Characterization of *Paecilomyces cinnamomeus* from the camellia whitefly, *Aleurocanthus camelliae* (Hemiptera: Aleyrodidae), infesting tea in Japan

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1 Characterization of *Paecilomyces cinnamomeus* from the camellia whitefly,
2 *Aleurocanthus camelliae* (Hemiptera: Aleyrodidae), infesting tea in Japan

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7
8 ABSTRACT

9 The whitefly, *Aleurocanthus camelliae* Kanmiya & Kasai (Hemiptera: Aleyrodidae), is an invasive
10 species in Japan that was first discovered in 2004 on tea in Kyoto. Soon after its arrival epizootics of
11 an entomopathogenic fungus were observed in populations of the whitefly in many tea-growing
12 regions. Here we identify this fungus as *Paecilomyces cinnamomeus* (Petch) Samson & W. Gams
13 (Hypocreales: Clavicipitaceae) based on morphological characteristics and molecular analyses. This
14 is the first record of *P. cinnamomeus* in Japan and also the first time it has been recorded from the
15 genus *Aleurocanthus*. A isolate of *P. cinnamomeus* caused greater than 50% and 90% infection in
16 whitefly nymphs at 1×10^6 and 1×10^7 conidia/ml respectively, while the commercial
17 mycoinsecticides Preferd[®] (*Isaria fumosorosea*) and Mycotol[®] (*Lecanicillium muscarium*) caused <
18 10% infection at their recommended field rates (5×10^6 and 9×10^6 conidia/ml, respectively),
19 suggesting that *P. cinnamomeus* may be more useful as a control agent than the currently available
20 mycoinsecticides. Optimum and upper limit temperatures for *in vitro* growth of *P. cinnamomeus*
21 isolates were 22.5-25°C and 32.5°C, respectively. At field rates, the fungicide thiophanate-methyl
22 caused some inhibition of *in vitro* growth of *P. cinnamomeus* isolates, and the bactericide copper
23 oxychloride and the insecticides tolfenpyrad and methidathion were strongly inhibitory. The findings
24 obtained in this study will be useful in the development of microbial control programs using *P.*
25 *cinnamomeus* against *A. camelliae*.

26
27 **Keywords:**

28 Tea

29 *Aleurocanthus camelliae*

30 *Paecilomyces cinnamomeus*

31 *Conoideocrella luteorostrata*

32 Molecular analyses

33 Pesticides

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1. Introduction

In 2004, heavy whitefly infestations were first recorded on tea in Kyoto Prefecture, central Japan, and quickly spread to other Prefectures (Yamashita and Hayashida, 2006; Kasai et al., 2010). Infested tea plants were weakened due to sap loss and to the growth of sooty mold on honeydew-covered leaf surfaces during heavy infestations. Recently this whitefly has been described as a new species, *Aleurocanthus camelliae* Kanmiya & Kasai (Hemiptera: Aleyrodidae). Originally it had been thought to be *A. spiniferus* (Quaintance), a species infesting tea in China (Kanmiya et al., 2011). It is believed that *A. camelliae* was introduced to Japan from China (Kasai et al., 2010).

Simultaneously with outbreaks of the whitefly, fungal epizootics were also observed in *A. camelliae* nymphs in many tea fields. Infected whitefly nymphs all had the common symptom of being covered with a cinnamon-colored mat of mycelium. In this study, the fungus was identified based on morphological characteristics and phylogeny of the ITS rDNA, the β -tubulin gene and the translation elongation factor 1 α gene. In addition, the virulences of the fungal isolates were determined and compared with the mycoinsecticides Preferd[®] (*Isaria fumosorosea*) and Mycotol[®] (*Lecanicillium muscarium*), which are used commercially to control vegetable whiteflies such as *Bemisia tabaci* (Gennadius) B biotype (formerly *B. argentifolii*) and *Trialeurodes vaporariorum* (Westwood). Moreover, the effects of temperature and pesticides (a bactericide, fungicides and insecticides) on fungal growth were also evaluated. Together, these data inform our understanding of how this fungus might be encouraged under the environmental conditions found in Japanese tea-growing regions and integrated with existing whitefly control strategies.

2. Materials and Methods

2.1. Morphological observation and isolation of the fungus

Infected nymphs were collected from commercial tea plants growing in Kyoto, Shiga and Mie Prefectures, Japan, between August 2009 and February 2010 when heavy infections were observed. Twelve isolates were established as pure cultures on Sabouraud dextrose agar (SDA) (Difco Laboratories Inc., MI, USA) from conidia on cadavers collected in different fields in each Prefecture (Table 1), and deposited in the culture collection of FFPRI (Forestry and Forest Products Research Institute), Tsukuba, Ibaraki, Japan. Infected cadavers were observed and photographed using a stereo microscope (MZ-16, Leica Microsystems GmbH, Wetzlar, Germany) and a scanning electron microscope (SEM; NeoScope JCM-5000, Jeol, Tokyo, Japan). The sizes of conidiogenous cells and conidia of all the isolates were measured using a light microscope (S-Ke, Nikon, Tokyo, Japan).

