Hapalocystis occidentalis - a new species of *Diaporthales* from North America and a key to the species of *Hapalocystis*

Walter M. Jaklitsch[1*] and Hermann Voglmayr[2]

[1]*Ratschkygasse 46/28, A-1120 Vienna, Austria;* [2]*Institute of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria*

Correspondence: Walter M. Jaklitsch, jaklitsc@mail.zserv.tuwien.ac.at

Abstract: *Hapalocystis occidentalis* is described as a new species in the *Diaporthales* based on morphological observations and phylogenetic analyses of partial nuclear large subunit ribosomal DNA sequences. A key to the six species recognised in the genus *Hapalocystis* Auersw. ex Fuckel is presented.

Taxonomic novelty: *Hapalocystis occidentalis* W.M. Jaklitsch & Voglmayr sp. nov.
Key words: *Diaporthales*, *Hapalocystis*, phylogeny, *Prosthecium*, pyrenomycetes, taxonomy.

INTRODUCTION

The genus *Hapalocystis* Auersw. ex Fuckel was established by Fuckel (1863) based on two species, *H. berkeleyi* Auersw. ex Fuckel and *H. bicaudata* Fuckel. In 1870, Fuckel himself recombined *H. berkeleyi* as *Calospora hapalocystis* (Berk. & Broome) Fuckel and *H. bicaudata* as *Melanconis berkeleyi* Tul. (Fuckel 1870). As a consequence, *Hapalocystis* disappeared from the taxonomic landscape. Winter (1887) and Ellis & Everhart (1892) filed these species under *Pseudovalsa* Ces. & De Not. Wehmeyer (1941) grouped a number of taxa including these two species in his subgenus *Pseudoprosthecium* of the genus *Prosthecium* Fresen., which is distinguished from other members of the *Diaporthales* by brown or hyaline phragmospores and reduced, light-coloured (ento-) stroma. Barr (1978) took note of a remark by Holm (1975), that the name *Hapalocystis* was valid and available, and that Clements & Shear (1931) had chosen *H. berkeleyi* as the lectotype species. She combined all species of *Pseudoprosthecium* into *Hapalocystis* and characterised the genus as differing from *Prosthecium* in having fused beaks forming a reduced stromatic disc and in having ascospore appendages that are elongate and strap-like.

Later Barr (1979) added *Hapalocystis corni* (Wehm.) M.E. Barr to the genus. This species has circularly arranged perithecia sharing a single ostiole. Ascospores are brown, 2-celled; with short pulvinate appendages (similar to those of e.g. *Melanconis spodiaea* Tul. & C. Tul.). This fungus differs substantially from the concept of *Hapalocystis* and is therefore not included in the key to the species below.

The anamorphic state of *Hapalocystis* is given as *Stilbospora* Pers., *Hendersonia* E.J. Butler or *Dothiorella* Sacc. (Wehmeyer 1941), or as *Stilbospora*-like

(Barr 1978). These proposed connections have been deduced merely from associations seen on the substrate. Glawe (1985) produced a *Phoma*-like anamorph for *H. berkeleyi* in culture.

We here describe a new species of *Hapalocystis* on *Platanus* from North America. This species is closely related to the type species, as is apparent in study of ascospore morphology and of phylogenetic placement determined via nucleotide sequence comparisons.

MATERIALS AND METHODS

Sample sources used for sequencing

Single spore isolates were prepared and grown on 2 % malt extract agar. *Hapalocystis berkeleyi*: **Germany**, Baden-Württemberg, Tübingen, Anlagensee, MTB 7420/3, on *Platanus hybrida*, 20 Oct. 2002, W. Jaklitsch, WU 24707. *Hapalocystis occidentalis*: **U.S.A.**, Tennessee, Great Smoky Mountains National Park, Townsend, Cades Cove, on *Platanus occidentalis*, 22 May 2003, W. Jaklitsch & H. Voglmayr, WU 24705 (**holotype**), living culture deposited as CBS 115156 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). *Prosthecium ellipsosporum* Fresen.: **Austria**, Niederösterreich, Rekawinkel, MTB 7862/1, on *Carpinus betulus*, 20 Oct. 2001, W. Jaklitsch, WU 24708, living culture deposited as CBS 115073. The GenBank accession numbers of the corresponding partial nuclear large subunit (nLSU) ribosomal DNA (rDNA) sequences are given in the tree (Fig. 1).

