth to slightly roughened. Conidiogenous cells hyaline, smooth to slightly roughened, (9.1–)12.4–18.0(–20.8) $\times$ 1.7–2.4 μ m. Conidia ellipsoid, hyaline to pale brown, smooth to roughened, 3.6–5.1 $\times$ 2.1–3.1 μ m.

Culture: Colonies on OA covering a 6 cm Petri dish after 3 weeks, at first whitish becoming Apricot (42) powdery with scarce white mycelium that form rays towards the margins; reverse, Orange (7), Siena (8). Sporulating regions scattered over entire surface of colony in Apricot (42) areas.

Secondary metabolites: The stromatal extracts of *H. hinnuleum* contain BNT, minutellin D and various unknown azaphilones of the cohaerin type (FIG. 8). Liquid cultures produce demethoxyviridin and demethoxyviridiol in CM medium (FIG. 9).

Specimens examined: USA. TEXAS: San Jacinto Co., Sam Houston National Forest, Big Creek Scenic Area, Big Creek Trail, on dead hardwood, 5 Sep 2016, E. Kuhnert EKSHN16003 (GLM-F116098 and LIL), culture DSM 107932; Harris Co., Houston, Memorial Park, Houston Arboretum & Nature Center, on a trail between Willow Oak Trail and Arboretum Lake, on dead hardwood, 30 Oct 2016, E. Kuhnert EKMP16005 (GLM-F116106 and LIL), culture DSM 107926.

Other specimens compared: USA. GEORGIA, Rabun Co., Mountain City, on decorticated wood of *Liriodendron tulipifera*, J. H. Miller, 6610, (GAM 2197, HOLOTYPE of *H. croceum*); Rabun Co., Barron Playhouse, on rotten wood of *Liriodendron tulipifera*, 17 Aug 1932, J. H. Miller (GAM 2198), Rabun Co., on rotten decorticated oak log, 7 Aug 1929, J. H. Miller (GAM 2199). VENEZUELA: Mountain forest, Rio Aguada, above Valencia, 7.8.1932, Chardon, C.E. & Stelling 856, dead wood (GAM 4109, ISOTYPE of *H. semitruncatum*).

Notes: The material presented here has teleomorphic features that clearly suggest close relationships to *H. croceum* and *H. minicroceum*. A detailed comparison of the morphological features is listed in TABLE 3. *Hypoxylon hinnuleum* can be mainly separate from *H. croceum* by its ascospores size and extractable pigments. In addition, *H. hinnuleum* forms virgariella-, nodulisporium and periconiella-like conidiophores and contains minutellin D and BNT in stromatal extracts, both of which lack in the herbaria material of *H. croceum* (with the exception of *H. semitruncatum*). In contrast, *H. minicroceum* differs by smaller perithecia, larger and light brown ascospores with long germ slit and umber/cinnamon pigments.

Our initial investigation of the Texan material led to the conclusion that the specimens EKSHN16003 and EKMP16005 represent typical collections of *H. croceum*, which mainly varied in the color of the KOH-extractable pigments from the description provided by Ju & Rogers (1996). Even though the pigment color is usually a reliable stable character within species of the Hypoxylaceae (Stadler and Hellwig 2005), they have to be treated with care for the following reasons. First the perception of certain color tones is a subjective criterion differing between authors and second the color reaction can be depending on the age of the specimens as pigments start to degrade over time. All specimens studied by Ju & Rogers (1996) were collected between 1896 and 1932, which in our opinion was a reasonable explanation for the observed differences in pigment color. However, the chemical analyses of the stromatal constituents of the type material of *H. croceum* and various other

