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FUNGAL DISEASES

Morphological and molecular characterization of *Oidium* subgenus *Reticuloidium* (powdery mildew) newly occurred on cucumber in Japan

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Abstract In 2002, a powdery mildew with catenate conidia lacking fibrosin bodies was found on cucumber in a greenhouse in Kanagawa Prefecture, Japan. Morphological observation revealed that the fungus belongs to *Oidium* subgenus *Reticuloidium*, anamorph of the genus *Golovinomyces*. Molecular phylogenetic analyses of the nucleotide sequences of the rDNA ITS regions and D1/D2 domains of the 28S rDNA indicated that the fungus belongs to the clade of *G. orontii* with other *Golovinomyces* fungi from a wide range of host plants, suggesting that the fungus was newly transported from abroad. Because there has been no prior report of cucumber powdery mildew caused by *Reticuloidium*, further research on the physiology, epidemiology, control and resistant cucumber varieties is required.

Keywords *Cucumis sativus* · Erysiphaceae · *Golovinomyces orontii* · Molecular phylogeny · Morphological observation · rDNA sequence

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Introduction

The Phytopathological Society of Japan (2000) recorded four powdery mildew fungi on *Cucumis sativus* in Japan: *Erysiphe polygoni*, *Oidiopsis sicula* (anamorph of *Leveillula taurica*; Saito and Kurata 1975), *Oidium* sp. of polygoni-type (*Oidium* subgenus *Pseudoidium*; Sato et al. 1996), and *Sphaerotheca cucurbitae* (=*Podosphaera xanthii*; anamorph: *Oidium* subgenus *Fibroidium*). Because the anamorph of *E. polygoni* belongs to *Oidium* subgenus *Pseudoidium*, the powdery mildews of *C. sativus* may be reduced to three. Because the occurrence of *Oidiopsis sicula* and *Oidium* subgenus *Pseudoidium* has been reported only once in Japan, the major causal agent of the cucumber powdery mildew seems to be *S. cucurbitae*. Based on morphological observations of the conidial stage and on molecular phylogenetic analyses, the genus *Sphaerotheca* was merged into the genus *Podosphaera* (Braun and Takamatsu 2000), and the name of the cucumber mildew was revised to *Podosphaera xanthii* (Braun et al. 2001). We therefore named the *Podosphaera* species on cucumber in the present study as *Podosphaera xanthii* and its anamorph as *Fibroidium*.

In 2002, three powdery mildews with conidial stages of different morphologies were found on cucumber plants in a greenhouse in Hiratsuka-shi, Kanagawa Prefecture, Japan. Two of the fungi were identified as *Oidium* subgenus *Fibroidium* and *Oidium* subgenus *Pseudoidium*. The third mildew with catenate conidia and lacking fibrosin bodies belonged to *Oidium* subgenus *Reticuloidium*, anamorph of the genus *Golovinomyces*. *Golovinomyces orontii* is a common powdery mildew of cucurbits worldwide, including Europe, North America, South America, Africa, and West and Central Asia (Amano 1986). In East Asia, although *E. cucurbitacearum* (=*G. orontii*) has been

reported to occur in China, there is no record of this fungus in Korea and Japan (Braun 1987; Shin 2000; Phytopathological Society of Japan 2000). Therefore, we characterized the fungus through morphological observations and molecular phylogenetic analyses.

Materials and methods

Fungal isolation

Leaf disks with a diameter of 9 mm were cut from cotyledons of *C. sativus* (cv. Tokiwa Hikari-3P) with a cork borer. One hundred leaf disks were put on a wet filter paper in a Petri dish (150 mm in diameter) and exposed for 3 h at two locations in a greenhouse with diseased cucumber plants. Then, the leaf disks were moved to small Petri dishes (60 mm in diameter; a single leaf/dish) and incubated for 6 days at 20°C under 2,000–2,500 lux with a photoperiod of 12/12 h (day/night) in an incubation room. A single spore was picked up with an endodontic file from a colony that developed on the leaf disk and placed on a healthy cucumber cotyledon, then incubated under the same conditions. Powdery mildew of *Lamium amplexicaule* in the same greenhouse, was also isolated by the same procedure. Cucumber seedlings (cultivar Tokiwa Hikari-3P) at the 2–3 leaf stage were sprayed with conidia of the isolates every 2 weeks and kept in a plastic box (20 × 20 × 30 cm) until the pathogenicity test.

