Studies on *Hypoxylon ferrugineum* (Xylariaceae), a rarely reported species collected in the urban area of Graz (Austria)

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**Abstract:** *Hypoxylon ferrugineum* has been collected repeatedly on branches of *Tilia* in the urban area of Graz. It is described and illustrated based on this newly collected material as well as the previously known collections from Austria. An overview of the known distribution of *H. ferrugineum* is given. Its ecology, host specificity, and some newly observed as well as previously rarely reported morphological characters are discussed. *Hypoxylon ferrugineum* appears to be a distinct and well-characterized species based on morphology as well as the results of phylogenetic analyses of β-tubulin, α-actin, and ITS sequences.

**Keywords:** Ascomycota, ecology, host specificity, pyrenomycetes, *Tilia, Xylariaceae*.


**Schlüsselwörter:** Ascomycota, Ökologie, Pyrenomyceten, *Tilia, Wirtsspezifität, Xylariaceae*.

**Introduction**

The family *Xylariaceae* contains over 1300 accepted species in about 76 genera (*Lumbsch & Huhndorf, 2010; Stadler et al., 2013*) and can be divided into two large groups, i.e. the *Xylarioidae* (*xylarioid Xylariaceae*) and the *Hypoxyladoideae* (*hypoxylloid Xylariaceae*) represented mainly by the genera *Xylaria* Hill ex Schrank and *Hypoxylon* Bull., respectively. Thirty *Hypoxylon* species have been reported from Europe (*Fourrier et al., 2010; Fourrier, 2014*), including two new species recently described from the French Pyrenees (*Fourrier, 2014*). Some *Hypoxylon* species are known to be endophytes in their asexual state (*Petrini & Petrini, 1985; Petrini & Muller, 1986*), whereas the sexual states of known European taxa grow almost exclusively on dead wood of dicotyledons. Molecular studies help to shed light on the connection of endophytic strains to sexual morphs, e.g. in the case of the tropical insecticide-producing *H. pulicidum* J. Fourn., *Polishok & Bills* (*Bills et al., 2014*).

European *Hypoxylon* species show great intrageneric variation regarding host specificity and could be roughly divided into three groups based on their host preferences: i) species that are highly restricted to a single host genus, e.g. *H. cedricola* (Berk. & M.A. Curtis ex Peck) Y.M. Ju & J.D. Rogers and *H. fraxinophilum* Pouzar which are confined to *Fraxinus*; ii) species that show strong preference for a single host but are also known from other woody plants, e.g. *H. fragiforme* (Pers.) J. Kickx f. which mainly grows on *Fagus* but is occasionally encountered on *Alnus, Betula, Tilia*, etc. (*Petrini & Muller, 1986*); iii) plurivorous species such as *H. fuscos* (Pers.) Fr. which is known from various deciduous trees. *Hypoxylon ferrugineum* G.H. Oth appears to be host-specific to *Tilia* (*Scheuer et al., 2001; Stadler et al., 2008*) although a few collections on different substrates are mentioned in literature (*Jong & Rogers, 1972; Stadler et al., 2004; Stadler et al., 2008*) (see discussion below).

In the course of this study we collected and searched for *H. ferrugineum* from 2011–2015 in Graz (Styria, Austria) and surrounding areas. Our study shows that *H. ferrugineum* is a rather common fungus on branches of *Tilia* in certain parts of the urban area of Graz, which is a surprising result given that overall there exist only relatively few collections of *H. ferrugineum* worldwide and it is, consequently, reported to be an uncommon species (*Scheuer, 2001; Stadler et al., 2008*). We collected *H. ferrugineum* most frequently after strong winds and heavy snowfalls which caused the branches to fall on the ground. The plentiful, freshly collected material allowed detailed study of the morphology and ecology as well as the first phylogenetic analyses of *H. ferrugineum*.

**Material and methods**

A part of the collected branches with stromata of *H. ferrugineum* was stored outdoors for more than one year in order to observe a possible further development or decay of the stromata. For extraction of the KOH-soluble pigments a piece of the outer part of a stroma was placed in 10% KOH on a microscope slide and observed on white background after 1 minute and again after 25 minutes following the suggestions by *Fourrier & Lechat* (*2015*).

For morphological studies an Olympus SZX10 stereomicroscope with an Olympus KL. 2500 LCD light source, and an Olympus BX51 light microscope with an Olympus DP72 camera were used. Colour names follow *Ridgway* (*1912*). Measurements are reported as given in *Friebes et al.* (*2016*). Microscopic observations were made in tap water (to measure and observe the colour and shape of the ascosporas, germ slits, and asci), Melzer’s reagent (to test the amyloidity of the apical ring and to measure it), and in 5% KOH (to test the dehiscence of the perispore). Microphotos were made of material in tap water if not indicated otherwise. For SEM photomicrographs the stromata were cut open and sputtered with gold. The photomicrographs were taken at the Centre of Natural History of Universalmuseum Joanneum, Graz, using a SEM of the type Jeol 6610 LV (*10 kev, working distance of 9 mm, HV mode, secondary electron images*).