collections of this species deposited at GAM compared with our Texan material revealed striking differences that cannot be explained with instability of compounds. Rather we recognized three distinct chemotypes (FIG. 8) between the type of *H. croceum* (and other conspecific collections from Georgia, USA), the isotype of *H. semitruncatum* (synonym of *H. croceum*) and *H. hinnuleum*. All chemotypes contain a different combination of cohaerin-like azaphilones as dominating metabolites. Of these compounds only minutellin D (Kuhnert et al. 2017b) can be identified, which solely appears in *H. hinnuleum* and *H. semitruncatum*. We therefore concluded that *H. croceum* is a species complex containing at least three species. Based on the present data, it appears plausible to reconstitute *H. semitruncatum* as a separate species, also because the type material originates from Venezuela. However, we refrain from this for the time being until fresh material has been re-collected from this area and cultures can be obtained for molecular analysis. In a previous survey of Texan Hypoxylons, *H. minicroceum* was proposed as a new species resembling *H. croceum* in most parts except for the size of stromata and KOH-extractable pigments (Vasilyeva and Stephenson 2014). Both characters are in agreement with our material but the size of the perithecia, ascospores and germ slit morphology differ. Our attempts to acquire the holotype for comparison failed as the original collector passed away and the respective herbaria (VAL) is not responding to loan requests. Furthermore, no viable cultures have been obtained from this species. Based on the morphological differences of the teleomorph according to the literature we concluded that our Texan collections represent a different, putatively undescribed species.

A BLAST search analysis based on the ITS region of the corresponding strains revealed high similarity with the ITS sequence of the type strain of *Nodulisporium hinnuleum* (GenBank Acc. No. NR_145212, CBS 286.62), which was isolated from a *Sorghum bicolor* seed in Kansas (USA) (Smith 1962). The genus *Nodulisporium* is the “asexual state” of all known Hypoxylaceae and has been synonymized with *Hypoxylon* in the context of the “one fungus – one name” concept (Réblová et al. 2016). Thus, the name *N. hinnuleum* was likely typified by a seed-borne strain of a member of the *H. croceum* complex. To verify this assumption, we re-sequenced the type strain and included it into the multigene phylogeny. The very close relationship of *N. hinnuleum* with the Texan strains was confirmed by the respective phylogenetic analysis (see FIG. 1). Furthermore, the dimensions of the conidiogenous structures of *N. hinnuleum* according to literature (Smith 1962) and our isolates are in agreement (conidia $4\text{--}5 \times 2\text{--}2.2$ vs $3.6\text{--}5.1 \times 2.1\text{--}3.1$ μm ; conidiogenous cells $10\text{--}25 \times 2$ vs $12.4\text{--}18.0 \times 1.7\text{--}2.4$ μm). Therefore, we concluded that *N. hinnuleum* is the asexual state of the Texan collections and created a new combination, *Hypoxylon hinnuleum* comb. nov. In addition, the secondary metabolite profiles of the corresponding cultures were compared under various conditions revealing the production of identical compounds (FIG. 9). In case of the CM medium, these metabolites were identified as the known steroids demethoxyviridin and demethoxyviridiol, which are potent inhibitors of phosphatidylinositol 3-kinases (Woscholski et al. 1994). The latter compound has been originally reported from a different *N. hinnuleum* strain (ATCC 24911), which was obtained from a peanut seed in Virginia, USA. The investigation of the biosynthetic pathway of viridin-type metabolites was subject of a recent study (Wang et al. 2018). The respective genome-sequenced producing strain has been identified as *Nodulisporium* sp. and has been isolated from an *Everniastrum* lichen collected in the Yunnan province in China. We obtained sequences of the fungus from the corresponding authors and included them in our multigene phylogenetic reconstruction, revealing the close relationship of the Chinese *Nodulisporium* strain with *H. hinnuleum* (FIG. 1). Moreover, the BLAST search analysis of the ITS locus of *H. hinnuleum* retrieved more than 50 unnamed hits with an identity between 97 % and 99 %. These sequences are mainly derived from endophytic and endolichenic isolates from the southern USA (U'Ren et al. 2012, 2016), which is consistent with the known distribution of *H. croceum* and allies. The corresponding isolates were obtained from a broad host range of trees (e.g., *Quercus*, *Taxodium*, *Pinus*), lichens (e.g. *Cladonia*,

Diploschistes, *Parmotrema*, *Sticta*) and even mosses. Whether, all of those organisms represent real hosts of *H. hinnuleum* and related species in a biological context has to be questioned, but cannot be answered in the course of this study. To confirm that the strains belong to the *H. croceum* species complex, we included them into the ITS phylogeny, where they formed a highly supported clade with *H. hinnuleum*. Interestingly, besides the Chinese *Nodulisporium* sp. further sequences originate from East Asian environmental samples (China, South Korea, Japan) pointing towards a more global appearance of the species complex. However, whether those isolates belong to *H. hinnuleum*, *H. croceum* or a different related species cannot be verified without authentic teleomorphic collections from these regions.