Morphological observations

Hyphae, conidiophores and conidia were stripped off the surface of a detached leaf with clear adhesive tape, mounted on a microscope glass slide with the fungal mycelium uppermost and examined in water. To observe conidial germ tubes, we inoculated the inner cell layer of onion scales using the method of Hirata (1942, 1955).

Pathogenicity test

An isolate of *Fibroidium* B-7SF and two isolates of *Reticuloidium* No. 2–7 (from cucumber) and B-7H2 (from *Lamium amplexicaule*) were used to test pathogenicity. Potted cucumber plants (cvs. Sharp 1, Haru-no Megumi and Tokiwa Hikari-3P) were grown in Kureha horticultural compost (Kureha Chemical, Tokyo, Japan) at 25°C under 3,000–5,000 lux with a photoperiod of 12/12 h (day/night) in a phytotron for 2–3 weeks. These plants were then sprayed with a conidial suspension [ca. 10^5 conidia/ml, amended with 1/5,000 (v/v) Tween 20] of an isolate. The inoculated plants were put into a plastic box and incubated for 21 days at 20°C under 2,000–2,500 lux at the photoperiod of 12/12 h (day/night). The percentage of infected area of each of the powdery-mildewed cucumber leaves was determined by the following scale: 0 = no colony, 0.5 = percentage of area infection ≤10%, 1 = >10–20%, 2 = >20–40%, 3 = >40–60%, 4 = >60–80%, 5 = >80%. Disease severity was calculated as $[(5A + 4B + 3C + 2D + E + 0.5F)/5G] \times 100$, where A, B, C, D, E and F are the number of leaves corresponding to the scale at 5, 4, 3, 2, 1 and 0.5, respectively, and G is the total number of leaves assessed.

Molecular phylogenetic analyses

Whole-cell DNA was extracted from mycelia by the chelex method (Walsh et al. 1991) as described in Hirata and Takamatsu (1996). The rDNA ITS region and the 5′ end of the 28S rDNA, including the D1 and D2 regions, were separately amplified two times by a polymerase chain reaction using nested primer combinations, and then sequenced directly as described in Matsuda and Takamatsu (2003).

The sequences were initially aligned using the Clustal_X program (Thompson et al. 1997) and further analyzed to improve the alignment with a word processing program with colour-coded nucleotides and deposited in TreeBASE (http://www.treebase.org/) with accession number S2265. Phylogenetic trees were obtained from the data by the maximum-parsimony method using the heuristic search option in the program PAUP* 4.0b8 (Swofford 2001). This search was repeated 100 times with different random starting points, using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. Transversions and transitions were treated as equal weight. All sites were treated as unordered, with gaps treated as missing data. The branch-swapping algorithm was the tree bisection and reconstruction method, the MulTrees option was in effect, and zero-length branches were collapsed. The strength of the internal branches from the resulting trees was tested by bootstrap analysis (Felsenstein 1985) using 1,000 replications.

Results

Morphological observation

To investigate the powdery mildew species occurring in a greenhouse of cucumber of Hiratsuka-shi, Kanagawa Prefecture, we examined all 106 powdery mildew colonies that appeared on 52 naturally infected leaves of 80 cucumber plants (cv. Sharp-1) in the greenhouse in June 2002. As a result, 83, 22 and 1 colonies were identified as *Oidium* subgenera *Reticuloidium*, *Fibroidium* and *Pseudoidium*,

respectively, based on Braun et al. (2002). The morphology of *Reticuloidium* was as follows: white mycelium on leaves, amphigenous, powdery-like. Leaf surface with colony slightly swelled out (Fig. 1a). Conidiophores erect, foot-cells slightly curved at the base, conidia produced in chains following 2–4 mother cells, semicylindrical or doliform, germ tubes arising from the middle portion of the conidia, tips unlobed, slightly enlarged with nipple-shaped appressoria (cichoracearum-type) (Table 1; Fig. 1c–e). The morphology of single-spore isolate No. 2–7 was consistent with these characteristics.

Powdery mildews also occurred on *Lamium amplexicaule* (Fig. 1b) and *Oxalis carniculata*, common weeds both inside and outside of the greenhouse where cucumber powdery mildew was found. Powdery mildew on *Oxalis carniculata* belonged to subgenus *Pseudoidium* based on the characteristics of having noncatenate conidia and polygoni-type conidial germ tubes and is regarded as an anamorph of *Erysiphe russellii* (Nomura 1997). The fungus on *Lamium amplexicaule* belonged to subgenus *Reticuloidium*, from which a single-spore isolate, B-7H2, was established (Table 1).

