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Distinction of the Asiatic brown rot fungus *Monilia polystroma* sp. nov. from *M. fructigena*

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Monilinia fructigena isolates from Japan were compared with isolates from Europe. General colony characteristics, stroma formation, growth rate and conidial dimensions were determined for six isolates each from both groups, as well as sporulation intensity on potato dextrose agar (PDA) and germ tube features. Potential differences in pathogenicity were tested on the pear cultivars 'Conference' and 'Doyenné du Comice', and on the apple cultivar 'Cox's Orange Pippin'. A marked difference in stroma formation occurred, the area of stromatal plates ranged from 4.1 to 5.2 cm^2 in the Japanese group, and from 0 to 0.9 cm^2 in the European. The mean growth rate was significantly higher for Japanese isolates (*t*-test, $P = 0.01$). Length and width of conidia were significantly greater in European isolates (*t*-test, $P = 0.01$). Conidia measured on average 19×11.5 μm in European isolates, and 16×10 μm in Japanese ones when grown on cherry agar. On fruits, the difference in conidium size was even more pronounced. Sporulation intensity on PDA and germ tube features did not differ between both groups. No differences were found in latency period, lesion growth rate or sporulation intensity on apple and pear fruits between both groups. Together with previously published differences in the ITS region of ribosomal DNA, our results show that the Japanese isolates belong to a distinct species, *Monilia polystroma* sp. nov. A description of the anamorph is given, as well as a table summarising key features for all four brown rot associated *Monilia* species.

INTRODUCTION

Since Honey (1928) erected the genus *Monilinia* within the *Sclerotiniaceae*, the brown rot fungi within this genus have comprised three distinct species: *M. fructicola*, *M. laxa* and *M. fructigena*. Within *Monilinia*, these three species constitute the section *Junctoriae*. In contrast with the section *Disjunctoriae*, they do not possess disjunctors between the mature conidia within the macroconidial chains (Honey 1936). Identification of the brown rot fungi is based on morphology and general colony characteristics on natural media (Hewitt & Leach 1939, Byrde & Willetts 1977, Batra 1991). Recently, van Leeuwen & van Kesteren (1998) developed an identification protocol based on quantitative cultural and morphological features. Lately, delineation of the three species on the basis of sequence divergence within the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) has been given attention (Carbone & Kohn 1993, Holst-Jensen *et al.* 1997, Snyder & Jones 1999). Five base-pair substitutions were found between *M. fructicola* and *M. laxa*, while *M. fructigena* showed sequence divergence at 13 positions compared with both other species in the ITS region (Holst-Jensen *et al.* 1997, Fulton *et al.* 1999). Intraspecific sequence variation was found in *M. fructicola* as well as *M. laxa*, consisting of one to three base substitutions (Holst-Jensen *et al.* 1997, Snyder & Jones 1999). While working with a larger set of *M. fructigena* isolates, Fulton *et al.* (1999) found distinct intraspecific variation in *M. fructigena*. Isolates from Japan had four base substitutions in ITS 1 and one substitution in the ITS 2 region compared with the sequence found in European *M. fructigena* isolates. The sequence for isolates from Europe concurred with that reported by Holst-Jensen *et al.* (1997). No further variation was detected within neither the Japanese nor the European group.

To what extent genetic variation in the ITS region of

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rDNA corresponds to morphological and biological differences, varies considerably within the *Sclerotiniaceae*. Within the section *Disjunctoriae* in *Monilinia*, *M. gaylussaciae* and *M. vaccinii-corymbosi* have identical ITS sequences (Holst-Jensen *et al.* 1997), but are distinct morphologically and biologically (Batra 1991). Phylogenetic analysis on the basis of ITS and partial sequencing of the small (18S) and large (28S) subunits of rDNA, has shown that the section *Junctoriae* forms a monophyletic lineage with three outgroup taxa, *Botrytis cinerea*, *Ciboria caucus* and *Sclerotinia sclerotiorum* (Holst-Jensen *et al.* 1997). The level of sequence heterogeneity within this lineage is low, while morphologically these are well-delimited genera. For example, *M. fructicola* and *B. cinerea* differ in only four nucleotide sites within the ITS 1 region (Carbone & Kohn 1993). Given the genetic differences found between Japanese and European *M. fructigena* isolates, we expected to find cultural and morphological differences between these two groups. So far, however, a direct comparison of cultural and morphological characteristics between Japanese and European *M. fructigena* isolates has not been made.