It is noteworthy that a collection of *H. croceum* has been reported from driftwood in a mangrove estuary in Florida and the corresponding culture produced sordarin-type antifungals (Daferner et al. 1999). Furthermore, various binaphthyls (hinnulin A – D) have been obtained from a strain designated as *N. hinnuleum* (ATCC 36102) (Schlingmann et al. 2011). Our isolates did not produce any of these compounds under the tested conditions. In conclusion, it can be stated that the *H. croceum* complex is widely distributed across the Northern hemisphere ranging from the tropics to the southernmost areas of the temperate zones with hot spots in the Southeast USA and Southeast Asia. An extensive search within our secondary metabolite screening library including more than 1000 extracts derived from over 300 Xylariales strains (mainly of the family Hypoxylaceae and Xylariaceae) showed that the production of viridin-type steroids is restricted to members of the *H. croceum* complex. Therefore, these compounds can be used as chemotaxonomic markers for this species complex.

Hypoxylon fendleri

FIGS. 10-11

MB206066

Morphology: Stromata effused-pulvinate; 15–60 × 10–12 × 0.5–1 mm thick; with conspicuous to inconspicuous perithecial mounds, in some case with wrinkles between the perithecia; surface Brown Vinaceous (84) with orange tones, pruinose; orange granules immediately beneath the surface and between perithecia; with KOH extractable pigments Orange (7); tissue below the perithecial layer inconspicuous, black. Perithecia obovoid 0.35–0.6 mm high × 0.3–0.5 diam, ostioles umbilicate. Asci fragmentary, with amyloid apical apparatus, discoid, 0.5–1.1 µm high × 2–2.5 µm broad. Ascospores brown to dark brown, ellipsoid-inequilateral, with narrow rounded ends, (7.8–)8.8–10.7(–12.2) × (3.8–)4.1–5.1(–5.9) µm (N=60, Me=9.8 × 4.6 µm), with sigmoid germ slit spore-length on the convex side; perispore dehiscent in 10% KOH, smooth to faintly striated; epispore smooth.

Conidiogenous structure on natural substrate and artificial medium with virgariella-like and nodulisporium-like branching patterns (FIG. 11C-G). Conidiophores hyaline, smooth to slightly roughened, Conidiogenous hyaline, smooth to slightly roughened, (23.5–)23.9–30.3(–30.8) × (2.1–)2.7–3.3(–3.4) µm. Conidia ellipsoid, hyaline, smooth, (5.0–)5.6–5.8(–5.9) × (3.1–)3.2–3.8(–3.9) µm.

Culture: Colonies on OA covering a 6 cm Petri dish after 3 weeks, at first whitish and Yellow-green (71) becoming white and Isabelline (65) velvety to felty, azonate, irregular entire margin; reverse, Isabelline (65), Umber (9) and Citrine (13). Sporulating regions scattered over entire surface of colony.

Secondary metabolites: Stromatal extracts of the Texan specimens contain mitorubrinic acid as main compound. In addition, mitorubrinol and mitorubrinol acetate can be found (FIG. 14).

Specimens examined: USA. TEXAS: Harris Co., Houston, Memorial Park, Houston Arboretum & Nature Center, Willow Oak Trail, on dead wood, 30 Oct 2016, *E. Kuhnert* EKMP16001 (GLM-F116103, LIL; culture DSM 107923 & NRRL 66877) and EKMP16007 (GLM-F116107, LIL; culture DSM 107927 & NRRL 66881).