DNA extraction, PCR and sequencing

Mycelium for DNA extraction was grown in shaker flasks at 125 rpm in 100 mL 1 % malt extract solution at room temperature for 7–14 d. Mycelium was harvested with a sterile forceps, put into 2 mL reaction-

tubes, and freeze-dried. The dried samples were subsequently ground in a Retsch 200 mixer mill (Retsch, Haan, Germany) for 10 min using 2–3 mm glass beads. Subsequently, DNA was extracted using the modified cetyltrimethylammoniumbromide (CTAB) protocol described in Riethmüller *et al.* (2002).

The D1, D2 region of the nLSU rDNA region was amplified with primers LR0R (Moncalvo *et al.* 1995) and TW14 (White *et al.* 1990). PCR products were purified using the QIAquick Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, U.K.) with LR0R and TW14 as primers and an automated DNA sequencer (ABI 377, Applied Biosystems).

Data analysis
For the phylogenetic analysis, sequences were selected from Castlebury *et al.* (2002). Taxa from all major lineages were included; if possible, generic type species were selected. The GenBank accession numbers of the sequences are given in the tree, following the taxon names (Fig. 1).
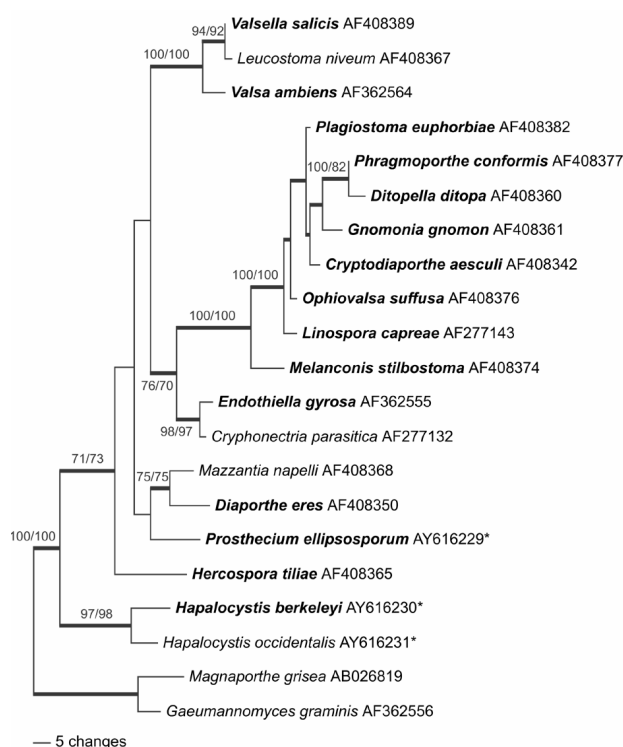
Fig. 1. One of the five most parsimonious trees (length = 329) of a phylogenetic analysis of the partial nuclear large subunit ribosomal DNA region. GenBank accession numbers marked with an asterisk (*) represent sequences obtained during the present study. Bootstrap values greater than 70 % are shown as first (maximum parsimony) and second (neighbour-joining) number on each branch. Taxa in bold represent type species of their respective genera. Thickened lines indicate branches that appeared in the strict consensus of the five trees.

The sequence alignment was initially produced with ClustalX (Thompson *et al.* 1997), version 1.81, and visually checked and refined with BioEdit (Hall 1999), version 4.8.6. In the subsequent phylogenetic analyses 874 characters of the resulting partial nLSU alignment were included.

Heuristic maximum parsimony (MP) analysis was performed with PAUP v. 4.0b10 (Swofford 2002), using 1000 replicates of random addition of sequences and subsequent Tree Bisection and Reconnection (TBR) branch swapping (MULTREES option in effect, steepest descent option not in effect). The same settings were also used for MP bootstrap analysis, with 20 rounds of random sequence addition and subsequent branch swapping during each of the 1000 bootstrap replicates. Gaps were treated as missing data.