Morphological and biological characteristics may differ between Japanese and European *M. fructigena* isolates. Biological characteristics such as host plant range, rate of development and formation of survival structures, form an important aspect in pest risk analysis (OEPP/EPPO 1997). The host range reported for Japanese *M. fructigena* (Harada 1998), is similar to that reported for European isolates (Byrde & Willetts 1977), and includes species of *Malus*, *Pyrus*, *Cydonia*, and *Prunus*. In the life-cycle of both Japanese and European *M. fructigena* isolates, the teleomorph hardly plays a role. In Europe, Solkina (1932) found apothecia of *M. fructigena* in the field, other workers obtained apothecia only after experimental incubation of mummified fruits (Aderhold & Ruhland 1905, Johansen 1945). Also in Japan apothecia are seldom found in the field (Batra & Harada 1986), but Harada (1977) managed to obtain apothecia *in vitro* with Japanese *M. fructigena* isolates.

Here we present a comparative study of cultural, morphological and biological characteristics of Japanese and European *M. fructigena* isolates. We provide evidence that Japanese *M. fructigena* isolates should be considered a new species, and discuss the possible evolutionary history of speciation. Finally, potential consequences of our findings for plant health and quarantine aspects are discussed.

MATERIALS AND METHODS

Isolates

Japanese *Monilinia fructigena* isolates were kindly provided by Y. Harada (Faculty of Agriculture and Life Science, Hirosaki University). A corresponding set of six isolates representative of European *M. fructigena* isolates was selected from a culture collection established by the cooperation of several European institutes and universities. Isolates were used without single spore culturing. Initially, *M. fructigena* isolates were lyophilised and subsequently used for experiments. Later, cultures were maintained on V-8-juice agar slants stored at 10 °C. Vitality of the cultures was guaranteed by stimulating sporulation under nuv light after every transfer in storage. Host, geographical origin and year of isolation of the cultures are given in Table 1. Cultures are deposited in CBS as CBS 101504, 102686, 102687, and 102688.

Table 1. List of *Monilinia* isolates used in this study.

Isolate	Host	Origin	Year of isolation
JAP 1145	*Malus pumila*	Japan	1987
JAP 1815	*Malus pumila*	Japan	1991
JAP 2314	*Malus pumila*	Japan	1994
JAP 2315	*Malus pumila*	Japan	1994
JAP 2316	*Malus pumila*	Japan	1994
JAP 2317	*Malus pumila*	Japan	1995
PD 4.96	*Malus pumila*	The Netherlands	1996
PD 8.96	*Prunus persica*	The Netherlands	1996
ES-48	*Prunus domestica*	Spain	1996
CC 752	*Prunus domestica*	Poland	1993
HU 1.96	*Prunus* sp.	Hungary	1996
FR-8	*Prunus armeniaca*	France	199?

Colony morphology, growth rate and conidial dimensions

General colony characteristics commonly used to describe *Monilinia* cultures (Byrde & Willetts 1977, Batra 1991) were determined for Japanese and European *M. fructigena* cultures grown on potato dextrose agar (PDA, Oxoid, Basingstoke) in 9 cm diam, plastic Petri dishes for 10–12 d at 22 ° in 12 h light/12 h dark regime. Light during incubation was provided by two nuv tubes (Philips TLD, 18W/08) at approximately 15 cm above the Petri dishes.

Formation of stroma on agar plates was quantified by image analysis. For this, isolates were grown on cherry decoction agar (CHA) prepared according to Gams *et al.* (1998) but with the cherry juice at half strength. Dishes were inoculated with *M. fructigena*, and incubated in darkness at 22 ° for 6 d. Plugs of 4 mm diam were taken from the edge of the colony and placed at the centre of new CHA dishes (six replicates per isolate). Subsequently, plates were incubated at 22 ° in darkness and sealed with parafilm after one day incubation to avoid dehydration of the plates. After 21 d incubation, the area of stroma formation per Petri dish was quantified using an image analyser (Quantimet 570, Cambridge Instruments). From the underneath of plates, the black stromatal plates contrasted well with the yellowish colour of the colony, and this allowed precise selection of stroma for measurement.

Colony growth rates of Japanese and European *M. fructigena* isolates were compared on PDA. Dishes were inoculated from stock cultures, and incubated at

22 ° in 12 h light/12 h dark regime (nuv). After 8 d incubation, plugs taken from the edge of the colony were placed in the centre of new PDA plates. Four replicate plates were incubated at 22 ° in darkness, and four were placed at 22 ° in 12 h light/12 h dark regime (nuv). Mean colony diameter, including the 4 mm plug, was determined after 3 and 6 days incubation. Subsequently, the absolute growth rate was calculated in mm day^{-1} between day 3 and 6. The experiment was conducted twice.

