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First report of *Monilia polystroma* on apple in Hungary

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Abstract A *Monilinia fructigena*-like isolate (UFT) was collected from apple shoots in northeastern Hungary (Újfehértó). Brownish dieback and buff-coloured stromata were observed on shoots and small fruits of cv. 'Ashton Bitter'. On potato dextrose agar (PDA) the colonies were yellowish in colour and irregular black stromatal crusts occurred. Conidia (16.6×10.1 μm) were slightly smaller than the average of *M. fructigena*. The fungus caused brown rot on inoculated apple fruits, and produced numerous sporodochia. The sequences of the rDNA internal transcribed spacer regions of the UFT isolate were almost identical to that of a previously described *Monilia polystroma* isolate, containing all five nucleotides that distinguish it from *M. fructigena*. Comparison of a genomic sequence of unknown function revealed that repetitive sequence motifs occurred in different numbers as insertions in the genomes of *M. fructigena*, *Monilia polystroma*, and the UFT isolate. Classical and molecular characterisation indicated that the UFT isolate belonged to *Monilia polystroma*. To our knowledge this is the first report of *Monilia polystroma* in Europe.

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Monilinia spp. are well-known pathogens of fruit trees in many fruit production regions in the world. Three species of *Monilinia* are particularly important with regard to fruit trees and ornamentals because they cause serious blossom and twig blight and brown rot on fruits: *M. fructicola*, *M. fructigena* and *M. laxa* (Bryde and Willetts 1977). In 2002, a new species, *Monilia polystroma* (van Leeuwen et al. 2002) was distinguished from *M. fructigena* based on morphological and molecular characteristics (Fulton et al. 1999; van Leeuwen et al. 2002). Other *Monilinia* species are known to cause disease in fruit trees, including *M. mali*, which attacks apple and Japanese crab apple (Shima 1936), and *M. laxa* f. sp. *mali*, which causes blossom wilt on apple trees (Wormald 1954). *Monilinia laxa* and *M. fructigena* are the main agents of brown rot in Europe (Bryde and Willetts 1977) and Hungary. *Monilinia fructicola*, which is widespread in the USA (OEPP/EPPO 2003), occurred in Hungarian orchards only in 2007. *Monilinia laxa* f. sp. *mali* is present in Europe; however, the pathogen is rarely observed in orchards, due to effective chemical prophylaxis (Willets and Bullock 1993). *Monilinia mali* and *Monilia polystroma* exist only in Japan, causing severe diseases (Shima 1936; van Leeuwen et al. 2002).

Identification of the three main *Monilinia* species is commonly based on morphology and colony

characteristics (Bryde and Willetts 1977; Batra 1991; Willetts and Bullock 1993). *Monilia polystroma* forms a large number of yellowish or buff-coloured stromata (van Leeuwen et al. 2002). Exogenous stromata of *M. laxa* f. sp. *mali* are similar to those of *M. laxa*, being both smaller and greyish (Wormald 1954). *Monilinia fructigena* has the largest macroconidia, whereas the conidia of *Monilia polystroma* are slightly smaller (van Leeuwen et al. 2002). Colonies of *M. fructigena* are creamy/yellow, their margins are not lobed, and they do not form rosettes on potato dextrose agar (PDA). Colonies of *Monilia polystroma* are similar to those of *M. fructigena*, but black stromatal plates occur on the colonies after incubation for 10–12 days (van Leeuwen et al. 2002; OEPP/EPPO 2003), and *Monilia polystroma* isolates grow faster than *M. fructigena* isolates under the same conditions (Holb 2004).

Several molecular methods have been developed to distinguish *Monilinia* species. Fulton and Brown (1997) established a PCR-based method of targeting the group I intron in the gene for the ribosomal small subunit. Ioos and Frey (2000) designed species-specific primer pairs for the ribosomal internal transcribed spacer (ITS) region. According to Fulton et al. (1999), isolates of Japanese *M. fructigena* differed from European *M. fructigena* isolates by five basepairs in the ITS and formed a separate group from *M. laxa*, *M. fructigena*, and *M. fructicola*. Later, Japanese *M. fructigena* isolates were identified as a new species under the name of *Monilia polystroma* (van Leeuwen et al. 2002). Multiplex PCR assays were developed to detect and identify *Monilinia* species based on the different lengths of fragments of a genomic region with unknown function (Côté et al. 2004). More recently, internal control based universal PCR protocol was developed for *Monilinia* spp., and species-specific primers were designed by using SCAR markers (Gell et al. 2007).