Phylogenetic analysis of α-actin, β-tubulin, and ITS sequence data was provided by Hui-Mei Hsieh and Yu-Ming Ju (pers. comm.) based on DNA extracts from a culture of GJO79301 (*Hypoxylon ferrugineum* 1721*) in Fig. 6; CBS accession number: CBS 141259; GenBank accession number ITS: KX090079; α-actin: KX090081; β-tubulin: KX090080) on scratch-malt extract agar (cultured by Yu-Ming Ju). Pablo Alvarado (pers. comm.) provided further phylogenetic analysis of ITS sequence data based on DNA extracts from a dry culture of GJO79316 (*Hypoxylon ferrugineum* ALV5880*) in Fig. 6; GenBank accession number KX090078) from perithecial contents on malt extract agar plates provided by Walter Buzina (Medical University of Graz).

The collected material is deposited in the herbarium of Universalmuseum Joanneum, Graz (GJO).
Taxonomy

_Hypoxylon ferrugineum_ G.H. Otth, _Mitth. naturf. Ges. Bern_, 1868 (Nr. 654–683): 41 (1869) – Fig. 1–5.


Stromata erumpent through the periderm, solitary or in small, crowded groups, sometimes confluent, when young often elongate with velvety surface, when older pulvinate to hemispherical, sometimes flattened and somewhat discoid, partly restricted at base, 1–7.5 mm diam., 1–2 mm high, Cinnamon (15’’) when young, later Wood Brown (17’’) or Bister (15’’), KOH-extractable pigments Orange (15) to Orange Rufous (11) after 1 minute, slightly duller after 25 minutes, with Yellow Ocher (17’’) to Ochraceous-Orange (15’) granules beneath the surface. Entostroma between the perithecia black, grey when young, lighter coloured to almost white towards the base, often rooting deeply into the bast, surrounded by black stromatic lines. Surface uneven, rough, sometimes cracked.

_**Perithecia**_ subglobose to ellipsoid, 300—500 μm high, 200–400 μm diam. (n=20). _**Ostioles**_ inconspicuous, black.

_Ascoli. 156.5–202.5 × 10–11.5 μm, _pars sporifera_ (88–)92–130.5 μm, stipe 39.5–72 μm (n=30), 8-spored (rarely 4-spored), ascospores uniseriate, apical ring amyloid, sometimes only weakly, discoid, 3–3.5 × 0.7–1 μm (n=15). _**Ascospores**_ (10.5–)13.4–16.1(–21) × (5-)6.5–7.9(10) μm. Q=(1.3–)1.8–2.3(–3.6) (n=695), dark brown in water, dark grey brown in KOH, inequilaterally ellipsoid, partly ± limoniform, ends narrowly rounded or sometimes pointed, in Melzer’s reagent often showing De Bary bubbles, germ slit spore-length, on the convex side of the ascospore, straight to sigmoid. Perispore dehiscent in KOH, appearing smooth to transversally striate or wrinkly by light microscopy, epispore smooth. _**Paraphyses**_ numerous, hyaline, filiform, filled with tiny droplets. _**Perithecial wall**_ often with violet tinges.

Asexual morph in nature: young stromata velvety but no clear conidiogenous structures observed.


Additional specimens examined: **Austria**: Styria, Deutschlandsberg, Soboth, next to chapel St. Leonhard, 21.VI.2006, 15°04’30’’ ± 10’’E 46°41’03’’ ± 5’’N, on branches of _Tilia_ on the ground, leg. H. Timmer, det. L.E. Petrini (GJO30278, GJO62452). Hochschwab area, ca. 26 km SSW of Mariazell, Allenz-Kurort, in the park below the swimming pool, 780 m alt., 01.X.2000, 47°32.7’ N 15°14.2’E, on dead, cor-

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*Fig. 1. – Hypoxylon ferrugineum.* a: dead branch with stromata, still attached to the tree. b: close-up of the same branch as in a. c–e: immature stromata (note the velvety surface). f–j: mature stromata (i: transverse section; j: surface with ostioles). k, l: KOH-extractable pigments after 1 (k) and 25 (l) minutes. a, b: GJO79306; c–e: GJO79303; f: GJO79259; g, h, j: GJO79297; i, k, l: GJO79300. Photos: I. Wendelin (a, b), G. Friebes (c–l).