The size of conidia obtained from agar cultures and from sporulating fruits was determined for both groups. After 9–13 d incubation at 22 ° in 12 h light/12 h dark regime (nuv), conidia were washed off CHA plates by adding 4–5 ml of distilled water and gently scraping the cultures with a needle. For each isolate, length and width of 35 conidia were measured in distilled water at ×400 magnification. Ripe pear fruits of cv. 'Conference' were inoculated and incubated in closed trays at 14–18 °. Light intensity was 10.000–12.000 lux in a 14 h light/10 h dark regime. Eight days after inoculation, the lids were removed from the trays to promote dehiscence of conidial chains at lower relative humidity (RH 60–70 %). Conidia were collected in distilled water 10 d after inoculation, and per isolate 35 conidia were measured as described above. Experiments *in vitro* as well as *in vivo* were conducted twice.

Mean area of stroma formation, growth rate and conidial dimensions were determined for each isolate, and subsequently the overall means for the Japanese and European group were compared with a *t*-test. For stroma formation, isolates that did not produce any stroma were excluded from the statistical analysis.

Increase in colony diameter, sporulation intensity and germ tube characteristics

In a former study, a protocol was developed to delineate *Monilinia fructicola*, *M. laxa* and *M. fructigena* on the basis of quantitative characteristics when grown on PDA (van Leeuwen & van Kesteren 1998). Increase in colony diameter (3–5 d incubation), sporulation intensity, and germ tube characteristics were included in this protocol. To determine whether these characters are also appropriate to distinguish Japanese from European *M. fructigena* isolates, a comparison was made between five isolates of each group. Characters were determined according to the method described in van Leeuwen & van Kesteren (1998). Distance from conidium to the first branch in the germ tube was not determined. Means of both groups were compared with a *t*-test, data for sporulation intensity were first log-transformed. Mean percentages of conidia with more than one germ tube were compared with a Wilcoxon-test.

Pathogenicity on fruits

Pathogenicity of Japanese and European *Monilinia fructigena* isolates was assessed on ripe pear (cv. 'Conference', cv. 'Doyenné du Comice') and both ripe and unripe apple (cv. 'Cox's Orange Pippin'). An agar plug from the edge of an actively growing colony on PDA or CHA was inserted into a small hole punched in the fruit skin (4 mm diam). Subsequently, fruits were incubated in closed trays with a transparent lid (l × w × h = 45 × 30 × 30 cm), with a layer of water underneath the plateau on which the fruits were placed. Six replicate fruits per isolate were used in each experiment, distributed over six trays (blocks). The trays were placed in a climate room. Temperature inside the trays ranged from 14 to 18 °, RH was 95–98 % and light intensity 10.000–12.000 lux (Philips, TD 32W/84HF) in a 14 h light/10 h dark regime. After inoculation, fruits were examined every day to score the appearance of conidial pustules (latency period). Width and height of the developing lesion were determined after 3 and 6 d incubation (ripe Conference, exp I), or, alternatively, after 2 and 4 d (in all other experiments). The lid was removed from trays after 4 d in experiments with unripe fruits, and after 5 d for ripe fruits, to promote dehiscence of conidial chains at lower RH (70–75 %). One day later, conidia were collected by shaking the fruits in 30–40 ml water at 200 rpm for 30 s in an orbital shaker. The suspension was filtered through a double layer of cheesecloth, and subsequently the number of conidia per ml determined using a haemocytometer. The total number of conidia produced per fruit was calculated by multiplying the number of conidia per ml with the collected volume in ml.

Lesion growth rate in mm d^{-1} was calculated from the difference in lesion size between the two assessment dates. The number of conidia produced per cm^2 lesion was calculated by taking the quotient of the number of conidia produced per fruit and the lesion area on day 4. Sporulation data were log-transformed for statistical analysis. The procedure PROC MIXED of the statistical program SAS (SAS Institute, Cary, NC) was used to test for significant differences between the Japanese and European groups of *M. fructigena* isolates (*F*-test). The factors 'block' and 'isolate' were included as random effects in the model.