Neighbour-joining (NJ) bootstrap analysis was performed with 1000 replicates, based on the general time-reversible model of DNA substitution, additionally assuming a portion of invariant sites with gamma-distributed substitution rates of the remaining sites (GTR+I+G; see Swofford *et al.* 1996). This model was implemented because it was chosen as most appropriate substitution model in Modeltest version 3.06 (Posada & Crandall 1998) under the Akaike Information Criterion (AIC).

RESULTS

The final alignments and the trees obtained were deposited in TreeBASE (http://www.treebase.org) and are available under study accession no. S1179.

The heuristic MP analysis revealed five most parsimonious trees of length 329, of which one is shown in Fig. 1. *Hapalocystis occidentalis* and *H. berkeleyi* form a highly supported clade (97 and 98 % bootstrap values in MP and NJ, respectively). Sister group relationship of this *Hapalocystis* clade to all other *Diaporthales* is moderately supported (71 and 73 % bootstrap value).

Taxonomical part

Hapalocystis occidentalis W.M. Jaklitsch & Voglmayr, **sp. nov.** MycoBank MB500064. Figs 2–7.

Species generis Diaporthalium, differt ab *Hapalocysti berkeleyi* ostiolis erumpentibus non-coniunctis, sporis hyalinis minibusque, magnitudine 17–23 × 7.5–11 μm et ascis 4-sporis.

Outward appearance: ostioles erumpent in loose clusters (Fig. 2) of 3–10 through fissures in the bark up to 150 μm apart, sometimes single, or sometimes convergent and closely crowded and then laterally