Notes: Ju & Rogers (1996) mentioned that *H. fendleri* is very common in the tropical regions. This fungus is characterized by having a vinaceous stromatal surface, orange stromatal granules, orange extractable pigments and ascospores with sigmoid germ slit. In this respect, the Texan collections are typical. The anamorph of *H. fendleri* was described as nodulisporium-like in culture (Ju and Rogers 1996). We found virgariella- and nodulisporium-like branching patterns on the natural substrate and in culture implying that this character is not diagnostic for the species. Hellwig et al. (2005) reported the presence of mitorubrinol and mitorubrinol acetate in the holotype of *H. fendleri*, both of which can be found in our specimens. However, the dominant compound in fresh Texan stromata seems to be mitorubrinic acid, which lacks in the type material. So far, this metabolite has only been detected in relatively fresh collections and therefore we assume it to be quite unstable, explaining the observed discrepancies. As no cultures or sequences of the type material of the fungus exist, it would be important to epitypify the species. In our opinion, the material from Texas does not serve this purpose as the holotype originates from Venezuela. We prefer epitypification of collections if possible from the same location or at least same geographical area (i.e. northern part of South America or Caribbean Islands).

Hypoxylon perforatum

FIGS. 12-13

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Morphology: Stromata effused-pulvinate; 4–7 mm long × 2–4.5 mm broad × 0.6–1 mm thick; with conspicuous to inconspicuous perithecial mounds; surface Brown Vinaceous (84), pruinose; orange-brown granules immediately beneath the surface and between perithecia; with KOH extractable pigments Luteous (12) to Citrine (13); tissue below the perithecial layer inconspicuous, black. Perithecia spherical to obovoid, 0.3–0.45 mm high × 0.26–0.3 diam, Ostioles umbilicate, fringed with a white layer and most often opening at the centre of a slightly raised disc, 0.2–0.3 mm diam. Asci 8-spored, cylindrical, the spore-bearing parts 65–85 µm long × 8–9(14) µm wide, stipes 36–74 µm long, with amyloid apical apparatus, discoid, 0.8–0.9(–1.3) µm high × 2–2.5 µm broad. Paraphyses filiform, copious. Ascospores brown to dark brown, ellipsoid-inequilateral, with narrow rounded ends, (10.3–)10.9–12.7(–13.0) × (4.1–)4.7–6.0(–7.0) µm (N= 60, Me= 11.8 × 5.4 µm), with straight germ slit spore-length on the convex side; perispore dehiscent in 10% KOH, inconspicuous to conspicuous striated; episore smooth. Conidiogenous structure on artificial medium with virgariella-like to nodulisporium-like branching patterns (FIG. 13D-H). Conidiophores hyaline to pale yellow, smooth to slightly roughened. Conidiogenous cells hyaline to pale yellow, smooth to slightly roughened, 18.2–25.2 × (1.7–)2.1–2.6 µm. Conidia ellipsoid, hyaline, smooth, 4.9–5.8 × 2.5–3.2 µm.

Culture: Colonies on OA covering a 6 cm Petri dish after 4 weeks, at first whitish becoming whitish with Yellow Green (18) areas, cottony, slightly zonate with entire margin; reverse, mainly uncolored with Isabelline (65) pale areas. Sporulation in Yellow Green (18) areas.

Secondary metabolites: The stromatal extract of the Texan specimen contained only hypomiltin (FIG. 14).

Specimens examined: USA. TEXAS: Hardin Co., Saratoga, Big Thicket National Preserve, Lance Rosier Unit, 12 November 2016, on dead wood, 12 Nov 2016, *E. Kuhnert EKB16006* (GLM-F116102 and LIL), culture DSM 107930 & NRRL 66884.