RESULTS

Colony morphology, growth rate and conidial dimensions

Small differences were found in general colony morphology between both groups. The development of aerial mycelium on which sporulation occurs, was more profuse in European *Monilinia fructigena* isolates. In two of the European isolates, aerial mycelium rose 4–5 mm above the colony's surface, while in Japanese isolates this was only 1–2 mm. The colour of sporogenous tissue was distinct buff/pale luteous (Rayner 1970) in both groups, and clear concentric rings of sporulation appeared in isolates with a high growth rate (e.g. JAP 2315, JAP 2317, ES-48). After 10–12 d

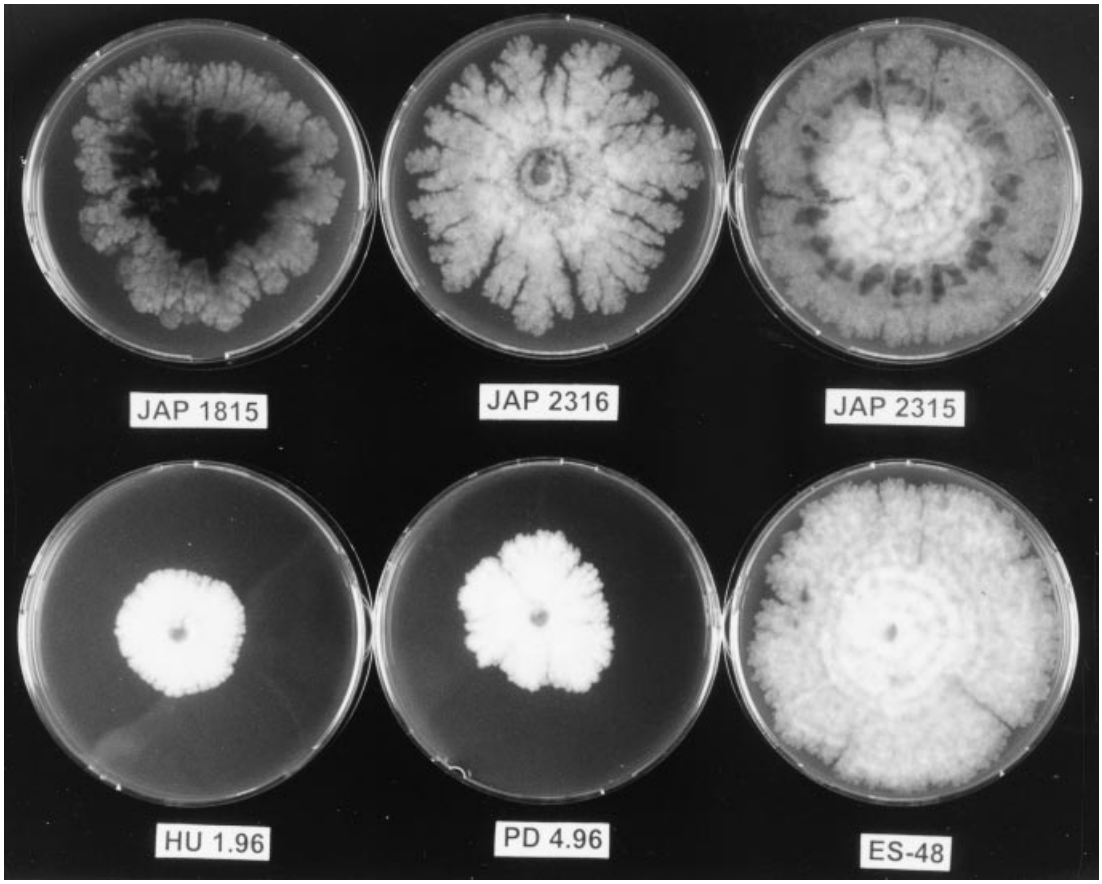

Fig. 1. *Monilinia fructigena* cultures on potato dextrose agar after 10 d incubation at 22 °C in 12 h light/12 h dark regime (nuv). Upper row Japanese isolates (i.e. *Monilia polystroma*), lower row European isolates.

incubation, stroma started to develop in Japanese isolates; especially in isolate JAP 1815 this was very pronounced (Fig. 1). At that time, there was no stroma visible in any of the European isolates.

The mean area of stroma formed after 21 d incubation ranged from 4.1 to 5.2 cm^2 in the Japanese isolates and from 0 to 0.9 cm^2 within the European group (Table 2). In two of the European isolates no stroma was observed at all (CC 752 and HU 1.96). The difference in stroma formation between the two groups was highly significant (*t*-test, $P = 0.001$).

Mean colony growth rate of Japanese *M. fructigena* isolates was significantly higher (*t*-test, $P = 0.01$) in both light regimes (Table 2). In 12 h light/12 h dark regime, the mean growth rate for Japanese isolates was 8.4 mm d^{-1} and 5 mm d^{-1} for European isolates; in continuous darkness this was 7 and 3.7 mm d^{-1}, respectively. Between individual isolates, some variation occurred within each group. Within the European group, isolate ES-48 showed a relatively high growth rate (8.3 mm d^{-1} in light/dark regime, 7.0 mm d^{-1} in darkness). Within the Japanese group, limited within-group variation occurred in the light/dark regime compared with the variation observed in continuous darkness (Table 2). In replicate experiments, similar results were obtained, though absolute growth rates were slightly lower than those in the first experiment (data not shown).