2.2. Molecular analyses

The twelve isolates were cultured on SDA for 5 days at 25°C. Mycelia were scraped from the plates using a 200-µl pipette tip and suspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Each suspension was homogenized with a hand homogenizer for 30 s and the DNA extracted using a PUREGENE Tissue DNA Purification kit (Qiagen, Tokyo, Japan). Morphological studies of the isolates suggested that they were likely to belong to the genus *Paecilomyces*. To confirm this attribution, or otherwise, DNA sequences and phylogenetic analyses were made on the same three sequence regions that had been used previously to analyze relationships among fungal species placed in *Paecilomyces* and the neighboring *Conoideocrella* and *Isaria* genera (Luangsa-ard et al., 2005; Johnson et al., 2009).

Polymerase chain reactions (PCRs) were performed to amplify the ITS rDNA with primers ITS4 and ITS5 (White et al., 1990), the partial β -tubulin gene (TUB) sequences with primers bt2a and bt2b (Glass and Donaldson, 1995) and the partial translation elongation factor 1 α gene (TEF) sequences with primers 983F and 2218R (Rehner and Buckley, 2005). Amplification reactions were made in volumes of 50 µl containing 200 µM dNTPs, 1.25 units of Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 0.4 µM primer, 1× Ex Taq buffer (Takara Bio Inc. Shiga, Japan) and 10 ng of template DNA. The ITS rDNA was amplified using the same PCR conditions, except for the annealing temperature which was 55°C. PCR conditions for the TUB were 1 cycle at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and then 1 cycle at 72°C for 7 min. Amplification of the TEF was made under the following conditions: 1 cycle at 94°C for 4 min followed by 37 cycles at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and then 1 cycle at 72°C for 7 min. Three µl of each PCR suspension was electrophoresed through a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light to check for amplification of bands of an expected size. Singly amplified products of 630 bp for ITS rDNA were purified using the QIAquick PCR Purification kit (Qiagen, Tokyo, Japan). The amplified products of 380 and 1030 bp for the TUB and TEF, respectively, were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan).

Purified PCR products were sub-cloned into the p3T cloning vector (MoBiTec GmbH, Göttingen, Germany). DNA sequence analysis of both strands was done with a capillary sequencer (ABI 3130XL, Applied Biosystems, CA, USA). Three clones were sequenced for each strand. DNA sequences were subjected to a Basic Local Alignment Search Tool (BLAST) search to identify sequences deposited in GenBank that had a significant homology. The sequences were aligned with the corresponding sequences of the reference isolates and species shown in Table 2 using ClustalW incorporated in MEGA 4 (Tamura et al., 2007). In the ITS rDNA and TUB phylogenies, the fungal isolates that were used previously to clarify relationships amongst fungal species placed in *Isaria* and *Paecilomyces* sect. *Paecilomyces* (Luangsa-ard et al., 2005) were included. A *Paecilomyces* sp. isolate from *A. spiniferus* on tea in China was also included in the ITS phylogeny to understand the relationship between this isolate and our isolates. In the TEF phylogeny, some fungal isolates belonging to Clavicipitaceae were included. These isolates were used in the Johnson et al. (2009) study on systematics of fungi including *Conoideocrella* spp. that had been referred to as species of

the genus *Torrubiella*. Alignment gaps were treated as missing data in the analyses. Phylogenetic trees were constructed by the neighbor-joining method of MEGA 4 (Tamura et al., 2007). Bootstrap analyses of 1000 replications were made to determine the supporting values of branches. *Isaria farinosa* and *I. tenuipes* were the designated outgroups.

2.3. Bioassays

The three isolates Kyoto-32, Shiga-22 and Mie-1 were used in bioassays because they all sporulated well on medium. Conidia of each isolate were harvested from 20 plate cultures that had been grown for 3 weeks on SDA at $25 \pm 1^\circ\text{C}$ in darkness by adding 10 ml of sterile 0.1% Tween 80 to each plate, agitating with a sterile spreader and pipetting the conidia suspension into a sterile flask. Conidial suspensions were filtered through sterile gauze, and conidial concentrations estimated using a Thoma haemocytometer and adjusted to provide suspensions containing 1×10^6 , 1×10^7 and 1×10^8 conidia/ml. The mycoinsecticides Preferd[®] (Tokai Bussan, Nagoya, Japan; active ingredient *I. fumosorosea*) and Mycotal[®] (Arista LifeScience, Tokyo, Japan; active ingredient *L. muscarium*) were also used at their recommended field application rates (1g/L). The actual conidial concentrations of these field rates were determined as 5×10^6 and 9×10^6 conidia/ml, respectively, using a Thoma haemocytometer.