Pathogenicity test

Reticuloidium as well as *Fibroidium* are pathogenic on the three cucumber varieties used in this study. However, the disease severities with *Reticuloidium* isolates No. 2–7 (disease severity = 16–45%) and B-7H2 (22–42%) were lower than that of *Fibroidium* isolate B-7SF (64–83%) (Table 2). The latent period of *Reticuloidium* was about 7 days longer than that of *Fibroidium*. In this study, we estimated disease severity 21 days after inoculation. The different disease severities between *Fibroidium* and *Reticuloidium* may be derived from the different latent period of these fungi. *Reticuloidium* isolate No. 2–7 from cucumber was pathogenic on *Lamium amplexicaule*.

ITS phylogeny

The rDNA ITS region and the D1/D2 domains of the 28S rDNA were sequenced for each isolate from *C. sativus* (cv. No. 1–16) and *Lamium amplexicaule* (cv. B-7H2). The sequences were deposited in the DDBJ DNA database with accession numbers AB427187 and AB427188, respectively. These two sequences were identical in both ITS and 28S rDNA regions. The two ITS sequences obtained in this study were aligned with 70 ITS sequences retrieved from the DDBJ database. The ITS data set consists of 72 taxa, of which 70 are members of the genus *Golovinomyces* (including *Oidium* subgenus *Reticuloidium*). Of the 70 *Golovinomyces* sequences, five sequences (shown by solid circle in Fig. 2) are from cucurbit powdery mildew. The ITS sequences of the isolates from *C. sativus* and *Lamium*

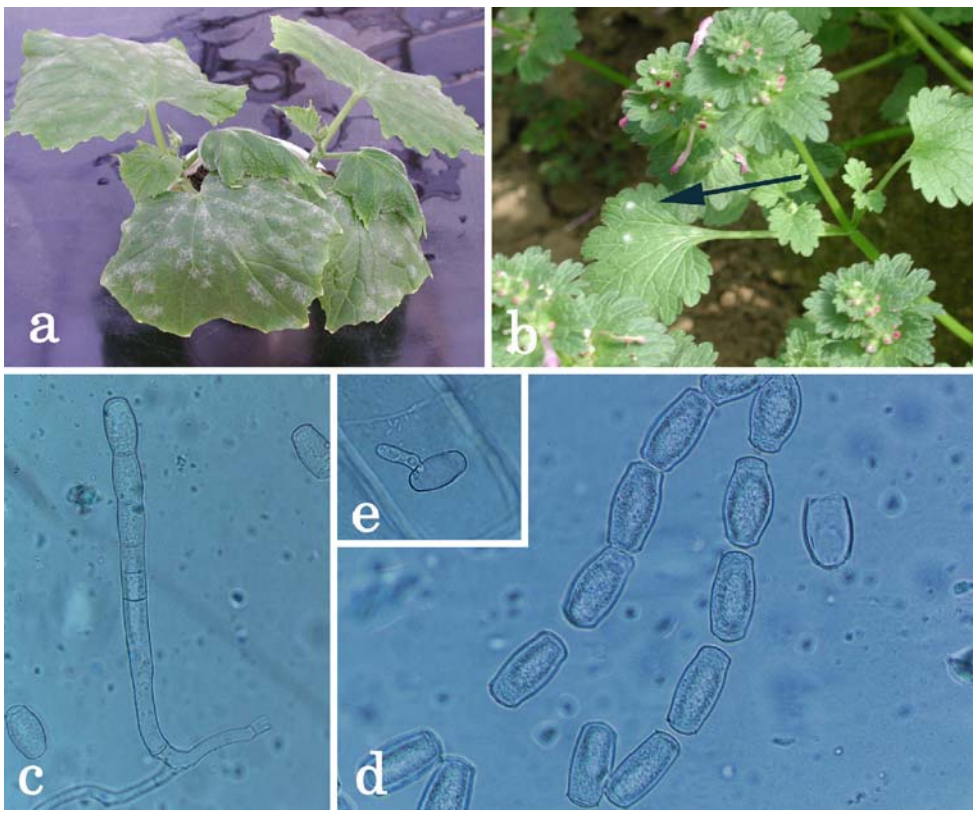

Fig. 1 Symptoms of powdery mildew on cucumber and *Lamium amplexicaule* and causal fungus *Oidium* subgenus *Reticuloidium*. Symptoms of cucumber (**a**) and *L. amplexicaule* (**b**). Conidiophore with catenate conidia (**c**), conidia (**d**), and conidial germ tube (**e**) of *Oidium* subgenus *Reticuloidium* (*bar* 20 μm)