Conidial dimensions of Japanese and European isolates were compared *in vitro* as well as *in vivo* (Table 2). Length and width of conidia obtained from CHA were significantly greater in European *M. fructigena* isolates compared with Japanese isolates (*t*-test, $P = 0.01$). The range in mean length and width calculated per individual isolate, showed no overlap between the groups, except for a slight overlap in mean width (Table 2). The difference in conidium size was even more pronounced for conidia taken from sporulating fruits. Mean length and width for European isolates was 21.5×13 μm, and 16×10.5 μm for Japanese isolates. Difference in mean length and width was highly significant (*t*-test, $P = 0.001$).

Increase in colony diameter, sporulation intensity and germ tube characteristics

A comparison of increase in colony diameter from 3 to 5 d incubation, sporulation intensity and germ tube characteristics between European and Japanese *Moliniinia fructigena* isolates was carried out according to the standardized protocol of van Leeuwen & van Kesteren (1998) for distinguishing between *Monilinia fructigena*, *M. fructicola* and *M. laxa*. Though the increase in colony diameter of Japanese isolates seemed higher than that of European ones, the difference was not significant (*t*-test, $P = 0.05$). No significant differ-

Table 2. Comparison of stroma formation, growth rate and conidial dimensions between European (Eur) and Japanese (Jap) *Monilinia fructigena* isolates.

	European strains	Japanese strains
Stromatal plates (cm²)		
Mean	0.4 ± 0.25**	4.6 ± 0.25**
Range	0–0.9	4.1–5.2
Colony growth rate (mm d⁻¹		
12 h Light/12 h Dark		
Mean	5 ± 0.9*	8.4 ± 0.4*
Range	2–8.3	7.5–10.4
Darkness		
Mean	3.7 ± 0.7*	7 ± 0.8*
Range	2.6–7	3.7–9.3
Conidial size (μm)		
Grown on CHA		
Mean L × W[b]	19 × 11.5*	16 × 10*
Range	17.5–20.5 × 10.5–12.5	13–17 × 9–10.5
Grown on pear		
Mean L × W	21.5 × 13**	16 × 10.5**
Range	20.5–23.5 × 12.5–13.5	14.5–19 × 12

Significantly different values (*t*-test) at $P = 0.01$ (*) and $P = 0.001$ (**) are indicated in superscript.

[a] Mean ± S.E.M.

[b] Length × width (average of 35 conidia).

ences could be detected in sporulation intensity, germ tube length and percentage of conidia with more than one germ tube either (not shown).

Pathogenicity on fruits

Minimal variation in latency period occurred between individual fruits in the experiments with unripe fruits. Three days after inoculation, the first pustules appeared in Japanese as well as European isolates on cv. 'Conference' and cv. 'Cox's Orange Pippin'. More variation in latency period between isolates was found in ripe fruits. In Japanese isolates, the latency period ranged from 3 to 4.7 d on cv. 'Conference' in the first experiment, whilst in European isolates this was 3 to 3.5 d. In the second experiment with cv. 'Conference', values ranged from 2.3 to 3.0 d and 2.2 to 3.8 d for Japanese and European isolates, respectively. The difference between both groups was not significant (Wilcoxon-test, $P = 0.05$). Results for lesion growth rate and sporulation intensity on ripe fruits are shown in Table 3. In the first experiment with ripe 'Conference' fruits (Exp I), we found a significantly higher lesion growth rate for Japanese isolates compared with European isolates (*t*-test, $P = 0.05$), namely 13.6 and 16 mm d^{-1}, respectively. However, in later experiments no significant difference in lesion growth rate was found (Exp II and III, Table 3). No differences were found in the number of conidia produced per cm² lesion between Japanese and European isolates. In the other pear cultivar, 'Doyenné du Comice', neither differences in lesion growth rate nor in sporulation intensity were observed between the *M. fructigena* groups (data not shown). Mean lesion growth rate on cv. 'Cox's Orange Pippin' was equal for both groups, as was sporulation intensity. Similar experiments with unripe fruits did not show significant differences in measured characters between the groups (data not shown).