Field-collected nymphs of *A. camelliae* on tea shoots were used. Tea shoots were collected from an experimental field at the Kyoto Prefectural Tea Experiment Station in Uji, Kyoto, on March 11, 2010. No pesticides had been applied for more than one year, and entomopathogenic fungi infecting *A. camelliae* had not been observed. Each tea shoot collected was approximately 15 cm in length with four or five leaves and approximately 150 (ranging from 135 to 171) overwintering mature nymphs (3rd and 4th instars) of *A. camelliae*. Before inoculation, the viability of each isolate was checked; germination rate of conidia was > 90% in all cases. Three replicate shoots were used for each treatment. The three shoots were inoculated by gentle agitation in 200 ml of the same conidial suspension for 10 sec. Control shoots were treated in the same way but with sterile 0.1% Tween 80 only. The base of each shoot was then inserted into a polyethylene tube containing water, enclosed in a polyethylene bag to maintain a high humidity and incubated under controlled conditions of 16L: 8D and $20 \pm 1^\circ\text{C}$. After inoculation the shoots were checked daily using a stereo microscope to record the first appearance of infection. One, 2 and 3 weeks after inoculation the proportion of nymphs that had become infected by the fungus was calculated from the number of dead nymphs exhibiting mycosis and the number of living nymphs remaining that did not exhibit mycosis, as observed under a stereo microscope.

2.4. Effect of temperature

The three isolates, Kyoto-32, Shiga-22 and Mie-1 used in the bioassays, were cultured on SDA at $20 \pm 1^\circ\text{C}$ in darkness for 2 weeks. Plugs were taken from the growing margin of each colony using a 3 mm diameter sterile cork borer and placed individually into the center of sterile 9 cm triple vented

Petri dishes containing 30 ml of SDA. Replicate plates for each isolate were placed into incubators at 10, 15, 20, 22.5, 25, 27.5, 30, 32.5 and 35°C and incubated in darkness for 3 weeks. Colony diameter was then measured in two perpendicular directions, and the mean determined after subtraction of 3 mm to account for the original inoculation plug. Five replicate plates for each isolate were used at each temperature.

2.5. Effect of pesticides

A total of 30 commercial pesticides including 12 fungicides, one bactericide and 17 insecticides were evaluated. These pesticides are in general use to control insect pests and diseases on tea in Japan. Each pesticide was added to sterilized SDA medium at the recommended application rate once it had cooled to below 50°C and mixed using a stirrer before pouring the agar and allowing it to set. Their effects on mycelial growth of Kyoto-32, Shiga-22 and Mie-1 used in the previous experiments were determined using the same procedures as described above for the temperature experiment except that they were incubated at $25 \pm 1^\circ\text{C}$.

2.6. Statistical analyses

The proportion of nymphs infected in bioassays were arcsin square-root transformed and analyzed using ANOVA, followed by Tukey HSD tests to compare infection rates amongst treatments. Data from the pesticide experiment were analyzed using ANOVA, followed by a Dunnett test to detect differences between each pesticide and the control. All analyses were conducted using the software package SPSS (SPSS, 2009).

3. Results

3.1. Description of the fungus

The entomopathogenic fungus was observed infecting nymphs but not eggs or adults in the field. Infected nymphs were covered with a thick cinnamon-colored mat of mycelium that appeared brush-like in form (Fig. 1A). The stromata were raised, pulvinate, tomentose and 1–2 mm in diameter with a hypothallus of thin and smooth-walled hyphae expanding radially to, commonly, 2–3 mm from the host on the leaf surface (Fig. 1B). Large hypothalli expanded to 15–20 mm in total diameter. Conidiogenous cells were phialidic, flask-shaped, and erected loosely on the hypothallus and rarely on the stromata (Fig. 1C). Conidia were fusiform and in basipetal chains (Fig. 1C and D). Perithecia were lacking on the stromata.

All isolates developed flattened, cinnamon-colored colonies *in vitro* and released a deep purple-red diffusible pigment into the medium, with the exception of Mie-1 that produced white colonies and no diffusible pigmentation. Massed conidia were green and produced sparsely on the colony. The mean sizes of conidiogenous cells ($n = 30$ per isolate) and conidia ($n = 50$ per isolate) of

the 12 isolates ranged between $2.4\text{--}2.8 \times 9.8\text{--}12.5 \mu\text{m}$ and $2.3\text{--}2.6 \times 5.2\text{--}7.6 \mu\text{m}$, respectively (Table 1). Perithecia were also lacking *in vitro*.