Notes: *Hypoxylon perforatum* is one of the few Hypoxylaceae species with a cosmopolitan distribution (Ju and Rogers 1996, Stadler et al. 2008). The light greenish yellow KOH-extractable pigments and the ostiolar discs with a conspicuous white layer are diagnostic characters for the species. Most of the features of the Texan specimens are in accordance with previous descriptions (Ju & Rogers 1996), however their perithecia were slightly larger (0.1–0.3 mm vs. 0.3–0.45 mm) and had brighter subsurface granules. A virgariella-like anamorph was originally reported for *H. perforatum* (Ju & Rogers 1996), whereas the Texan strain exhibited a nodulisporium-like asexual morph in addition. In our opinion, this small differences do not merit the erection of a new species. Ju & Rogers (1996) designated two collections of *Sphaeria perforata* as lectotype and isolectotype of this taxon. The origin of these collections seems ambiguously as Pennsylvania (Bethlehem) as well as North Carolina (Salem) have been stated. Even though an epitypification of the *H. perforatum* is important because no type cultures (and sequences) exist, we recommend to recollect the species from Eastern USA for this purpose.

DISCUSSION

The polyphasic concept for species delimitation within the Hypoxylaceae led to the establishment of *H. olivaceopigmentum* and *H. texense* as new species and enabled the identification of the sexual morph of *N. hinnuleum*.

Thoughts on the *H. rubiginosum* complex.–

Whereas *H. olivaceopigmentum* can be easily distinguished from other *Hypoxylon* species by its morphological characteristics, *H. texense* falls into the continuously growing group of the *H. rubiginosum* complex. The original broad species concept of *H. rubiginosum* by Miller (1961) has meanwhile been resolved into more than 30 different species (Stadler et al. 2004, 2008). Members of the complex sensu stricto feature sepia to brown vinaceous (or dark brick) stromata with effused-pulvinate to pulvinate shape, yellowish brown to orange granules, orange KOH-extractable pigments and inequilateral ascospores with dehiscent perispore that is usually striated. This description comprises *H. canariense*, *H. lusitanicum*, *H. petriniae*, *H. retpela*, *H. rubiginosum*, *H. salicicola*, *H. texense* and *H. urriesii*. As the ascospore size ranges of these species often overlap, it becomes a difficult task to differentiate them based on morphological characters alone. The host-specificity of some European members is helpful to identify the respective species, such as *H. salicicola* (*Salix*) or *H. petriniae* (*Fraxinus*). In addition, the investigation of the stromatal metabolite extracts by HPLC has proved to be a valuable tool to distinguish taxa of the *H. rubiginosum* complex (Fournier et al. 2010). Especially, *H. retpela*, which produces an unknown azaphilone ($m/z = 442$) as dominating compound and *H. petriniae* containing BNT display unique profiles. Moreover, *H. canariense*, *H. lusitanicum*, *H. rubiginosum* and *H. texense* show rubiginosin A as major constituents, whereas *H. salicicola* and *H. urriesii* are recognized by mitorubrinol acetate as the main metabolite. The multigene phylogenetic analysis further demonstrates that *H. rubiginosum* and allies are a rather homogenous clade with still reasonable sequence variety to resolve relationships at the species rank as seen for *H. texense*. However, the majority of members from the complex are not represented in the tree as most of the

loci could not be obtained so far or corresponding cultures do not exist at all (e.g. *H. retpela*). *Hypoxylon cercidicola*, which is also part of the *H. rubiginosum* clade in our phylogeny, shares most of the typical characteristics of its sister taxa but forms erumpent discoid stromata as diagnostic feature. In order to get a better understanding of the speciation and species delimitation within the *H. rubiginosum* complex extensive sampling effort is necessary in order to collect multiple specimens of the different species for sequencing.