DISCUSSION

Our study clearly shows that genetic divergence within the non-coding rDNA ITS region between Japanese and European *Monilinia fructigena* isolates, reported by Fulton *et al.* (1999), is reflected in distinct and reproducible morphological differences between both groups. Each Japanese isolate formed more stroma than individual European isolates. In a comparison of *Monilinia* isolates from different geographical regions, Wormald (1927) noted that a Japanese *M. fructigena* culture produced zones of black stromatal plates in culture, a feature he never observed in European *M. fructigena* isolates. In his monograph on Japanese

Table 3. Lesion growth rate and sporulation intensity of European (Eur) and Japanese (Jap) *Monilinia fructigena* strains on ripe fruits of apple and pear.

	cv. 'Conference'		cv. 'Cox's Orange Pippin'	
	Lesion growth rate (mm d⁻¹)[a]	No of conidia cm⁻² (log-transformed)[b]	Lesion growth rate (mm d⁻¹)	No of conidia cm⁻² (log-transformed)
Exp I				
Eur	13.6 ± 0.49 (6) a[c]	nd		
Jap	16 ± 0.39 (6) b	nd		
Exp II				
Eur	16.4 ± 0.85 (5)	5.1 ± 0.03	13 ± 0.59 (5)	3.8 ± 0.09
Jap	20.7 ± 1.85 (5)	5.1 ± 0.07	12.8 ± 0.78 (5)	3.8 ± 0.18
Exp III				
Eur	12.4 ± 1.23 (5)	4.8 ± 0.11	16.7 ± 1.01 (5)	4.2 ± 0.06
Jap	15.9 ± 0.99 (5)	4.8 ± 0.24	17.8 ± 0.32 (5)	3.9 ± 0.31

[a] Mean ± S.E.M.; number of values used in calculating the mean given in parentheses.

[b] Conidia collected from fruits 6 d after inoculation of the fruits; lesion size data from day 4.

[c] Values followed by different letters significantly differ at $P = 0.05$ (*t*-test).

nd, not determined.

Table 4. Distinguishing *in vitro* characters of the *Monilinia* (and *Monilia*) brown rot fungi of fruit crops.

Species	Character	
Colonies on PDA (22 °C, 12 h light/12 h dark regime)		
fructicola	Growth rate high; sporulation intense (also in continuous darkness); colour of sporogenous tissue hazel/isabelline*	
laxa	Growth rate low, renewed outgrowth of mycelium at the edge of the colony taking place after growth is checked temporarily (rosetting, i.e. mycelium in distinct layers on top of each); sporulation sparse, colour of sporogenous tissue hazel/isabelline	
fructigena	Growth rate low-moderate; sporulation sparse, on distinct tufts/rings of aerial mycelium, colour of sporogenous tissue buff/pale luteous	
polystroma	Growth rate moderate; sporulation sparse; intense formation of black, stromatal plates initiated after 10–12 d incubation; colour of sporogenous tissue buff/pale luteous	
Conidial dimensions (mean and range)[a]		
fructicola	13 × 9 μm (8)[b]	12.5–14.5 × 8–10 μm
laxa	12 × 8.5 μm (4)	11–13 × 8–9.5 μm
fructigena	19 × 11.5 μm (6)	17.5–20.5 × 10.5–12.5 μm
polystroma	16 × 10 μm (6)	13–17 × 9–10.5 μm
Germ tube morphology[c]		
fructicola	long, straight, 750–900 μm, one per conidium	
laxa	short, twisted, 150–350 μm, one per conidium	
fructigena	long, straight, 600–900 μm, often two per conidium	
polystroma	long, straight, 700–1000 μm, often two per conidium	

* Colour notations follow Rayner (1970).

[a] Conidia grown on CHA at 22 ° under NUV light.

[b] 35 conidia measured per isolate in distilled water, number of isolates in parentheses.

[c] After 18 h at 22 ° on water agar plates.

Monilinia species, Harada (1977) confirmed the occurrence of intense stroma formation in Japanese *M. fructigena* isolates. Although we only quantified stroma formation *in vitro*, we found similar differences in stroma formation between both groups on infected fruits. Japanese isolates produced a thick hyphal mantle of (ecto)stroma on the host's cuticle, while European isolates produced none (van Leeuwen, unpubl.).