Based on the taxonomic keys of Samson (1974), the fungus was morphologically identical to *Paecilomyces cinnamomeus* (Petch) Samson & W. Gams.

3.2. Molecular analyses

All isolates used in the present study had an identical sequence for 586 bp of ITS rDNA. A BLAST search indicated that the ITS rDNA sequence of our isolates had 99% alignment with a *Paecilomyces* sp. isolate from *A. spiniferus* on tea in China. The second most significant alignment was 98% with the sequences of *P. cinnamomeus* and *Conoideocrella luteorostrata* (Zimm.) D. Johnson, G. H. Sung, Hywel-Jones & Spatafora (formerly *Torrubiella luteorostrata*) (Johnson et al., 2009). In the ITS rDNA phylogram, all of our isolates were grouped with *Paecilomyces* sp., *C. luteorostrata* and *P. cinnamomeus* and were well supported by a bootstrap value of 99% (Fig. 2).

The partial sequences of TUB for our isolates had significant alignments of 89–99% with *C. luteorostrata* and *P. cinnamomeus*. A TUB phylogram positioned our isolates in a clade that included *C. luteorostrata* and *P. cinnamomeus* and was supported by a bootstrap value of 100% (Fig. 3).

The partial sequences of TEF for our isolates had a 99% alignment with *C. luteorostrata* and the second most significant alignment was with the sequences of *Conoideocrella tenuis* (Petch) D. Johnson, G. H. Sung, Hywel-Jones & Spatafora (formerly *Torrubiella tenuis*) in the family Clavicipitaceae (Johnson et al., 2009). A phylogram of TEF indicated that our isolates were positioned with *C. luteorostrata* in a clade that was supported by a bootstrap value of 99% (Fig. 4).

The sequences were deposited in GenBank with accession numbers listed in Table 1.

3.3 Bioassays

Within 5 days of inoculation infected nymphs died and appeared white or cinnamon-colored with hyphae growing from the edges of their bodies. Subsequently the host body became covered with thick-walled cinnamon-colored hyphae. The appearance of the artificially induced infection was similar to that of natural infections in the field.

Bioassays were ended 3 weeks after inoculation when whitefly adults began to emerge (Fig. 5). No mycosis was recorded from nymphs in the control treatment. At week 3 there were significant differences among the isolates in the proportion of nymphs that became infected in the low ($F = 5.51$, $p = 0.024$), medium ($F = 71.13$, $p < 0.001$) and high conidial concentration treatments ($F = 83.80$, $p < 0.001$). At the low concentration (1×10^6 conidia/ml), Kyoto-32 caused the greatest percent infection (52.3%) which was significantly higher than that caused by Shiga-22 but not Mie-1 (Tukey HSD, $p < 0.05$). At the medium concentration (1×10^7 conidia/ml), Kyoto-32 and Shiga-22 infected 91.7% and 81.7% of nymphs respectively and these percentages were significantly greater than that for Mie-1 (Tukey HSD, $p < 0.05$). At the high concentration (1×10^8 conidia/ml), Kyoto-32, Shiga-22 and Mie-1 all infected more than 85% of nymphs and there were no significant differences

amongst the isolates (Tukey HSD, $p > 0.05$). Both mycoinsecticides (Preferd[®] at 5×10^6 conidia/ml and Mycotal[®] at 9×10^6 conidia/ml) infected $< 10\%$ of nymphs which was not significantly different to the control where there was 0% infection (Tukey HSD, $p > 0.05$).

3.4. Effect of temperature

The three isolates, Kyoto-32, Shiga-22 and Mie-1, grew most rapidly between 22.5°C and 25°C and ceased growth at 32.5°C (Fig. 6).

3.5. Effect of pesticides

There were significant differences in growth rates among treatments for Kyoto-32 ($F = 94.49$, $p < 0.001$), Shiga-22 ($F = 209.03$, $p < 0.001$) and Mie-1 ($F = 225.75$, $p < 0.001$). The bactericide copper oxychloride completely inhibited growth of all three isolates (Fig. 7). Most fungicides were harmful to the isolates, especially difenoconazole and tebuconazole, that completely inhibited growth of Shiga-22, while thiophanate-methyl had no effect on growth of Shiga-22 ($p = 0.78$) and showed only slight inhibition of growth of Kyoto-32 ($p = 0.03$) and Mie-1 ($p = 0.06$). Some insecticides, including tolfenpyrad and methidathion, were extremely inhibitory to all isolates, while imidacloprid, thiacloprid and pyridaben had no effect on growth of any isolate.