Status of the genus *Nodulisporium*.–

Currently, 37 validly described species of the hyphomycetous genus *Nodulisporium* are assigned to the Hypoxylaceae (according to Index Fungorum). Prior to this study, only two of them were linked to a sexual morph of the family namely *N. griseobrunneum* (*H. griseobrunneum*) and *N. ochraceum* (*H. fragiforme*). Whereas the first relationship was verified by extensive phylogenetic studies (Kuhnert et al. 2014a), the status of *N. ochraceum* was controversially discussed due to the lack of living cultures. Stadler et al. (2013) reported that the type material is housed in the fungarium B but is devoid of conidiogenous structures (pers. comm.) and can therefore not be synonymized with any *Hypoxylon* species with certainty. In contrast, Réblová et al. (2016) refers to Von Arx (1982) who seemingly proved the conspecificity by morphological comparison. However, it is doubtful whether an unambiguously identification based solely on micromorphological character is feasible in this case. A brief search in the databases of various culture collections (ATCC, CBS, IMI, MUCL, NRRL) revealed the presence of living strains of at least nine further species (*N. acervatum*, *N. africanum*, *N. cinnamomeum*, *N. didymosporum*, *N. gregarium*, *N. honiaraense*, *N. indicum*, *N. tuberum*, *N. verrucosum*). This offers the opportunity to sequence these isolates and presumably identify the respective sexual morph. As the amount of morphologically usable characters for *Nodulisporium* is rather limited and phylogenetic data have to be treated carefully when interpreting incomplete trees (i.e. sequence data is missing for certain species), it is very helpful to complement these criteria with information about the natural products produced by the strains. This polythetic approach was essential for finding the sexual state of *N. hinnuleum*.

The value of conidiophore branching patterns for species delimitation.–

Ju & Rogers (1996) established the type of conidiophore branching patterns within the form genus *Nodulisporium* (e.i. sporothrix-, virgariella-, nodulisporium-, periconiella-like) as a diagnostic feature at the species level for *Hypoxylon*. This system was adopted by many mycologists and has been applied to other Hypoxylaceae genera (e.g. Stadler et al. 2014). Recent studies showed that in many cases the branching of conidiophores is a plastic character that can change within species or even strains (Stadler et al. 2014, Sir et al. 2016, 2018, Kuhnert et al. 2017a) as exemplified here by *H. hinnuleum* where we observed a succession of conidiophore complexity from simple (virgariella-like) to strongly branched (periconiella-like). All other studied species herein showed at least two branching types. Therefore, it seems likely that the type of branching pattern may be an age-dependent developmental feature of the culture (artificial substrate) or the environmental conditions (natural substrate) rather than intrinsic to the taxon itself. Another factor influencing the assignment of the correct subtypes is the sample size of conidiophores. As more specimens and cultures of a species are investigated, the more likely it becomes to expand the range of branching patterns. Hence, it is strongly recommended to interpret this character carefully and to avoid erecting new species based on the subclassification of the asexual structures as a sole discriminative character.

Concluding remarks.–

This study expands the knowledge about the diversity of the genus *Hypoxylon* in the USA and provides new phylogenetic as well as chemotaxonomic data which help for faster and more reliable species identification. Moreover, the taxonomic position of the widespread *H. croceum* complex and its endophytic or environmental isolates was resolved for the first time enabling population studies and targeted search for new producers of demethoxyviridin and demethoxyviridiol, which are important natural products widely used in biological studies (Wang et al. 2018). The subtropical areas of southern USA are to a great extent underexplored for fungal diversity and offer a great opportunity in finding new taxa.

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Figure 1. Multigene phylogenetic reconstruction of the Hypoxylaceae dataset as inferred from a cured ITS, 28S, *RPB2* and *TUB2* MAFFT alignment. Likelihood (ML) bootstrap support values above 50%, from 1000 Bootstrap replicates are assigned to the tree topology of the most likely (lnL=-39763.56902) tree found by PhyML. Strains obtained during this study are highlighted in bold. Sequences derived from type material are labelled with T (holotype) or ET (epitype). Species complexes discussed in the context of this study are shown in grey boxes (HCC – *Hypoxylon croceum* complex, HRC – *Hypoxylon rubiginosum* complex). Scaling shows nucleotide substitutions per site.