The mean radial growth rate of Japanese *M. fructigena* isolates on PDA, measured between 3 and 6 d incubation, was significantly higher than that of European isolates. A similar trend was seen when the growth rate was measured between the third and fifth day of growth, according to the protocol of van Leeuwen & van Kesteren (1998) for distinguishing between *M. fructigena*, *M. fructicola* and *M. laxa*. That differences were not significant in the latter experiment is likely due to the shorter growth period. Mean lesion growth rates on Conference fruits were higher for Japanese than for European strains in one experiment, but not in two others in which growth was measured after 2 and 4 d incubation instead of after 3 and 6 d (Table 3). It seems that initially, in the first days of colony expansion, no difference in growth rate exists between both groups, but after this period colony/lesion expansion rate is higher in Japanese isolates. That less time is taken for Japanese *M. fructigena* isolates to colonise cv. 'Conference' fruits completely than for European isolates, supports this interpretation (van Leeuwen, unpubl.). Harada (1977) found that a Japanese *M. fructigena* isolate had an even higher growth rate on PDA than *M. fructicola*, whereas van Leeuwen & van Kesteren (1998) showed that growth rate on PDA under similar conditions was consistently lower in European *M. fructigena* isolates than in those of *M. fructicola*. Though overall means, averaged over isolates, significantly differed between both groups, the European isolate ES-48 showed a growth rate similar to Japanese isolates (Fig. 1). Obviously, the distributions of both groups of strains show some overlap in growth rate.

Clear differences in the size of conidia were found between the geographical groups (Table 2), conidia being smaller in isolates of the Japanese group. Khokhriakova (1978) found differences in conidium size between *M. fructigena* populations from different regions in the former USSR. Mean length and width of conidia for the European population was 19.4 × 11.5 μm; for the Central Asian and the Far Eastern populations this was 17.2 × 11.9 μm and 18 × 11.4 μm, respectively. Thus, conidia tended to be smaller in Asian regions. On apple fruits incubated at 15 °, Batra & Harada (1986) measured a range in conidium length × width of 14–20 × 9.5–14.5 μm for Japanese material, concurring perfectly with our findings.

The colony and germ tube characteristics which enabled separation of *M. fructicola*, *M. laxa* and (European) *M. fructigena* (van Leeuwen & van Kesteren, 1998), were not useful for separating Japanese and European *M. fructigena* isolates. Good discriminating morphological characters between Japanese and European isolates of *M. fructigena* are the intensity of stroma formation and conidium size. An overview of distinguishing characters of the brown rot fungi is given in Table 4. *M. fructicola* and *M. laxa* are distinguished on the basis of differences in sporulation intensity, shape of the colony and growth rate (Table 4). European and Japanese *M. fructigena* isolates differ in stroma

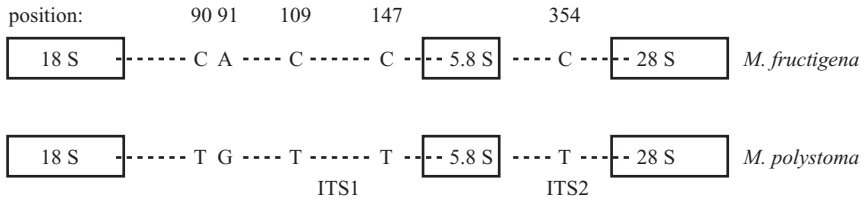

Fig. 2. Sequence divergence in the ITS region of ribosomal DNA (rDNA) between *Monilinia fructigena* (EMBL Z73779) and *Monilia polystroma* (EMBL Y17876). After Fulton *et al*. (1999).

formation and conidial dimensions. Moreover, European and Japanese *M. fructigena* isolates show basepair substitutions in the ITS region in five positions (Fulton *et al*. 1999; schematically represented in Fig. 2), equal to the number of substitutions found between *M. fructicola* and *M. laxa*. Therefore, we conclude that isolates from Japan, formerly described as *M. fructigena*, belong to a distinct species, which is here named as *Monilia polystroma* as only the anamorph is known.

Monilia polystroma van Leeuwen, **sp. nov.**

Etym.: *polystroma* , after the species' intense stroma formation

Coloniae in agaro PDA dicto post 6 dies 22 °C sub luce variabili 50–60 mm diam; margo regularis; mycelium fertile modice elevatum, bubalino-luteum. Stroma in vitro 22 ° sub luce variabili post 10–12 dies incipit; laminae stromaticae maturae primum discretae, deinde confluentes. Macroconidia globosa vel ovoidea vel limoniformia, levia, in vitro 22 ° 12–21 × 8–12 μm (media 16.4 × 9.1 μm), in vivo 15 ° 11–20 × 8–11 μm; massa conidialis bubalina vel brunneo-grisea.

Habitat: fructus arborum pomiferarum putrefaciens in Japonia.

Typus: Japonia; *Praefectura Aomori*: isolata e *Malo* (*sylvestri*) *pumila* in Honshu, *Y. Harada* (L 998.171 549-holotypus; CBS 102688-cultura viva).