In April 2006, unusual symptoms were observed on 'Ashton Bitter' apple trees in Újfehértó (Hungary). Brownish die back was present on the leaf petioles and laminas and on small fruits and fruit pedicels. Infected areas were covered with yellowish exogenous stromata (Fig. 1).

We measured the length and width of 100 conidia from the infected area. Ellipsoid conidia developed in chains on the surface of the yellowish stromata. Conidia were single-celled and hyaline. No disjunctors were observed. The conidia were smaller in size (mean: 16.6×10.1 μm) than those of *M. fructigena* (mean: 19×11.5 μm), similarly stated by van Leeuwen et al. (2002). UFT isolate was cultured on PDA (Difco Laboratories) to describe its cultural characteristics and colony growth rates (mm/24 h) at 22°C in 12 h light/12 h dark. Colonies of the UFT isolate grown on PDA were yellowish in colour and irregular black stromatal crusts occurred on the edges of the colonies after 10–12 days of incubation. The margins of the colonies were slightly undulate. The growth rate of the UFT isolate was higher (8.2 mm/24 h) than *M. fructigena* isolates (5.0±0.9 mm/24 h) as observed by van Leeuwen et al. (2002).

Fig. 1 Brownish dieback on leaf petioles, laminas, fruit pedicels and small fruits covered with yellowish exogenous stromata on apple cv. 'Ashton Bitter' caused by the UFT isolate (Foto: Szabó, T)

Identification of UFT isolate also was confirmed by PCR using species-specific primers. DNA extraction was based on the CTAB method of Dellaporte et al. (1983). Proteins were removed with chloroform:isoamyl alcohol, and DNA was precipitated in isopropanol (Maniatis et al. 1989) The pellet was resuspended in 50 μl TE buffer containing 10 μg ml^{-1} RNase. Approximately 100 ng of DNA was amplified in 1× PCR reaction buffer (Fermentas), 250 μM of each dNTP, 7.5 mM $MgCl_2$, 20 pM primers, and 2.5 units *Taq* DNA polymerase (Fermentas) in a total volume of 50 μl. Primers (synthesised by Biomi Kft. Gödöllő, Hungary) were used to amplify the ITS regions of ribosomal DNA (ITS1-5.8S-ITS2): ITS4Mfgn reverse primer (5′- GGTGTTTTGCCAGAAGCACACT-3′ by Ioos and Frey (2000)) and ITSMonilia forward primer (5′- GGTAGACCTCCCACCCTTGTGTA-3′)

designed by us based on sequence data from NCBI database, are specific to *M. fructigena*, *M. laxa, M. fructicola* and *Monilinia polystroma*.

To recover a genomic sequence with unknown functions, we designed primers using sequence data from Côté et al. (2004). This genomic region was chosen because it was the only available sequence data for *Monilinia polystroma* except for the ITS regions; in addition, this region is more diverse among the different *Monilinia* species. UNIMON.rev is a universal reverse primer for four *Monilinia* species: *M. fructigena*, *Monilinia polystroma*, *M. laxa*, and *M. fructicola* (5′-GAGCAAGGTGT CAAAACTTCCAT-3′); the MFG.forw forward prim-

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16FG      1 TTAAAGTCCATCC---CATCTAACAATCAAAGAAGTGTAAGTAATAAACCCTTAAACTTT
17FG      1 ............---.............................................
39FG      1 ............---.............................................
3FG       1 ............---.............................................
40FG      1 ............---.............................................
FG        1 ............---.............................................
MP        1 ............CAT..........A.G.......C.......................
UFT       1 ............CAT..........A.G.......C.......................

16FG     58 CTCAACCGCTTTTCTCTCCCCTTTCTTTACCCAGACACCA---CCTCCTCTCTAGCACTT
17FG     58 ........................................---..................
39FG     58 ........................................---..................
3FG      58 ........................................---..................
40FG     58 ........................................---..................
FG       58 ........................................---..................
MP       61 ..........................................CCT..................
UFT      61 ..........................................CCT..................