adhering, occupying areas 0.3–0.7(–2) × 0.2–0.5 mm. Most ostioles terminate at the level of the bark surface, but some clusters protrude up to 250 µm beyond the bark surface. Stromatic tissues lacking or rarely present as few hyaline thick-walled hyphae 1.5–3.5 µm diam growing between the bark and the upper parts of perithecia or ostioles. *Ostioles* (Fig. 3): black, cylindrical, with a diameter of 80–120 µm or subconical and then tapered apically to 40–70 µm, sometimes broadest in the middle and sometimes compressed laterally, centrally perforated by a circular pore 10–20(–50) µm diam, 80–350 µm long, arising laterally at the upper part of the perithecium, often convergent and clustered, never fused. Interior packed with numerous flexuous hyaline periphyses, (8–)10–33 × 1–2 µm, in a gelatinous matrix; exterior an olive-brown *textura intricata* of hyphae 5–8 µm thick in face view, at the base gradually turning into the coarse *textura angularis* of the perithecial wall. *Perithecia*: in valsoid, often more or less circularly and densely aggregated groups of approximately 1 mm diam embedded in the bark; no entostroma present. Perithecial groups topped by a central cluster of ostioles; non-clustered perithecia bearing a single distinctly lateral ostiole. Perithecia depressed globose to lenticular; in dry state, basal wall often concave; height 120–200 µm, diameter (130–)190–420 µm. *Peridium*: black, surface verruculose, usually covered by light brownish granules of bark tissue, approximately 20–40 µm thick, consisting of a thin, hyaline to light brown inner layer, 6–16 µm thick, of strongly compressed thin-walled cells, and a dark (reddish-) brown outer layer 18–30 µm thick, seen in surface view as a coarse *textura angularis* with cells (8–)10–21(–32) µm diam and walls 0.5–1.5 µm thick. Cells filled with large guttules. *Hamathecium*: hyaline bands 3–8 µm thick, collapsing at maturity. *Asci* (Figs 4, 5): detached from the perithecial wall at maturity, fusoid with four ascospores biseriately arranged in the middle, (57–)60–74(–79) × (12–)14–20(–26) (n = 25), with a short stipe and an inconspicuous flat, refractive ring on the lower end of the thickened apical wall (Fig. 6), usually concealed by ascospore appendages. At maturity, release of ascospores is initiated by the penetration of an appendage through this ring.
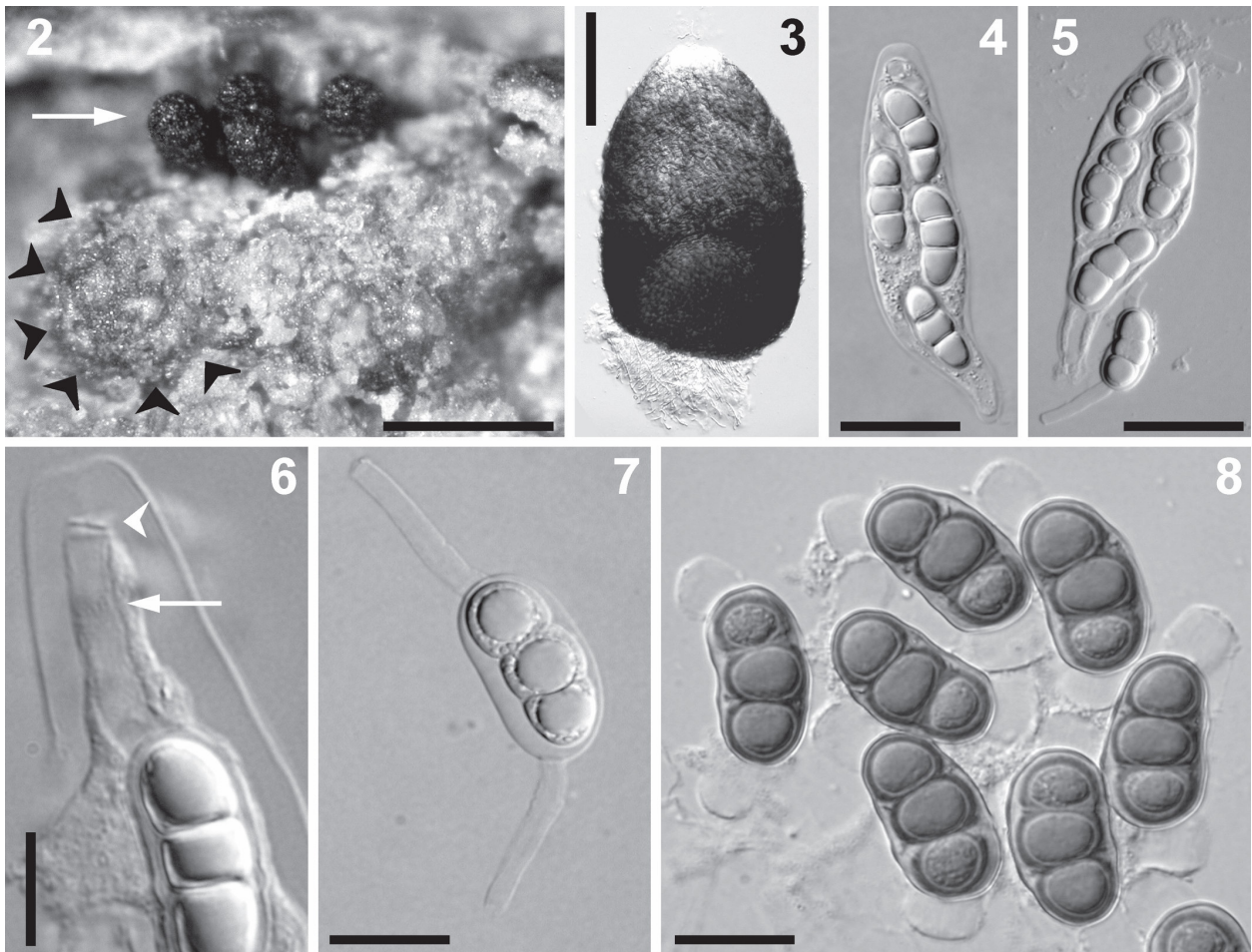
Figs 2–8. *Hapalocystis occidentalis*. 2. Cluster of ostioles (arrow) and perithecia; perithecial outlines are indicated by arrowheads. 3. Single ostiole with periphyses in a gelatinous matrix on the basis. 4, 5. Asci with four ascospores and an ascospore appendage penetrating the apex (5). 6. Apical part of an ascus showing the apical ring (arrowhead) and an ascospore appendage (arrow). 7. Ascospore with long hyaline strap-like appendages. 8. Ascospores of *Hapalocystis berkeleyi*. (Scale bars: 2 = 200 µm, 3 = 50 µm, 4, 5, 8 = 20 µm, 6, 7 = 10 µm).