Figure 2. *Hypoxylon olivaceopigmentum*. A: Stromatal habit on wood. B: Stromatal habit on old stromata (arrows). C, D: Detail of stroma on wood. E: Detail of stroma on old stromata layers (arrows). F: Close-up view on stromatal surface showing smaller black papillae. G: Stroma in section showing perithecia. H: Stroma in section, showing perithecia and three stromatal layers (arrows). I: Stromatal granules in water. J: KOH-extractable pigments. K: immature and mature asci under

polarized light. L, M: Ascus tips in Melzer's reagent (arrows). N: Ascospores in water. O: Ascospores in 10% KOH, showing germ slit (arrows). P: Ascospore under SEM. Scale bars. A, B: 2 mm. C-E: 1 mm. F-H: 0.5 mm. K, N, O: 10 μ m. L, M: 5 μ m, P: 2 μ m.

Figure 3. Macromorphology of culture and anamorphic structures of *Hypoxylon olivaceopigmentum*. A, B: Culture on 6 cm OA plates after 3 weeks of incubation, A surface of colony, B reverse of colony. C, E, F, G: General view of anamorph structure with virgariella-like branching patterns. D: Detail of periconiella-like branching patterns. H: Mature conidia. Scale bars. C, G: 20 μ m. E-F: 10 μ m. D, H: 5 μ m.

Figure 4. *Hypoxylon texense* (A, B, D, E, H-O from isotype; C and F from EKBT16003 and G from EKBT16001). A: Stromatal habit on wood. B: Close-up view on wrinkled to reticulate stromatal surface. C, G: Stromatal habit on bark. D: Stromatal granules in water. E: KOH extractable pigments. F: Close-up view on stroma showing inconspicuous perithecial mounds and ostioles with white layer (arrows). H: Stroma in section showing perithecia and ostiolar white layer (arrow). I: Ascus tips in Melzer's reagent (arrows). J: Immature and mature asci under polarized light. K: Anamorph from

substrate with nodulisporium-like branching patterns. L: Anamorph from substrate with virgariella-like branching patterns. M: Ascospores. N: Ascospores showing slightly sigmoid and straight germ slits (arrows). O: Ascospores in 10% KOH showing dehiscent faintly striate perispore (arrow). P: Ascospore under SEM. Scale bars. A, C: 10 mm. B: 2 mm. F-H: 1 mm. J, K: 20 μ m. L-O: 10 μ m. I, P: 5 μ m.

Figure 5. Macromorphology of culture and anamorphic structures of the *Hypoxylon texense*. A, B: Culture on 6 cm OA plates after 3 weeks of incubation, A surface of colony, B reverse of colony. C, D: General view of anamorph structure with virgariella-like branching patterns, showing intercalary conidiogenous cell in D (arrow). E: Detail of nodulisporium branching patterns and conidia. F: conidiogenous cells and immature conidia. Scale bars. C: 20 μ m. D-F: 10 μ m.

Figure 6. *Hypoxylon hinnuleum*. A, C: Stromatal habit. B: Close-up view on stromatal surface. D: Perithecioid stroma showing black papillae (arrows). E: Immature and mature asci under polarized light. F: Stroma in section showing perithecia. G: Stromatal granules in water. H: KOH-extractable pigments. I: Ascospores in water. J: Anamorph from substrate. K: Ascus tips in Melzer's reagent (arrows). L: Ascospore under SEM. M: Pyriform ascospores in water. N: Ascospores in 10% KOH showing germ slit. Scale bars. A, C: 1 mm. B, D, F: 0.5 mm. E, J: 20 μ m. I, K, M: 10 μ m. L, N: 5 μ m.

Figure 7. Macromorphology of culture and anamorphic structures of *Hypoxylon hinnuleum*. A, B: Culture on 6 cm OA plates after 3 weeks of incubation, A surface of colony, B reverse of colony. C, D: General view of anamorph structure. E: Detail of whorled conidiogenous cells. F: Details of virgariella-like branching patterns, G: Details of nodulisporium-like branching patterns. H: Detail of periconiella-like branching patterns. I: conidia. Scale bars. C, D: 20 μ m. E-H: 10 μ m. I: 5 μ m.