16FG    115 GCATTCTTCCTTCACGATCTGCCTCCCTAGCCTAGTCCA--------------TAGTCCC
17FG    115 ....................................------------------.......
39FG    115 ....................................------------------.......
3FG     115 ....................................------------------.......
40FG    115 ....................................------------------.......
FG      115 ....................................------------------.......
MP      121 ..........C.........................TAGTCCCTAGTCCC.......
UFT     121 ....................................-------TAGTCCC.......

16FG    161 TAGTCCCTAGTGACTATTACCGATTGCCTACGGAGCACTTAGCCATCTTACCACGCTTAT
17FG    161 ............................................................
39FG    161 ............................................................
3FG     161 ............................................................
40FG    161 ............................................................
FG      161 ............................................................
MP      181 ........................A...................................
UFT     174 ......................G...A...................................

16FG    221 TGTACTCGCTGTGCTAAATACTAATTCGATGCTAAACGTGTAACTATCTATCATTTGCCT
17FG    221 ............................................................
39FG    221 ............................................................
3FG     221 ............................................................
40FG    221 ............................................................
FG      221 ............................................................
MP      241 .T.............C......G.................A.....................
UFT     234 .T.....................G.................A.....................

16FG    281 AGGCAAAAAGTACTACTGTACACACATACATCCAGACCCATCAATAGCCAAAAATGTAAG
17FG    281 ............................................................
39FG    281 ............................................................
3FG     281 ............................................................
40FG    281 ............................................................
FG      281 ............................................................
MP      301 ............................................................
UFT     294 ............................................................

16FG    341 TGGGGGGGGG---AAGGGAGAGA
17FG    341 ..........---..........
39FG    341 ..........---..........
3FG     341 ..........---..........
40FG    341 ..........---..........
FG      341 .........----..........
MP      361 ..........GG-........A.
UFT     354 ..........GGG..........
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Fig. 2 Sequence alignment of a genomic region with unknown function (Côté *et al*., 2004) of *Monilia* species. Identical nucleotides are represented by *dots*, and absent nucleotides are shown by *hyphens*. Three repetitive sequences (CAT, CCT, TAGTCCA or TAGTCCC) are marked with *grey background*. (**Isolates** and GeneBank Accession numbers: *Monilinia fructigena*: **3FG** AM937115, **16 FG** AM937116, **17FG** AM937117, **39FG** AM937118, **40FG** AM937119, **FG** AF506701, *Monilia polystroma*: **MP** AY456197, **UFT** AM937120)

er is specific to *M. fructigena* and *Monilia polystroma* (5′-CTAGATCAAACATCGTCCATCTTC-3′). In both cases the reactions were carried out in a Perkin-Elmer 9700 thermocycler (Applied Biosystems) with an initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, elongation at 72°C for 1.5 min, and a final elongation at 72°C for 10 min. Amplified products were cloned into pGEM-T Easy (Promega) and sequenced by using M13 reverse and M13 forward primers and the ABI PRISM BigDye Terminator Cycle Sequencing kit on ABI PRISM 310 instrument (Applied Biosystem). Sequence analysis was performed using Wisconsin Package Version 10.0 (Genetics Computer Group GCG).

The sequence of ITS region was deposited in the GenBank database (accession number: AM937114). The sequence of the UFT isolate was almost identical (only one base difference) to that of *Monilia polystroma*, containing all five nucleotides that distinguish it from *M. fructigena* (Fulton et al. 1999; van Leeuwen et al. 2002). The only difference detected between the UFT isolate and the published sequence of *Monilia polystroma* occurred at nucleotide 372, where the published *Monilia polystroma* sequence contained an extra 'T'. The genomic sequence with unknown function was also deposited in the GenBank database (accession number: AM937120), and compared with other isolates. Comparing *M. fructigena* and *Monilia polystroma* sequences, insertions and substitutions were detected in the *Monilia polystroma* sequence (Fig. 2). The Hungarian UFT and the published *Monilia polystroma* sequences were almost identical. Three repetitive sequence motifs (CAT, CCT, TAGTCCA or TAGTCCC) were identified. The CAT and CCT motifs occurred twice in *M. fructigena* isolate and three times in *Monilia polystroma* and UFT isolates, while the TAGTCCA or TAGTCCC motif occurred three times in *M. fructigena* isolate, five times in *Monilia polystroma*, and four times in the UFT isolate (Fig. 2).