Table 1 Morphology of the *Reticuloidium* species found in a greenhouse on *Cucumis sativus* and *Lamium amplexicaule*

	Reticuloidium			*Erysiphe orontii*[a] (syn. *Golovinomyces orontii*)	*Sphaerotheca fusca*[a] (syn. *Podosphaera xanthii*)
	Combined 106 colonies (52 cucumber plants in greenhouse)	Isolate No. 2–7 (*Cucumis sativus*)	Isolate B-7H2 (*Lamium amplexicaule*)		
Conidia					
Size (μm)	26–40 × 12–18	28–4 × 12–18	32–42 × 14–18	25–40 × 15–23	24–45(–50) × 14–22(–26)
Shape	Cylindric-doliiform	Cylindric-doliiform	Cylindric-doliiform	Ellipsoid-ovoid to doliiform-subcylindric	Ellipsoid-ovoid to doliiform
Color	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Conidiogenesis	Catenate	Catenate	Catenate	Catenate	Catenate
Fibrosin body	Absent	Absent	Absent	Absent	Present
Conidiophore					
No. of cells	2–4	3–4	2–4	2–4	2–4
Length (μm)	76–200	106–158	124–288	40–100	(25–)40–80
Width (μm)	8–14	8–12	8–12	10–13	9–14.5
Appressoria	Nipple-shaped	Nipple-shaped	Nipple-shaped	Nipple-shaped	Indistinct

[a] Braun (1987)

Table 2 Disease severity on three cucumber varieties after inoculation with conidia of three powdery mildew isolates

Fungal isolate	Disease severity on cucumber variety[a]		
	Sharp 1	Haru-no Megumi	Tokiwa Hikari-3P
Fibroidium B-7SF	74.2 b	64.6 b	82.5 b
Reticuloidium No. 2-7	44.2 a	16.7 a	45.0 a
Reticuloidium B-7H2	41.7 a	22.4 a	NT

NT not tested

[a] Cucumber plants at the 2–3 leaf stage were sprayed with ca. 10^5 conidia/ml distilled water, amended with 1/5,000 (v/v) Tween 20. Disease severity (DS) was calculated as $[(5A + 4B + 3C + 2D + E + 0.5F)/5G] \times 100$, where A, B, C, D, E and F are the number of leaves with disease severity at a scale of 5, 4, 3, 2,1 and 0.5, respectively, and G is the total number of leaves assessed

Disease severity with the same letter(s) are not significantly different ($P < 0.05$) by the Duncan's multiplex test

amplexicaule were identical to the sequences of *G. orontii* from *Arabidopsis thaliana* (AF031282) and *Veronica arvensis* (AB077652) and differed by one base from the sequences of *G. orontii* from *Cucurbita pepo* (AF229017 and AB077670), *Cucurbita* sp. (AB077696), *Nicotiana tabacum* (AB022413); *G. cichoracearum* from *Sonchus* sp. (AB077669), *Cichorium intybus* (AB077695, AB077666 and AF011294), *Lactuca serriola* (AB077688), and *Mycelis muralis* (AB077661); and *Oidium* sp. on *Dahlia pinnata* (AB077677).

The data set consisted of 534 characters, from which 39 sites were removed to circumvent ambiguous alignments. Of the 495 remaining characters, 138 sites were variable, and 116 sites were phylogenetically informative for parsimony analyses. Two sequences from *Arthrocladiella mougeotii* were used as the outgroup based on Mori et al. (2000). The parsimony analysis using PAUP* generated 640 equally parsimonious trees of 303 steps (consistency index [CI] = 0.6469, retention index [RI] = 0.8981, rescaled consistency index [RC] = 0.5809). One of the 640 trees with the highest log-likelihood value is shown in Fig. 2, which shows nine clades differentiated within the *Golovinomyces* taxa investigated in this study. Each clade was strongly supported by bootstrap values of 94% or more. Of the nine clades, four clades, I, V, VI and VII, comprise isolates from each of the host tribes of the Asteraceae: clade I from tribe Cardueae, clade V from Anthemideae, clade VI from Astereae, and clade VII from Heliantheae. Clade II comprises isolates from Lamiaceae and Ranunculaceae. Clade III comprises a single isolate from *Lapsana* (Asteraceae), clade IV comprises isolates from *Lycopersicon* and *Solanum* (Solanaceae), and clade VIII comprises isolates from *Phlox* (Polemoniaceae) and *Physalis* (Solanaceae). Clade IX is large and comprises isolates from the tribe Lactuceae of the Asteraceae, and many other plant families such as Plantaginaceae, Solanaceae, Brassicaceae and Cucurbitaceae. Sequences from the isolates No. 1–16 (from *C. sativus*) and B-7H2 (from *Lamium amplexicaule*) belonged to the clade IX. All the nine clades were commonly appeared in all 640 parsimonious trees, although the branching order of the clades differed among the trees.