*Fig. 2. – Hypoxylon ferrugineum.* a–f: vertical sections of stromata (note the rooting entostromatic tissue). a–c: GJO79344; d, e: GJO79262; f: GZU000312389. Photos: G. Friebes.
Fig. 3. – *Hypoxylon ferrugineum*. a–c: asci and ascospores. d, e: 4-spored asci in Congo Red. f: ascogenous hypha in Congo Red. g: dehiscent perispore in 5% KOH + Congo Red. h: stromatal granules. i, j: sigmoid germ slits. k, l: transversally striate to wrinkly perispore in 5% KOH. Scale: a=30 μm; b–d, f, g=15 μm; e, h=20 μm; i–l=5 μm. a, b, d–i: GJO79300; c, j: GJO79304; k, l: GZU000312391. Photos: G. Friebes.

**Habitat:** on at least partially dead, attached or fallen, corticated, rather dry branches of *Tilia*. Diameter of the branches generally (1)2–4(5) cm, one branch up to 10 cm thick. Stromata mainly observed on one half of the branch (presumably the upper half when the branch was still attached to the tree). Development of young stromata was observed throughout the whole year in nature. Fresh stromata were most commonly encountered during spring. Altitude of the studied collections ranges from approx. 350 m to 1070 m.

**Distribution:** Central Europe: Switzerland (OTTH, 1869; PETRINI & MÜLLER, 1986; STADLER et al., 2004), Slovakia (STADLER et al., 2004), France (The BR Herbarium Catalogue, 2014), Austria (SCHEUER et al., 2001; present study); North America (MILLER, 1961).

**Discussion**

We mainly found *H. ferrugineum* in the inner city of Graz on *Tilia* alongside the biggest river, Mur, where it was only collected on branches of trees with high amounts of dead branches in the crown. The reasons for this concentrated occurrence nearby a river in the urban area are unclear. The following discussion about possible ecological influences on the growth of *H. ferrugineum* remains only speculation at this point of our study.

One factor that might contribute to the occurrence of *H. ferrugineum* alongside the river Mur might be the increased air humidity. However, we searched for *H. ferrugineum* in humid alluvial forests close to the same river in the south of Graz and were not able to find it there, suggesting that microclimatic differences, which are not easily apparent, might play an important role. It also seems possible that the extreme environmental conditions to which the trees are exposed in the inner city are somehow related to the growth of *H. ferrugineum*. Stress factors for the trees could be fumes, extreme weather, extensive lopping, road salt, insufficient water supply, etc., which may contribute to the dieback of single branches and, ultimately, the whole tree. Since fallen branches get removed quickly in the urban area it can be concluded that the stromata develop on branches that are still attached to the tree. This assumption is confirmed by our observation of a dead, still attached branch which was covered with well-developed stromata of *H. ferrugineum* (see Fig. 1a). Since our year-long observations of branches stored outdoors did not reveal any macroscopically apparent changes to the stromata it could be assumed that *H. ferrugineum* stops growth once the branches with the stromata make contact with the soil, like it is known from e.g. *H. fraxinophilum* (FOURNIER & MAGNI, 2004; as *H. intermedium* (Schwein.) Y.M. Ju & J.D. Rogers).

At least three different *Tilia* species could be possible hosts of *H. ferrugineum* in Graz (*T. americana*, *T. cordata*, and *T. platyphyllos*, see Tree Cadastre Graz, 2016) but, unfortunately, it was not possible to verify exactly which collection of *H. ferrugineum* grew on which *Tilia* species. Several fungi were found in association with *H. ferrugineum*, e.g. *Corynespora olivacea* (Wallr.) M.B. Ellis, *Helicogermisita gaudefroyi* (Fabre) Læssøe & Spooner, *Lachnellia alboviolascens* (Alb. & Schwein.) Fr., *Merismodes* sp., *Patellaria atrata* (Hedw.) Fr., *Peniophora rufomarginata* (Pers.) Bourdot & Galzin, *Schizophyllum commune* Fr., and *Tremella globispora* D.A. Reid.

**Fig. 4.** – *Hypoxylon ferrugineum*. SEM photograph of ascospores (GJO79304). Scale: a–c=2 μm; d=5 μm. Photos: H.-P. Bojar.
Before this study five collections of *H. ferrugineum* had been known from Austria, all located in Styria (see additional specimens examined). Furthermore, *H. ferrugineum* has been reported from Switzerland (Otth, 1869; Petrini & Müller, 1986; Stadler et al., 2004), Slovakia (Stadler et al., 2004), France (The BR Herbarium Catalogue, 2014), and North America (Miller, 1961). It has been reported from North America also in Jong & Rogers (1972) and Stadler et al. (2008) but none of these collections might actually represent *H. ferrugineum*; the collection studied by Jong & Rogers (1972) (as *Hypoxylon rubiginosum var. ferrugineum* (G.H. Otth) J.H. Mill., on *Acer macrophyllum*) probably does not correspond to *H. ferrugineum* in the more modern sense of Ju & Rogers (1996) (J.D. Rogers, pers. comm.), and Stadler et al. (2008) studied a collection from Canada on cf. *Populus* which showed a different HPLC profile compared to European material and therefore might be an undescribed taxon.