4. Discussion

Morphological characteristics of the fungal isolates from *A. camelliae* on tea agreed with those for *P. cinnamomeus* (Samson, 1974) that was reported to be the anamorph (without perithecia) of *C. luteoestrata* (the teleomorph with perithecia) by Hywel-Jones (1993). The relationship between both states was confirmed by phylogenetic analyses using the 28S rDNA (Artjariyasriping et al., 2001), the 18S rDNA (Luangsa-Ard et al., 2004) and the ITS rDNA and TUB (Luangsa-Ard et al., 2005). These findings are supported by the present study which demonstrated that our isolates fell into a clade with *P. cinnamomeus* and *C. luteoestrata* in the phylogenetic analyses of the ITS rDNA and TUB. Though recent phylogenetic work indicates that *C. luteoestrata* and *C. tenuis* are closely related species forming a sister group within the family Clavicipitaceae (Johnson et al., 2009), phylogenetic analysis using TEF also positioned our isolates with *C. luteoestrata* in a 99% supported clade. In addition, no perithecia could be found on cadavers from the field or from bioassays or *in vitro* cultures. These findings suggest that the fungus should be placed as the anamorph *P. cinnamomeus* of *C. luteoestrata*. Some *Paecilomyces* species (including *P. cinnamomeus*) still have taxonomic issues (Luangsa-Ard et al., 2005, 2011), and these issues remain unresolved. Therefore, here we refer to our isolates as *P. cinnamomeus*.

Paecilomyces cinnamomeus can be distinguished from the other *Paecilomyces* spp. by their

277 fusiform conidia, pigmented hyphae and deep purple-red diffusible pigment in the medium (Samson,
278 1974; Hywel-Jones, 1993). One of our isolates, Mie-1, produced a white colony and no pigmentation
279 *in vitro*, although the isolate did produce cinnamon-colored stromata *in vivo* on hosts as did the other
280 isolates. This suggests that these isolates have morphological and physiological variation in response
281 to media.

282 Since the relationship between *P. cinnamomeus* and *C. luteorostrata* has only recently been
283 discovered, the two species have generally been studied separately. *Paecilomyces cinnamomeus* has
284 been recorded in Thailand, North and Central America, Cuba, Mexico and Ghana, and is known as a
285 pathogen on scale insects and the citrus whitefly, *Dialeurodes citri* (Ashmead) (Samson, 1974;
286 Hywel-Jones, 1993). In contrast, *C. luteorostrata* has been recorded from scale insects and whiteflies
287 in Thailand, Java, Seychelles, Ceylon, Samoa, New Zealand and the Far East of Russia (Petch, 1924;
288 Dingley, 1953; Kobayasi, 1982; Kobayasi and Shimizu, 1982; Hywel-Jones, 1993). Our record of *P.*
289 *cinnamomeus* is the first record of this species in Japan and the first time that the host was from the
290 genus *Aleurocanthus*. However, the fungus possibly occurs on *A. spiniferus* infesting tea in China,
291 because phylogeny of the ITS rDNA of our isolates fell into the clade of a *Paecilomyces* sp. that was
292 isolated from *A. spiniferus* on tea in China (Huang et al., 2002). Moreover, a fungal species reported
293 as *P. aleurocanthus*, which was isolated from *A. spiniferus* on tea in China (Tang et al., 2003),
294 appears to be *P. cinnamomeus* according to attributes such as pigmented hyphae, deep purple-red
295 pigment and green colored conidia. There are also other Chinese records of *Paecilomyces* sp. from *A.*
296 *spiniferus* on tea (e.g. Han and Li, 2001; Tang, 2001; Guo et al., 2006).

297 In Thailand, the *P. cinnamomeus* and *C. luteorostrata* states occur throughout the year apart from
298 the hot dry season, and both were discovered simultaneously on stroma emerging from infected
299 insects (Hywel-Jones, 1993). Moreover, a second strain from a multiple-conidia isolation of the *P.*
300 *cinnamomeus* state produced perithecia *in vitro* after 6 months (Hywel-Jones, 1993). In Japan,
301 however, infected whiteflies attached to tea foliage were found throughout the year including the hot
302 and wet season (summer) and cold dry season (winter), and perithecia could not be found on hosts in
303 the field or in our bioassays. Furthermore, no perithecia formation was observed on 6 month old *in*
304 *vitro* cultures (data not shown). These findings suggest that maybe only the *P. cinnamomeus* state
305 occurs in Japan. Further research directed toward factors inducing both *P. cinnamomeus* and *C.*
306 *luteorostrata* states is necessary.