28S phylogeny

The two 28S rDNA sequences obtained in this study were aligned with 49 28S rDNA sequences retrieved from the

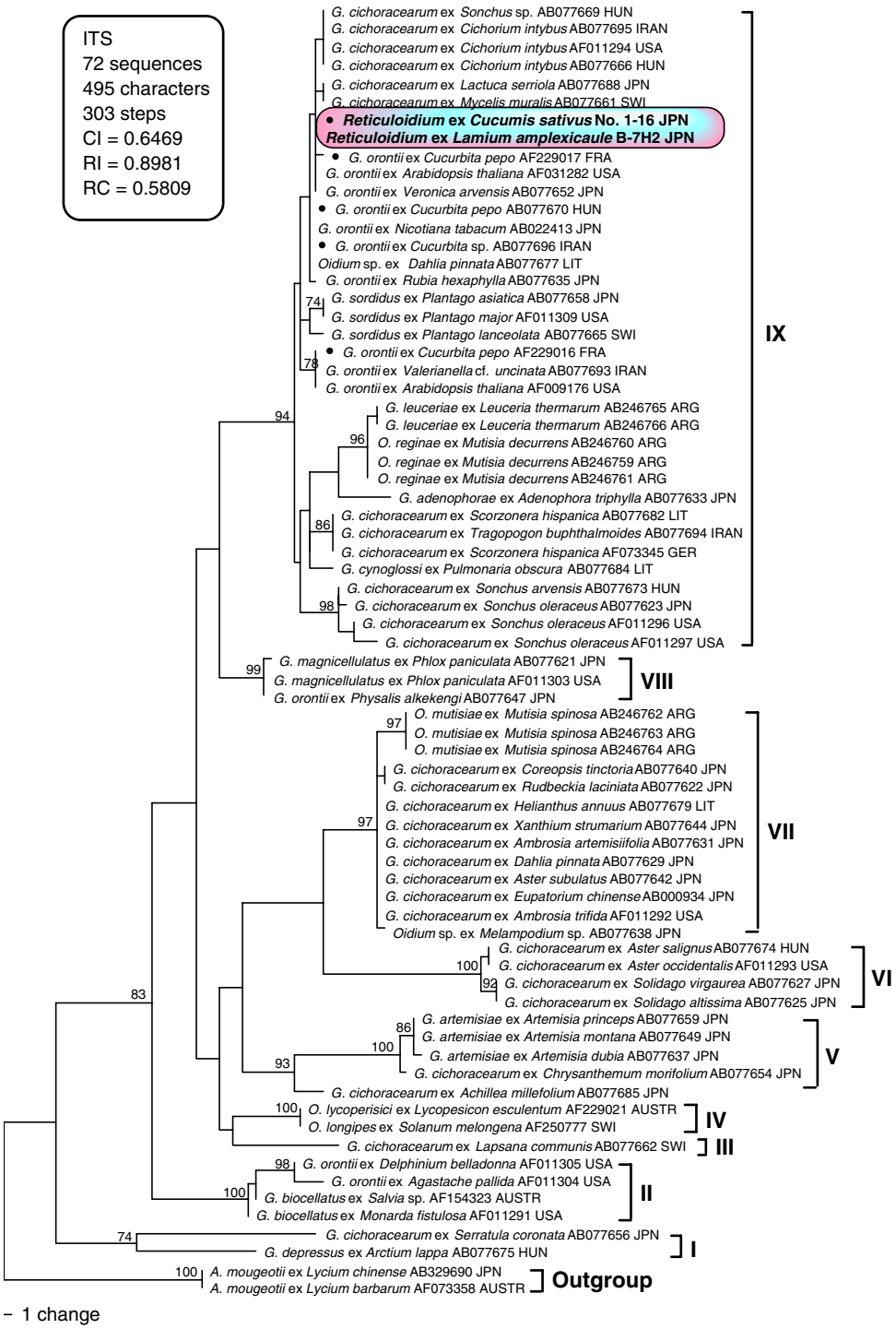

Fig. 2 Phylogenetic analysis of the nucleotide sequences of the ITS region including the 5.8S rDNA for 70 sequences from the genus *Golovinomycetes* (including *Oidium* subgenus *Reticuoidium*) and two outgroup sequences. The tree is a phylogram of the maximum likelihood tree among the 640 most parsimonious trees with 303 steps, which was obtained by a heuristic search employing the random stepwise addition option in the program PAUP*. Gaps were treated as missing data. *Horizontal* branch lengths are proportional to the number of nucleotide substitutions that were inferred to have occurred along a particular branch of the tree. Percentage bootstrap support (1,000 replications; $\geq$70%) are shown on the branches. *Solid circle* before a taxon label indicates a sequence from a cucurbit powdery mildew

DDBJ database. The 28S rDNA data set consists of 51 sequences, of which 50 are from members of the genus *Golovinomyces*. These 50 sequences include 11 *Golovinomyces* species from 44 different plant species that represent 13 plant families, of which three sequences shown by solid circle in Fig. 3 are from cucurbit powdery mildews. The two 28S rDNA sequences determined in this study differed one base from *G. cichoracearum* on *Lactuca serriola* (AB077687) and *Picris hietacioides* (AB077686), and two bases from *G. cichoracearum* on *Sonchus* sp. (AB077668), *Cichorium intybus* (AB077667), *Mycelis muralis* (AB077660) and *Lactuca serriola* (AB077651), and *G. orontii* on *Veronica arvensis* (AB077651). There was no sequence identical to these two 28S rDNA sequences.

The data set consists of 718 characters, all of which were used for tree construction because all their sites were

aligned unambiguously. Of the 718 characters, 65 sites were variable, and 45 were phylogenetically informative for parsimony analyses. A sequence from *Arthrocladiella mougeotii* was used as an outgroup based on Mori et al. (2000). The parsimony analysis using PAUP* generated 48,109 equally parsimonious trees of 121 steps (CI = 0.6694, RI = 0.8964, RC = 0.6001). Because the tree topologies were almost identical among the 48,109 trees except for branching order of terminal branches, one of the trees is shown in Fig. 3. All trees support the respective clades recognized in the ITS analysis, although clades III and IV were not included in the 28S tree due to the lack of DNA sequence data. The two sequences from isolates No. 1–16 (from *C. sativus*) and B-7H2 (from