Ascospores (Fig. 7): inequilaterally ellipsoidal, with broadly rounded ends, hyaline at maturity (i.e., when germinable), (1–)2(–3)-septate, with cells of equal length, thick-walled, smooth, (14–)17–23(–24) × (7–)7.5–11(–13) µm with a length/width ratio (l/w) of (1.6–)2–2.5(–2.8) (n = 30); long, hyaline strap-like appendages situated on both ends cylindrical or slightly attenuated towards truncated (or less commonly rounded) ends, (10–)13–19(–28) × (2.5–)2.9–3.6(–4) µm with l/w = (2.9–)3.7–6(–8) (n = 32). Senescent ascospores dilute brown and of the same size as hyaline ascospores, mostly lacking appendages.

Ascospore septa are interpreted as distosepta, as they do not penetrate the outer wall. The latter is often very variable in thickness even in individual ascospores, a feature that may suggest that what appears to be a wall is actually a sheath. In that case, septa would be eusepta rather than distosepta. In any case, measurements given here include the outer wall or sheath.

Anamorph: none observed.

Habitat: In thin dead corticated twigs (5–7 mm diam) of *Platanus occidentalis* L.

Distribution: North America (Tennessee).

Material examined: **U.S.A.**, Tennessee, Great Smoky Mountains National Park, Townsend, Cades Cove, on *Platanus occidentalis*, 22 May 2003, W. Jaklitsch & H. Voglmayr, WU 24705 (**holotype**), culture ex-type CBS 115156.

Key to the species recognised in *Hapalocystis*:

Data are compiled from the literature. All species show strap-like gelatinous hyaline appendages on both ends of ascospores. Only *H. berkeleyi* and *H. occidentalis* have been studied using molecular data. Other species described in the genus may not be congeneric with these species phylogenetically.

1. Ascospores hyaline (senescent ascospores may become pale brown) 2
1. Ascospores brown 3

2. Asci 4-spored, ascospores with 2 distosepta, 17–23 × 7–11 µm, on *Platanus occidentalis* in North America ....***H. occidentalis*** n. sp.
2. Asci 8-spored, ascospores with 3 distosepta, 30–45 × 12–15 µm, on *Ulmus* spp. in North America...***H. ulmi*** (Wehm.) M.E. Barr ....(syn. *Pseudovalsa caudata* (Ellis & Everh.) Sacc.)

3. Asci 8-spored, ascospores with 2 distosepta, 28–36(–45) × 13–18(–22) µm, on *Platanus* spp. in temperate regions...***H. berkeleyi*** Auersw. ex Fuckel **var. *berkeleyi*** .... (syn. *Pseudovalsa hapalocystis* (Berk. & Broome) Sacc.)
3. Ascospore septation different...4

4. Asci 4-spored; ascospores with 5 distosepta, 55–72 × 22–27 µm, on *Platanus* spp. in Europe.. ***H. berkeleyi*** **var. *kickxii*** (Westend.) M.E. Barr ....(syn. *Pseudovalsa kickxii* (Westend.) Sacc.)
4. Asci 8-spored, ascospores with 3 distosepta 5

5. On *Ulmus* spp. in Europe; ascospores 38–55 × 15–18 µm ....***H. bicaudata*** Fuckel ....(syn. *Pseudovalsa berkeleyi* (Tul.) Sacc.)
5. On *Platanus* spp. in North America; ascospores 46–70 × 13–20 µm.............. ***H. corticalis*** (Schwein.) M.E. Barr .... (syn. *Pseudovalsa bicornis* (Cooke) Sacc.)

Note: The spelling as 'corticale' in Barr (1978) is incorrect because of the feminine gender of *Hapalocystis*, it is therefore changed to 'corticalis'.

Three additional species have been described under the generic name of *Hapalocystis*, *H. corni* (Wehm.) M.E. Barr, *H. vexans* Speg. (Spegazzini 1925) and *H. mirabilis* Sorokin (1875). *H. corni* belongs to the *Diaporthales* but is not considered to be a member of this genus (see comments above). The last two species belong to the chytridiaceous genus *Hapalocystis* Sorokin (1874), a later homonym of *Hapalocystis* Auersw. ex Fuckel.