307 When the relationship between temperature and growth of *P. cinnamomeus* was unknown,
308 Hywel-Jones (1993) suggested that *P. cinnamomeus* should be considered as an entomopathogen
309 only in tropical and subtropical regions. However, in this study, *P. cinnamomeus* grew most rapidly
310 between 22.5°C and 25°C and ceased growth at 32.5°C, suggesting that the fungus prefers moderate
311 temperatures. In Kyoto, Shiga and Mie Prefectures where our isolates were collected, the lowest air
312 temperature is below 0°C in the winter and the highest air temperature is above 35°C in the summer.
313 However, the microclimate of commercial tea bushes, where many plants are grown closely together,
314 may provide not only the moderate temperatures but also the high humidity necessary to promote
315 infection by *P. cinnamomeus* and the development of epizootics in whitefly populations. In China,
316 entomopathogenic fungi are important natural mortality factors of whiteflies in tea-growing areas

(Han and Lin, 2003).

The citrus whitefly, *D. citri*, has been recorded as a host of *P. cinnamomeus* (Samson, 1974). This whitefly is a common pest on Satsuma mandarin, *Citrus unshiu* Marc., which is widely grown in Japan. However, *P. cinnamomeus* has not been found on *D. citri* in Japan. Moreover, *P. cinnamomeus* was highly virulent to the tobacco whitefly, *B. tabaci* B biotype, in the laboratory (Saito, unpublished data), which is also widespread in Japan, but again, infection by *P. cinnamomeus* in the field has never been recorded. It is possible either that *P. cinnamomeus* had been restricted to forests before invasion of *A. camelliae* since the primary habitat of the fungus is forests (Hywel-Jones, 1993), or that it was introduced simultaneously into Japan together with *A. camelliae*.

Preferd[®] and Mycotol[®], mycoinsecticides currently used to control whiteflies, caused very little infection (< 10%) in *A. camelliae* at recommended field application rates (5×10^6 and 9×10^6 conidia/ml, respectively), suggesting that neither are useful as control agents of this whitefly. In contrast, when isolate Kyoto-32 was used at the lower concentration of 1×10^6 conidia/ml, the proportion of nymphs becoming infected (> 50%) was greater than that achieved by the mycoinsecticides. At a concentration of 1×10^7 conidia/ml, the proportion of nymphs infected by isolate Kyoto-32 reached > 90%. These findings suggest that *P. cinnamomeus* may be more useful as a control agent for augmentation into tea fields than the currently available commercial mycoinsecticides.

Whether *P. cinnamomeus* can be used as a control agent for augmentative control or natural control will be influenced by interactions with pesticides that are currently in use in tea. For example, previous studies have shown that fungicides may disrupt control by entomopathogenic fungi, thereby resulting in extremely high populations of insect pests (Steinkraus, 2007). In this study, all the fungicides evaluated significantly inhibited growth of *P. cinnamomeus* although thiophanate-methyl was relatively safe for the fungus. The high susceptibility of the fungus to the bactericide copper hydroxide is interesting since this bactericide has different effects depending on the fungus species and whether *in vitro* growth or germination are being considered; it strongly affects *Lecanicillium* spp. (Saito, 1988; Saito and Yabuta, 1996) but not *Beauveria bassiana* (Saito, 1984). It is necessary to note that some insecticides e.g., tolfepryad and methidathion, were also highly inhibitory to the fungus. Further studies will be needed to determine what effect these pesticides may have on fungal efficacy in the field.

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Captions of Figures

Fig. 1. *Paecilomyces cinnamomeus* as an entomopathogenic fungus on the camellia spiny whitefly, *A. camelliae*. (A) Naturally infected larvae in a tea growing field, (B) cinnamon-colored stroma on the host and conidia on hypothalli expanding on a tea leaf, (C) conidiogenous cells and conidia, and (D) conidia.

Fig. 2. Neighbor-joining tree based on analysis of the ITS rDNA sequences showing the link between isolates from *A. camelliae* and *Paecilomyces* sp., *C. luteorostrata* and *P. cinnamomeus*. Numbers above or below the nodes indicate bootstrap values (> 50%) generated after 1000 replications. Branch termini are labelled according to species and GenBank accession numbers. Species names were expressed as the anamorph or teleomorph according to the morph of the analyzed samples.

Fig. 3. Neighbor-joining tree based on analysis of the partial β -tubulin gene sequences showing the link between isolates from *A. camelliae* and *C. luteorostrata* and *P. cinnamomeus*. Numbers above or below the nodes indicate bootstrap values (> 50%) generated after 1000 replications. Branch termini are labelled according to species and GenBank accession numbers. Species names were expressed as the anamorph or teleomorph according to the morph of the analyzed samples.

Fig. 4. Neighbor-joining tree based on analysis of the partial translation elongation factor 1 α gene sequences showing the link between isolates from *A. camelliae* and *C. luteorostrata*. Numbers above or below the nodes indicate bootstrap values (> 50%) generated after 1000 replications. Branch termini are labelled according to species and GenBank accession numbers. Species names were expressed as the anamorph or teleomorph according to the morph of the analyzed samples.