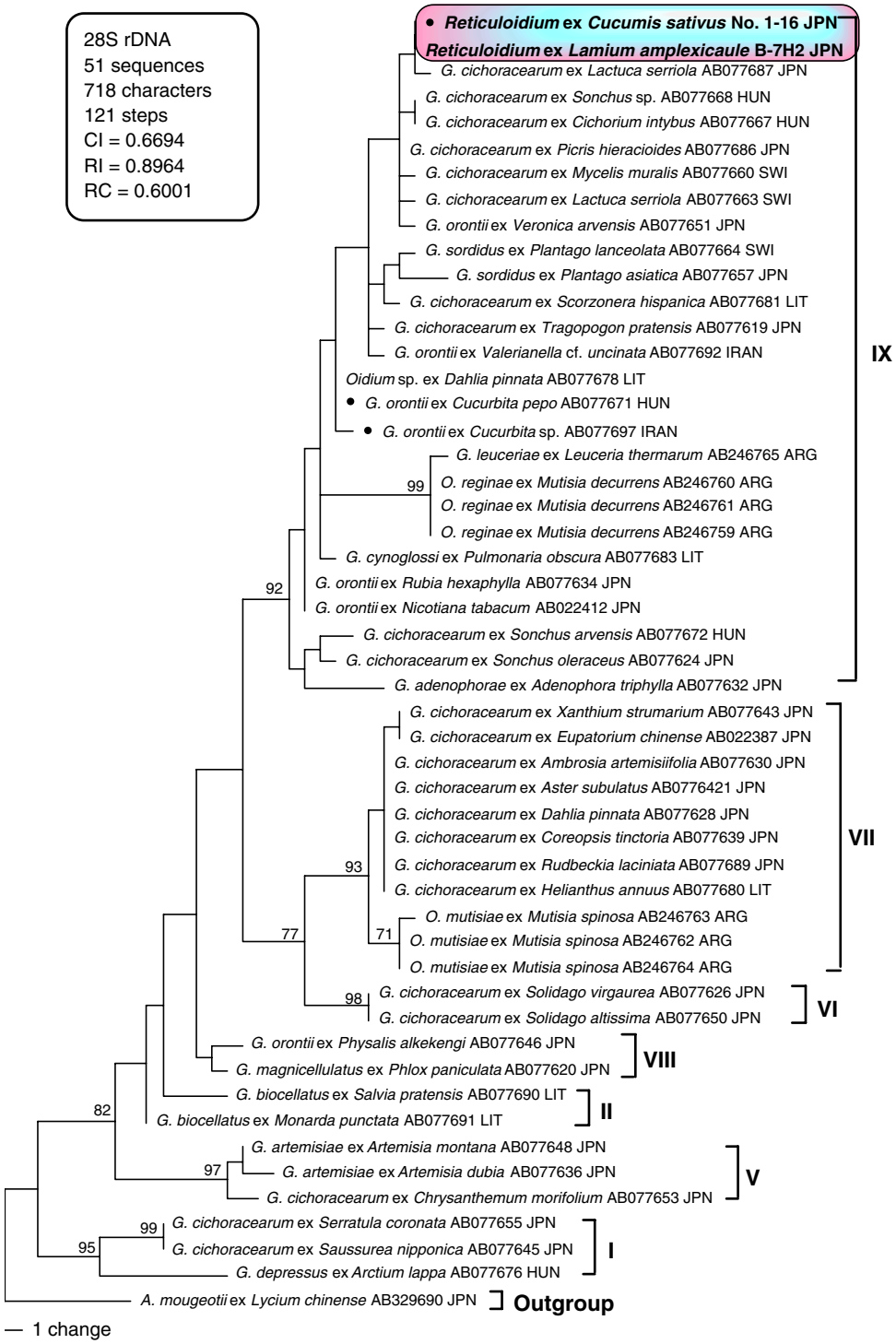

Fig. 3 Phylogenetic analysis of the divergent domains D1 and D2 sequences of the 28S rDNA for 50 sequences from the genus *Golovinomycetes* (including *Oidium* subgenus *Reticuloidium*) and one outgroup sequence. The tree is one of 48,109 most parsimonious trees with 121 steps and was obtained by a heuristic search using PAUP*. Gaps were treated as missing data. *Horizontal* branch lengths are proportional to the number of nucleotide substitutions that were inferred to have occurred along a particular branch of the tree. Percentage bootstrap support (1,000 replications; ≥70%) is shown on the branches. *Solid circle* before a taxon label indicates a sequence from a cucurbit powdery mildew

Lamium amplexicaule) belonged to the large terminal clade IX.

Discussion

Four powdery mildew species, i.e., *Erysiphe cichoracearum* (=*E. cucurbitacearum*, *E. polyphaga*; present name: *G. orontii*; anamorph: *Oidium* subgenus *Reticuloidium*), *E. communis* (anamorph: *Oidium* subgenus *Pseudoidium*), *Sphaerotheca fuliginea* (present name: *Podosphaera xanthii*; anamorph: *Oidium* subgenus *Fibroidium*) and *Leveillula taurica* (anamorph: *Oidiopsis siculae*) are listed as causal agents of cucumber powdery mildew in the world (Amano 1986). Three powdery mildews, i.e., *Oidium* subgenus *Pseudoidium, Podosphaera