Figure 8. Comparison of the HPLC-UV/vis (200-600 nm) profiles of stomatal extracts from *H. hinnuleum* (A, B), *H. croceum* (C, D) and its synonym *H. semitruncatum* (E). Compounds that are specific for the each species are highlighted (green – *H. hinnuleum*, yellow – *H. croceum*, purple – *H. semitruncatum*) and compounds only occurring in *H. hinnuleum* and *H. semitruncatum* are

highlighted in blue. Type specimens are indicated (T- holotype, IT – isotype). 1 – BNT, 2 – minutellin D, C – unknown cohaerin-type azaphilones, P – preserving agent.

Figure 9. HPLC-UV profiles of crude extracts generated from 14 days old mycelia of *H. hinnuleum* and the type strain of *N. hinnuleum* grown in CM fermentation medium. Grey frames highlight identical secondary metabolites produced by all strains. 1 – demethoxyviridiol, 2 – demethoxyviridin.

Figure 10. *Hypoxylon fendleri*. A: Stromatal habit on wood. B: Stromatal granules in water. C: KOH extractable pigments. G: Stromatal habit on bark. D, E: Close-up view on stromatal surface: F: Stroma in section showing perithecia G: Close-up view on stromatal surface showing inconspicuous perithecial mounds and white ostiolar discs (arrows). H: Close-up view on stromatal surface showing wrinkles between the perithecia mounds. I: Ascus tips in Melzer's reagent (arrows). J: Ascospores in water. K: Ascospores showing sigmoid germ slit (arrows). L: Ascospores in 10% KOH showing

dehiscent perispore (arrows). M: Ascospore under SEM. N: Detail of perispore under SEM. Scale bars. A: 10 mm. D, E: 2 mm. F-H: 1 mm. I-L: 10 μ m. M, N: 2 μ m.

Figure 11. Macromorphology of culture and anamorphic structures of *Hypoxylon fendleri*. A, B: Culture on 6 cm OA plates after 3 weeks of incubation, A surface of colony, B reverse of colony. C, D: General view of anamorph structure with virgariella-like branching patterns. E: Detail of terminal conidiogenous cells. F: nodulisporium-like branching patterns. G: conidiogenous cells and conidia (arrow). H: Mature conidia. Scale bars. C: 20 μ m. D-H: 10 μ m.

Figure 12. *Hypoxylon perforatum*. A: Stromatal habit on twig. B, E: Detail of stromata. C: Stromatal granules in water. D: KOH extractable pigments. F: Close-up view on stromatal surface showing ostiolar discs. G: Stroma in section showing perithecia and slightly raised disc (arrows). H: Detail of white ostiolar layer in section (arrow). I: Immature and mature asci under polarized light. J: Ascospores in water. K: Ascospores showing germ slit (arrows). L: Ascospores in 10% KOH showing dehiscent perispore (arrows). M: Detail of striated perispore (arrows). N: Ascospore under SEM. O: Ascospore showing germ slit (arrow).

Amyloid apical apparatus in Melzer's reagent (arrow). Scale bars. A, B: 3 mm. E: 1 mm. F, G: 0.5 mm. H: 0.1 mm. I: 30 μ m. J-M: 10 μ m. N, O: 5 μ m.

Figure 13. Macromorphology of culture and anamorphic structures of *Hypoxylon perforatum*. A, B: Culture on 6 cm OA plates after 4 weeks of incubation, A surface of colony, B reverse of colony. C: Detail of yellow sporulation areas (arrows). D, G: General view of anamorph structure. H: Detail of conidiogenous cells and immature conidia. I: Mature and immature conidia. Scale bars. C: 5 mm. D, E: 20 μ m. F-I: 10 μ m.

Figure 14. HPLC-UV profiles of stromatal extracts from Texan *Hypoxylon* species and characteristic DAD spectra of major constituents. A – *H. olivaceopigmentum*, B – *H. texense*, C – *H. fendleri*, D – *H. perforatum*, BNT – binaphthalene tetrol, H – hypomiltin, M – mitorubrinol, MA – mitorubrinol acetate, MCA – mitorubrinic acid, RA – rubiginosin A, UC – unknown compounds.

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