*Hypoxylon ferrugineum* seems to occur almost exclusively on *Tilia*; however, Stadler et al. (2004) studied *H. ferrugineum ss. str.* based on a Swiss collection on *Sorbus aucuparia var. edulis*. Most other mentions of *H. ferrugineum* growing on different hosts probably represent other species (see above).

The collected material is readily identifiable as *H. ferrugineum* using comprehensive keys of the genus *Hypoxylon* (Petrini & Müller, 1986; Ju & Rogers, 1996; Fournier et al., 2010). *Hypoxylon ferrugineum* is mainly recognized by more or less pulvinate and brownish stromata with orange KOH-extractable pigments, large ascospores, and its occurrence on *Tilia*. However, our collections of *H. ferrugineum* show a few deviations to the descriptions in literature. The most distinct difference is the form of the germ slit which is reported to be straight in literature but is at least partly sigmoid in the material studied by us, meaning that one of the reported distinguishing features between *H. ferrugineum* and *H. fuscum* (Scheele et al., 2001) is shown to be of limited importance. Furthermore, the perispore of the studied collections of *H. ferrugineum* is often conspicuously transversally striate or wrinkly by light microscopy, which is usually readily noticeable on the detached perispore (Fig. 3 k, l). SEM images of the ascospores congruently reveal a conspicuously wrinkly to coil-like striation (Fig. 4). Most of the descriptions of *H. ferrugineum* in the literature (for references see Descriptions/Illustrations above) do not mention such a striate perispore with the exception of Ju & Rogers (1996): “with inconspicuous coil-like ornamentation”. Striate perispore ornamentation has been reported from various *Hypoxylon* taxa and is regarded as an important morphological character at species level by Ju & Rogers (1996). Among the European *Hypoxylon* species the perispore varies from apparently smooth by light microscopy but striate by electron microscopy (e.g. *H. petriniae* M. Stadler & J. Fourn.; see Stadler et al., 2004), to faintly striate by light microscopy (e.g. *H. fuscoideas* J. Fourn., P. Leroy, M. Stadler & Roy Anderson or *H. lusitanicum* J. Fourn., M. Stadler & Priou; see Fournier et al., 2010), to even conspicuously striate by light microscopy (e.g. *H. gibriacense* J. Fourn., M. Stadler & Gardiennet; see Fournier et al., 2010). The latter corresponds to our observations of *H. ferrugineum*.

Another interesting feature apparent in the studied collections of *H. ferrugineum* is the distinctive entostromatic tissue with black stromatal lines which roots deep into the bast (Fig. 2). These dentoid roots are apparent in vertical section and can likely be considered a further distinctive character of *H. ferrugineum*. Interestingly, Otth (1869) describes rooting stromata in the protologue (“stromata quasi radicula ad corticis fundum descendente instructum”), but the
Fig. 6. Phylogenetic tree resulting from ITS and β-tubulin sequence data of *Hypoxylon ferruginum* and other *Hypoxylon* taxa. The values next to the nodes represent Bayesian posterior probability and maximum likelihood bootstrap percentage. GenBank accession numbers are given preceding the taxon names. The position of *Hypoxylon ferruginum* within the tree is highlighted in yellow. Tree provided by P. Alvarado.
more recent descriptions of *H. ferrugineum* do not mention this feature.

The stromata of *H. ferrugineum* collected in Graz often show a close association to other fungi. We frequently observed young stromata that develop directly on stromata of old podyremycetes. Similarly, some stromata of *H. ferrugineum* were found to enclose old perithecia and ostiolar canals of a different (probably diaporthal) fungus.

While previously published phylogenetic trees based on ITS and β-tubulin sequence data show incongruities when compared to each other and the right genes for phylogenetic studies in *Hypoxylon* remain yet to be found (Kuhnert et al., 2014), the phylogenetic results of combined β-tubulin and α-actin as well as combined β-tubulin and ITS sequence data support the position of *H. ferrugineum* as a distinct species. This is in agreement with the morphological results of this study. The sequence data of combined α-actin and β-tubulin genes position *H. ferrugineum* in clade H2 sensu Hsieh et al. (2005), clustering with *H. croceoporum* Berk. & M.A. Curtis, *H. fendleri* Berk. ex Cooke, and *H. erythrostoma* J.H. Mill. (phylogenetic tree not shown). A phylogenetic tree based on combined β-tubulin and ITS gene sequences is provided in this paper.

The rather common occurrence of *H. ferrugineum* in some parts of the urban area of Graz may be an indicator that it is actually not as rare as previously thought but does indeed have a very narrow host range and is probably restricted to rather specific ecological conditions.

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