Fig. 5. Proportion of nymphs infected 1, 2 and 3 weeks after inoculation with isolates Kyoto-32, Shiga-22, Mie-1 and the mycoinsecticides (Preferd[®] and Mycotal[®]). The isolates were inoculated at three different concentrations of conidia, the low concentration (1×10^6 conidia/ml), the medium concentration (1×10^7 conidia/ml) and the high concentration (1×10^8 conidia/ml). The mycoinsecticides Preferd[®] and Mycotal[®] were inoculated at their recommended field application rates that were measured as conidial concentrations of 5×10^6 and 9×10^6 conidia/ml, respectively. Error bars represent one standard error of 3 replicates. Columns followed by different letters in the same week of each inoculum are significantly different at the 5% level using Tukey HSD test.

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477 Fig. 6. Effect of temperature on growth of the three isolates, Kyoto-32, Shiga-22, Mie-1. Colony
478 diameter was determined 3 weeks after inoculation. Error bars represent standard error of 5
479 replicates.

480

481 Fig. 7. Effect of pesticides on growth of the three isolates, Kyoto-32, Shiga-22 and Mie-1. Colony
482 diameter was measured 3 weeks after inoculation. Error bars represent one standard error of 3
483 replicates. Asterisks indicate significant differences between each pesticide and the control at the 5%
484 level using the Dunnett test.

485

Table 1. List of isolates from *Aleurocanthus camelliae* in this study

Isolates	Sites and dates of collection	Cultural observation		Size (μm , mean $\pm$ SE)		GenBank accession no.		
		Color of colony	Diffusible pigmentation	Conidiogenous cells	Conidia	ITS rDNA	β -tubulin gene	TEF 1 α gene
Kyoto-26	Soraku, Kyoto, 2 Nov. 2009	Cinnamon	Yes	$2.5 \pm 0.12 \times 9.8 \pm 0.44$	$2.5 \pm 0.04 \times 6.7 \pm 0.18$	AB649298	AB663108	AB663120
Kyoto-31	Soraku, Kyoto, 14 Oct. 2009	Cinnamon	Yes	$2.6 \pm 0.12 \times 11.1 \pm 0.75$	$2.7 \pm 0.04 \times 6.2 \pm 0.16$	AB649299	AB663109	AB663121
Kyoto-32	Soraku, Kyoto, 14 Oct. 2009	Cinnamon	Yes	$2.5 \pm 0.06 \times 11.4 \pm 0.86$	$2.3 \pm 0.04 \times 5.9 \pm 0.16$	AB649300	AB663110	AB663122
Shiga-1	Koga, Shiga, 17 Sep. 2009	Cinnamon	Yes	$2.7 \pm 0.14 \times 12.5 \pm 0.77$	$2.6 \pm 0.04 \times 6.1 \pm 0.12$	AB649301	AB663111	AB663123
Shiga-2	Gamo, Shiga, 17 Sep. 2009	Cinnamon	Yes	$2.8 \pm 0.09 \times 10.5 \pm 0.77$	$2.5 \pm 0.05 \times 6.4 \pm 0.16$	AB649302	AB663112	AB663124
Shiga-4	Koga, Shiga, 19 Nov. 2009	Cinnamon	Yes	$2.9 \pm 0.13 \times 10.3 \pm 0.70$	$2.3 \pm 0.04 \times 5.6 \pm 0.11$	AB649303	AB663113	AB663125
Shiga-11	Koga, Shiga, 19 Nov. 2009	Cinnamon	Yes	$2.6 \pm 0.11 \times 12.1 \pm 0.80$	$2.5 \pm 0.04 \times 6.5 \pm 0.17$	AB649304	AB663114	AB663126
Shiga-12	Koga, Shiga, 19 Nov. 2009	Cinnamon	Yes	$2.8 \pm 0.08 \times 10.8 \pm 0.65$	$2.3 \pm 0.04 \times 5.2 \pm 0.08$	AB649305	AB663115	AB663127
Shiga-22	Koga, Shiga, 19 Nov. 2009	Cinnamon	Yes	$2.7 \pm 0.16 \times 12.1 \pm 0.58$	$2.5 \pm 0.03 \times 6.6 \pm 0.15$	AB649306	AB663116	AB663128
Mie-1	Taki, Mie, 20 Oct. 2009	White	No	$2.8 \pm 0.05 \times 12.2 \pm 0.94$	$2.6 \pm 0.03 \times 7.6 \pm 0.21$	AB649307	AB663117	AB663129
Mie-2	Watarai, Mie, 20 Oct. 2009	Cinnamon	Yes	$2.6 \pm 0.06 \times 12.3 \pm 0.57$	$2.4 \pm 0.04 \times 6.6 \pm 0.16$	AB649308	AB663118	AB663130
Mie-41	Kameyama, Mie, 11 Mar. 2010	Cinnamon	Yes	$2.4 \pm 0.12 \times 10.2 \pm 0.64$	$2.5 \pm 0.06 \times 6.2 \pm 0.15$	AB649309	AB663119	AB663131