Two repeated pathogenicity tests intended to verify Koch's Postulates were conducted in the laboratory. Apple fruits (cv. Idared) surface-disinfested by 70% ethanol and 1 year-old apple shoots (cv. Mutsu and Granny Smith) were inoculated with plugs (3 mm diam) of 7 day-old mycelia from fungal colony margins. Plugs of PDA were used as controls. Disease development was evaluated after 14 days by observing necrosis, shoot blight and appearance of stromata. Seven days after inoculation, the UFT isolate produced soft, brown rot lesions on the fruits. By the fourteenth day almost all of the fruits had rotted, and yellowish exogenous stromata appeared on the surface of infected apples. No symptoms developed on control fruit. Fourteen days after shoot inoculation, UFT isolate caused brownish die-back on the leaf-stalks, fruit-stalks and small fruits and yellowish stromata appeared on leaf-blades. None of the control shoots inoculated with the sterile agar plug was necrotic or wilted. The fungus was successfully reisolated from inoculated shoots and fruits.

The presence of this pathogen in Hungarian orchards might not have a substantial effect on fruit production, because the pathogen was observed only once in 2006 and was sporadic within the orchard. *Monilia polystroma* is currently not included in EPPO (European and Mediterranean Plant Protection Organisation) lists of quarantine pathogens. As a result of this report and the existence of this new pathogen in this region, EPPO might plan to perform a pest risk analysis to determine whether to place *Monilia polystroma* in their lists.

The pathogen identified in this study caused unique symptoms on apple shoots in Hungary and was confirmed as *Monilia polystroma*, a species previously reported only in Japan. To our knowledge this is the first report of *Monilia polystroma* in Europe.

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References

Batra, L. R. (1991). *World species of Monilinia (Fungi): Their ecology, biosystematics and control. Mycologia Memoir No. 16*. Berlin: Cramer.

Bryde, R. J. W., & Willetts, H. J. (1977). *The brown rot fungi of fruit. Their biology and control*. Oxford: Pergamon.

Côté, M. J., Tardif, M. C., & Meldrum, A. J. (2004). Identitification of *Monilinia fructigena, M. fructicola, M. laxa*, and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Disease, 99*, 1219–1225. doi:10.1094/PDIS.2004.88.11.1219.

Dellaporte, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter, 1*, 19–23. doi:10.1007/BF02712670.

Fulton, C. E., & Brown, A. E. (1997). Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from

M. laxa and *M. fructigena*. *FEMS Microbiology Letters, 157*, 307–312.

Fulton, C. E., van Leeuwen, G. C. M., & Brown, A. E. (1999). Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits. *European Journal of Plant Pathology, 105*, 495–500. doi:10.1023/A:1008711107347.

Gell, I., Cubero, J., & Melgarejo, P. (2007). Two different PCR approaches for universal diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees. *Journal of Applied Microbiology, 103*, 2629–2637. doi:10.1111/j.1365-2672.2007.03495.x.

Holb, I. J. (2004). Effect of acidity on growth rate and stroma formation of *Monilia fructigena* and *M polystroma* isolates. *International Journal of Horticultural Science, 10*, 63–67.

Ioos, R., & Frey, P. (2000). Genomic variation within *Monilinia laxa, M. fructigena* and *M. fructicola*, and application to species identification by PCR. *European Journal of Plant Pathology, 106*, 373–378. doi:10.1023/A:1008798520882.

Maniatis, T., Sambrook, J., & Fritsch, E. F. (1989). *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbor Laboratory.

OEPP/EPPO. (2003). EPPO standards, diagnostic protocols for regulated pests. *EPPO Bulletin, 33*, 245–247. doi:10.1046/j.1365-2338.2003.00629.x.

Shima, Y. (1936). Studies on the young fruit rot of apple tree. *Journal of the Faculty of Agriculture, Hokkaido University, 39*, 143–149.

van Leeuwen, G. C. M., Baayen, R. P., Holb, I. J., & Jeger, M. J. (2002). Distinction of the Asiatic brown rot fungus *Monilia polystroma* sp nov from *Monilia fructigena*. *Mycological Research, 106*, 444–451. doi:10.1017/S0953756202005695.

Willets, H. J., & Bullock, S. (1993). Cytology, histology and histochemistry of fruit infection by *Monilinia* species. In A. R. Biggs (Ed.), *Handbook of cytology, histology and histochemistry of fruit tree diseases*, pp. 113–136. Boca Raton: CRC.

Wormald, H. (1954). *The brown rot diseases of fruit trees*. London: Her Majesty's Stationery Office.