xanthii* (=*S. fuliginea*, *S. fusca*, *S. cucurbitae*) and *Leveillula taurica*, have been reported to occur in Japan (Phytopathological Society of Japan 2000; Saito and Kurata 1975; Sato et al. 1996). Of these, *Podosphaera xanthii* is the main causal agent and the other two fungi are rare. The fungus from Hiratsuka-shi morphologically resembled *Podosphaera xanthii* in producing catenate conidia, but differed from *Oidium* subgenus *Pseudoidium* and *Leveillula taurica*, which have noncatenate conidia. However, the present fungus was also distinguished from *Podosphaera xanthii* in not having distinct fibrosin bodies in its conidia. In addition, the conidial germ tube of *Podosphaera xanthii* is the fuligineatype, whereas that of the present fungus is the cichoracearum-type. These characteristics reveal that the present fungus differs from *Podosphaera xanthii* and belongs to *Oidium* subgenus *Reticuloidium*. This is the first record of cucumber powdery mildew caused by *Reticuloidium* in Japan. Because the teleomorph of *Reticuloidium* belongs to the genus *Golovinomyces* (Braun et al. 2002), the present fungus may be an anamorphic stage of *G. orontii*, a common powdery mildew of cucumber in the world except East Asia (Sitterly 1978; Braun 1987). Results of the molecular analysis showing that the fungus belongs to the clade of *G. orontii* support the assumption.