DISCUSSION

Three species and one variety of *Hapalocystis* have been described on *Platanus*. *Hapalocystis berkeleyi* and *H. corticalis* were collected together with *H.*

occidentalis at the same locality on the same twigs. Ascospores of *H. corticalis*, which were brown, 3-distoseptate and much larger (41–61 × 16–20 µm) than those of *H. occidentalis*, did not yield mycelium useful for DNA extraction; therefore *H. occidentalis* was only compared to the type species phylogenetically.

The fourth taxon occurring on *Platanus*, *H. berkeleyi* var. *kickxii*, differs from the type variety and all other species by possessing large 5-septate ascospores. This suggests it may be a species rather than a variety.

Hapalocystis occidentalis has the smallest ascospores seen in any species of *Hapalocystis*. These spores show a striking similarity in shape and septation to those of the type species, *H. berkeleyi*, indicating a close relationship to this species, but the ascospores remain hyaline at maturity and are distinctly smaller than those of *H. berkeleyi*. While the ascospore appendages of *H. berkeleyi* (Fig. 8) are broad and stout, approximately (5–)6–16(–23) × (4–)6–8(–11) µm, with l/w 0.6–2(–3), those of *H. occidentalis* have a l/w ratio of 4–6(–8). The integument of the ascospores is distinctly gelatinous, staining deeply pink in toluidine blue. In cotton blue/lactic acid the ascospore wall of *H. berkeleyi* collapses and becomes distinctly wrinkled, which suggests a sheath rather than a wall. This collapse does not occur with ascospores of *H. occidentalis*. A consistent interpretation of this integument (wall or sheath) can only be made possible by further study. At any rate, the high stability of this outer integument, which is usually present in herbarium specimens of *H. berkeleyi,* appears not to be found with ascospore sheaths of other diaporthalean fungi. The presence of this stable integument may therefore constitute a morphological characteristic of the genus *Hapalocystis*.

Hapalocystis occidentalis also differs markedly from the type species in its ostioles, which in *H. berkeleyi* are generally fused into a single broad central opening containing a gelatinous mass interspersed with periphyses. *Hapalocystis occidentalis* deviates from the morphological concept of the genus *Hapalocystis* (see Barr 1978) by forming individually erumpent and non-fused ostioles (Fig. 2). However, it should be noted that the new species *H. occidentalis* is based on a single specimen, a situation which does not allow us to assess the variability of this feature. On the other hand, also *H. berkeleyi* rarely shows 2–3 non-fused ostioles per perithecial group (e.g. *Sphaeria hapalocystis* Berk. & Broome of Fungi Britannici exsiccati; NY, No. 253). As a consequence, fusion of ostioles is not a reliable morphological characteristic for the definition of the genus. An interesting herbarium specimen deserving a comment in this context is No. 6782 from MASS (now in NY), collected and determined as *H. berkeleyi* by Margaret E. Barr (*Platanus wrightii*, Madera Canyon, Coronado National Forest, Santa Cruz Co., Arizona). Non-fused ostioles

and hyaline to light brownish ascospores with long appendages (20–32 × 4–6 µm) suggest *H. occidentalis*, but the size of ascospores, (23–)30–36 × 16–19 µm, is in accord with *H. berkeleyi*. The specimen is therefore intermediate between these two species, and possibly represents an additional taxon.

Phylogenetic analyses of the nLSU rDNA confirm a close relationship of *Hapalocystis occidentalis* with *H. berkeleyi*, the generic type (Fig. 1). On the other hand, the genetic distance of their sequences (5.4 %) confirms that the two species are clearly distinct. The *Hapalocystis* clade appears as sister group to the other *Diaporthales* included in the present analysis. The same result is obtained if all sequences of Castlebury *et al.* (2002) are included in the analysis (data not shown). *Prosthecium ellipsosporum*, the type species of *Prosthecium*, does not appear to be closely related to *H. berkeleyi*, which confirms independent generic status of *Hapalocystis* as proposed by Barr (1978).

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