Colonies on potato dextrose agar (PDA) reaching 50–60 mm diam after 6 d at 22 °C under 12 h light/12 h dark cycle. Colony margin even, sporogenous tissue slightly elevated above the colony surface (1–2 mm), colour buff/pale luteous. *Stromatal initials* formed 10–12 d after inoculation at 22 ° under 12 h light/12 h dark cycle; mature, black stromatal plates first discrete, later coalescing. *Macroconidia* globose, ovoid or limoniform, smooth, measuring 12–21 × 8–12 μm, av. 16.4 × 10.1 μm (distilled water) when grown on cherry agar (CHA) at 22 ° under NUV, and 11–20 × 8–11 μm, av. 14.9 × 9.1 μm on pear fruit at 15 °. On fruit, a thick hyphal layer of stroma appearing after the fruit is colonised; conidial tufts buff to brownish-grey.

Unfortunately, we were unable to induce the formation of apothecia in either European nor Japanese material. Following the method described by Willetts & Harada (1984), in Japanese material we only obtained minute apothecial stipe initials measuring 4–5 mm on stromatised pear discs. These initials shriveled before discs could develop. It is very likely that the description of a teleomorph in material from Japan given by Batra & Harada (1986) and designated as *Monilinia fructigena*, is an account of the teleomorph of *Monilia polystroma*. The authors describe the intense formation of stroma in the anamorph, and conidial size on sporulating fruits matches with our observations in *M. polystroma*. The description of the teleomorph given, did not differ from those of apothecia of *M. fructigena* found in Europe (Aderhold & Ruhland 1905, Johansen 1945). Because of uncertainties and no teleomorphic material being available to serve as type, we only describe here a new anamorph taxon.

Our study gives a clear example of speciation due to geographical isolation; although geographical isolation is particularly important in plants and animals, it is not always recognised as such in fungal evolution (Moore-Landecker 1996). Byrde & Willetts (1977) postulated that in the past *M. laxa* probably evolved first from an ancestral form of the brown rot fungi. Phylogenetic analysis of rDNA sequence data supports this hypothesis (Holst-Jensen *et al*. 1997). According to Leppik (1970), the Far East may be the place where the common ancestor was first associated with fruit plants. The occurrence of substantial genetic variation, not only in *M. fructigena*/*M. polystroma*, but also in Japanese *M. fructicola* isolates (Fulton *et al*. 1999), supports this view. A *M. laxa*-like ancestor presumably caused blossom- and twig blight on the wild progenitors of present day fruit crops in this region. When people started to select for cultivars with larger and more juicy fruits, the ancestor of *M. fructigena* and *M. polystroma* might have evolved in the Far East as a specialised fruit pathogen. Subsequently, due to geographical separation, once introduced into their own locality, two distinct forms evolved in Europe and Japan. Differences in the host gene pool, climatic conditions, and biotic environment may have played a role in morphological and genetic divergence (Moore-Landecker 1996). Harada (1998) mentions the occurrence of a mycoparasite, *Lambertella corni-maris*, on stroma of *M. fructigena* on apple fruits in Japan. A hostile biotic environment may have triggered defense mechanisms, with intensified stroma formation as a consequence.

Clear morphological (this study) and genetic differences (Fulton *et al*. 1999) exist between *M. fructigena* and *M. polystroma*, but biological differences seem small. Measurements of lesion growth rate and sporulation intensity on the pear cultivars 'Conference' and 'Doyenné du Comice' showed no significant differences.

The same held for the apple cultivars 'Cox's Orange Pippin' (Table 3), cv. 'Golden Delicious' and cv. 'James Grieve' (van Leeuwen, unpubl.). However, on cv. 'Conference' we found indications that lesion growth rate was higher in *M. polystroma* when determined later in the process of lesion expansion. Fruits infected in the field might be colonised faster by *M. polystroma* compared with *M. fructigena*. Moreover, the abundant stroma formation by *M. polystroma* is also expressed *in vivo*. The mantle of (ecto)stroma formed on the host's cuticle after conidial pustules have ruptured the cuticle, protects the colonised fruit against degredation and decomposition by abiotic and biotic factors. Because the pathogen survives in dried-out infected fruits (mummified fruits), inhibition of quick decomposition of infected fruits might enhance survival, and subsequently increase the amount of primary inoculum in the next season (Byrde & Willetts 1977). These aspects deserve attention in future research, in order to determine the risk of introduction of *M. polystroma* into areas outside Japan, using pest risk analysis schemes (OEPP/EPPO 1997). Furthermore, it would be of particular interest to collect *M. fructigena* isolates in other areas in East Asia (e.g. China, Russia), and characterise these morphologically and genetically. This would give a better understanding of the geographical distribution of *M. fructigena* and *M. polystroma*, and may clarify their evolutionary history.

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