Table 2. List of specimens used in phylogenetic analyses

Specimens	Host taxon	GenBank accession no.		
		ITS rDNA	β-tubulin gene	TEF 1α gene
<i>Aschersonia badia</i>	Scale insect (Hemiptera)			DQ522317
<i>A. placenta</i>	Scale insect (Hemiptera)			EF469056
<i>Balansia henningsiana</i>	<i>Panicum</i> sp. (Poaceae)			AY489610
<i>B. pilulaeformis</i>	Poaceae			DQ522319
<i>Chamaeleomyces viridis</i>	<i>Chameleo lateralis</i> (Chamaeleonidae)	AY624197	AY624235	
	<i>Chameleo lateralis</i> (Chamaeleonidae)		AY624236	
<i>Claviceps fusiformis</i>	Poaceae			DQ522320
<i>C. paspali</i>	Poaceae			DQ522321
<i>C. purpurea</i>	<i>Dactylis glomerata</i> (Poaceae)			AF543778
<i>Conioideocrella luteorostrata</i>	Hemipteran insect	AY624206	AY624237	
	Scale insect (Hemiptera)			EF468800
	Scale insect (Hemiptera)			EF468801
<i>C. tenuis</i>	Scale insect (Hemiptera)			EU369028
	Scale insect (Hemiptera)			EU369029
	Scale insect (Hemiptera)			EU369030
<i>Epichloe typhina</i>	<i>Festuca rubra</i> (Poaceae)			AF543777
<i>Hypocrella schizostachyi</i>	Scale insect (Hemiptera)			DQ522346
<i>Isaria farinosa</i>	Soil	AY624179	AY624217	
	Lepidopteran pupa			DQ522348
<i>I. tenuipes</i>	Lepidopteran larva	AY624195	AY624233	
	Lepidopteran pupa			DQ522349
<i>Metacordyceps chlamydosporia</i>	Egg of slug (Diplopoda)			DQ522327
<i>Metarhizium album</i>	<i>Cofana spectra</i> (Hemiptera)			DQ522352
<i>M. anisopliae</i>	<i>Oryctes rhinoceros</i> (Coleoptera)			AF543774
<i>M. cylindrospora</i>	Hemipteran adult	AY624204	AY624247	
			AF368270	
<i>M. flavoviride</i>	Hemipteran adult	AY624203	AY624248	
	<i>Nilaparvata lugens</i> (Hemiptera)		AY624249	DQ522353
<i>Metarhizium</i> sp.	Coleopteran larva		AY624246	
<i>Myriogenospora atramentosa</i>	<i>Andropogon virginicus</i> (Poaceae)			AY489628
<i>Nomuraea rileyi</i>	<i>Trichoplusia ni</i> (Lepidoptera)	AY624205	AY624250	
<i>Orbiocrella petchii</i>	Scale insect (Hemiptera)			EU369022
	Scale insect (Hemiptera)			EU369023
	Scale insect (Hemiptera)			EU369021
<i>Paecilomyces carneus</i>	Soil	AY624170	AY624210	
	Dune sand	AY624171	AY624209	
<i>P. cinnamomeus</i>	Coccidae insect		AY624214	
	Living leaf of <i>Syzygium jambos</i> (Myrtaceae)	AY624174	AY624213	
<i>P. marquandii</i>	Soil	AY624193	AY624229	
		AB099511		
<i>P. penicillatus</i>	Rotting mushroom	AY624194	AY624232	
<i>Paecilomyces</i> sp.	<i>Aleurocanthus spiniferus</i> (Hemiptera)	AF368806		
<i>Pochonia gonioides</i>	Nematode			DQ522354
<i>Purpureocillium lilacinum</i>	<i>Aethus</i> sp. (Hemiptera)	AY624190		
	Egg mass of Meloidogyne		AY624228	
	Egg mass of Meloidogyne	AY624188		
	Soil	AY624189	AY624227	
	Cydnid bug (Hemiptera)		AY624226	
<i>Regiocrella camerunensis</i>	Scale insect (Hemiptera)			DQ118743
<i>Rotiferophthora angustispora</i>	Rotifer (Rotifera)			AF543776
<i>Verticillium epiphytum</i>	<i>Hemileia vastatrix</i> (Uredinales)			DQ522361

1×10^6 conidia/ml

1×10^7 conidia/ml

1×10^8 conidia/ml

Field application rates