Reticuloidium mildew on cucumber was classified as *E. cichoracearum* in the monograph of Salmon (1900). *E. cichoracearum* sensu Salmon was reported from a wide range of plant families including Asteraceae, but considered as a complex of numerous races with narrow host ranges. Hammarlund (1945) inoculated 99 plant species from a wide range of families with an '*E. cichoracearum*' isolate from cucumber and reported that the fungus infected 62 plant species of 11 families such as Asteraceae, Crassulaceae, Cucurbitaceae, and Solanaceae. Thus, he concluded that this fungus has a wide host range, although it resembled *E. cichoracearum* in morphology, and called the fungus '*E. polyphaga*'. Blumer (1952, 1967) performed independent inoculation tests and confirmed the presence of such a fungus with a wide host range. Braun (1980) found that the foot cells of the conidiophore of '*E. polyphaga*' often curved at the base and proposed the name '*E. orontii*' (the oldest available name for this fungus). He confined the name '*E. cichoracearum*' to fungi on Asteraceae. The genus *Erysiphe* was then split into three genera based on the morphology of the anamorph and on molecular analyses, and *E. orontii* was revised as *G. orontii* (Braun 1999; Heluta 1988).

We used five cucurbitaceous mildew sequences in the present phylogenetic analysis. There is no sequence identical to the ITS sequence of the present Japanese isolates. AF229017 sequenced in France differs by only one base from the Japanese isolate at the 125th site from 5′-end of the ITS1 region. Recently, a new occurrence of *Reticuloidium* has been reported on several cultivated plants such as *Helianthus tuberosus*, *Viola tricolor* and *Zinnia elegans* (Hoshi et al. 2007; Tanda and Suga 2002). It is likely that these *Reticuloidium* mildews were newly imported from abroad.

We found *Reticuloidium* on *Lamium amplexicaule* in the same greenhouse in which cucumber *Reticuloidium* was found. Both the ITS and the 28S rDNA sequences of this isolate are identical to those of the cucumber mildew, and the isolate is pathogenic on cucumber. This result suggests that *Lamium amplexicaule* provided the primary inoculum for the cucumber mildew in this greenhouse. *Neoerysiphe galeopsidis* has been the only powdery mildew species to infect *Lamium* species (Amano 1986; Braun 1987). Blumer (1952) reported that *Reticuloidium* infects *Lamium galeobdolon*, and Garibaldi et al. (2007) reported the occurrence of *G. orontii* on *Lamium galeobdolon*. Uchida et al. (2002) found *Oidium* subgenus *Fibroidium* on *Lamium amplexicaule* near the cucumber greenhouses in Hiratsuka-shi. This *Fibroidium* was pathogenic to both cucumber and *Lamium amplexicaule*, suggesting that common weeds like *Lamium amplexicaule* and *Lamium purpureum* serve as hosts to produce primary inoculum of Fibroidium as well as Reticuloidium to cause cucumber mildew.

In Europe and other regions of the world, *G. orontii* is an important causal agent of cucurbit mildew with *Podosphaera xanthii* (Bardin et al. 1999; Jahn et al. 2002; Mohamed et al. 1995; Sitterly 1978; Sz Nagy 1970, 1972, 1976; Vakalounakis and Klironomou 1995; Vakalounakis et al. 1994). Based on Sz Nagy (1976), '*E. cichoracearum*' (=*Reticuloidium*) can germinate in wider temperature ranges than '*Sphaerotheca fuliginea*' (=*Podosphaera xanthii*; *Fibroidium*), and conidia of the former species are able to germinate at lower humidity. Hoshi et al. (2007) reported that *Reticuloidium* occurs earlier than *Fibroidium* in Japan. Thus, the two mildew fungi differ in some physiological

and ecological characteristics. Although evidence on cucumber mildew caused by *Fibroidium* has been accumulating for many years in Japan (Abiko 1978, 1982a, 1982b; Endo 1989; Hosoya et al. 1999, 2000; Kuzuya et al. 2003, 2006), there is no report of cucumber mildew caused by *Reticuloidium*. Further research on the physiology, epidemiology, chemical control of this fungus and resistant varieties of cucumber is needed.

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