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FULL PAPER

Taxonomic reevaluation of *Raffaelea quercivora* isolates collected from mass mortality of oak trees in Japan

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Abstract In this study, we reevaluated isolates of *Raffaelea quercivora* associated with Japanese oak wilt in Japan to clarify their taxonomy and to help researchers diagnose the cause of the mass mortality of oak trees in Asian countries more accurately. We examined the morphological and molecular characteristics of 15 isolates of *R. quercivora*, including an ex-holotype strain, obtained from a wide range of areas of Japan. Light microscopy showed that all the isolates had wider ranges of conidial and conidiophore sizes than previously recognized, but that their sizes and shapes did not differ among the isolates. A phylogenetic tree generated from sequences for a partial large subunit of ribosomal DNA showed that the new isolates and the ex-holotype formed a single clade within the *Raffaelea* clade, with a high bootstrap value. Scanning electron microscopy revealed multimodal conidial development in the isolates: sympodial or annellidic-percurrent proliferation or both, with delayed secession. These results suggest that the isolates examined and the ex-holotype strain have a different genetic identity from other known *Raffaelea* species. The diverse conidiogenesis and subtle characteristics in the conidium morphology of *R. quercivora* reflected in the emended description.

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Introduction

Mass mortality of oak trees in Japan was first reported in the southern part of Kyushu Island in 1934 (Kumamoto Regional Forest Office 1941). The decline and death of *Quercus crispula* Blume and *Q. serrata* Thunb. have been observed on the coast of Honshu Island and in the southern part of Kyushu Island since the late 1980s (Ito and Yamada 1998), and the areas of mortality continue to expand (Ito 2008). The causal agent was identified morphologically as a new species, *Raffaelea quercivora* Kubono & Shin. Ito (2002), which is carried by the ambrosia beetle *Platypus quercivorus* Murayama (Kinuura and Kobayashi 2006). Moreover, in an inoculation experiment using *R. quercivora*, the fungus caused significant wilting and death of *Q. crispula* and *Q. serrata* seedlings (Ito et al. 1998; Murata et al. 2005, 2007). Recent molecular analyses of *R. quercivora* isolates indicated that fungal isolates from the trees affected by the mass mortality of oak trees in Japan form a monophyletic group that is phylogenetically similar to *R. montetyi* Morelet (1998) based on three regions of their nuclear ribosomal DNA (Matsuda et al. 2010).

The genus *Raffaelea* was established by von Arx and Hennebert (1965). *Raffaelea* species are generally known to have a close symbiotic relationship with wood-boring ambrosia beetles in subfamilies Scolytinae and Platypodinae of the Curculionidae (Batra 1963; Farrell et al. 2001). Batra (1967) revised the taxonomic concepts of ambrosia fungi based on their morphological characteristics.

The genus *Raffaelea* is characterized by conidiophores that taper gradually toward the apex and which bear a series of cicatricial conidia scars and conidia (von Arx and Hennebert 1965; Batra 1967). The species of this genus were classified based on the size and shape of their conidia (Batra 1967), and these morphological traits are still crucial criteria for identification of the species. On the other hand, conidiogenesis of the genus *Raffaelea*, which is conventionally recognized as "sympodial," has recently been revised. Detailed observations of conidiogenesis using a scanning electron microscope (SEM) showed that the conidia of four *Raffaelea* species (*R. ambrosiae* Arx & Hennebert, *R. montetyi*, *R. arxii* Arx & Hennebert, and *R. albimanens* D.B. Scott & J.W. du Toit) were produced following annellidic-percurrent proliferation of conidiogenous cells (Gebhardt et al. 2004; Gebhardt and Oberwinkler 2005). However, a previous study using a light microscope (LM) concluded that the conidia were produced following sympodial proliferation (Batra 1967). Thus, light microscopy may not provide sufficient resolution to reveal key details that differentiate between the patterns of conidiogenesis of *Raffaelea* species. The conidiogenesis that is observed following the sympodial or annellidic-percurrent proliferation of conidiogenous cells supports the taxonomic placement of an isolate, which means that conidiogenesis of *Raffaelea* is identical to that of other anamorphs within the Ophiostomatales (Gebhardt et al. 2004; Gebhardt and Oberwinkler 2005).

The conidiogenesis of *R. quercivora* has been described as "sympodial" based on observations using an SEM (Kubono and Ito 2002). However, Gebhardt and Oberwinkler (2005) pointed out that additional studies would be necessary to clarify whether the flat lateral scars in *R. quercivora* that were accompanied by sympodial proliferation, as shown in the SEM micrographs in the protologue (Kubono and Ito 2002), are true scars or artifacts caused by shrinking of the conidiogenous cells during drying.

The aim of the present study was to reevaluate the isolates of *R. quercivora* that are associated with Japanese oak wilt in Japan so as to clarify their taxonomy and thereby to help researchers to more accurately diagnose the causes of the mass mortality of oak trees which is occurring in Asian countries. To accomplish this goal, we examined the morphological and molecular characteristics of these isolates of *R. quercivora*, including an ex-holotype strain.

Materials and methods

Fungal collection and isolates

We examined 15 *R. quercivora* isolates, including an ex-holotype strain (MAFF410918) that is maintained at the Laboratory of Forest Pathology and Mycology, Graduate School of Bioresources, Mie University, Mie, Japan. These isolates were obtained from dead or declining oak trees (*Q. crispula*, *Q. phillyraeoides* A. Gray, and *Q. serrata*), from other dead or declining trees (*Castanea crenata* Siebold & Zucc., *Castanopsis cuspidata* (Thunb) Schottky var. *sieboldii* (Makino) Nakai, *Castanopsis* sp.), and from ambrosia beetles (*P. quercivorus*) in Japan (Table 1). To obtain samples of the isolates, we surface-sterilized pieces of discolored sapwood of dead trees with 80% (v/v) ethanol and 1% (v/v) sodium hypochlorite, washed the samples in two changes of sterilized water, and inoculated the material onto potato dextrose agar (PDA) plates. Male and female adults of *P. quercivorus* were extracted from dead oak trees, surface-sterilized, washed in two changes of sterilized water, then inoculated onto PDA. Mycangia of female *P. quercivorus* were excised from their bodies using a sharp scalpel and inoculated directly onto PDA plates. All the PDA plates were incubated at 18°C for about 1 month in the dark. Then, colony morphologies that appeared to correspond to the description of Kubono and Ito (2002) were examined further. The isolates we obtained were re-isolated from a single spore and were maintained on PDA at 25°C until they were used for our morphological and molecular characterizations.

Morphological observations

For the LM observations, the isolates obtained were newly grown on either PDA, malt extract agar (MEA), or oatmeal agar (OMA) plates. We obtained 50 measurements at 1,000× magnification for conidia and at 400× magnification for conidiophores that were grown on PDA for 3–4 days at 25°C. Their morphological traits were described using the ranges of length and width, the length-to-width ratio of the conidia, and the conidial shapes. For the SEM observations, we cut agar discs with an 8-mm-diameter cork borer. All the isolates were then fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. The fixed materials were dehydrated in a graded ethanol series and then transposed into 100% *t*-butyl alcohol. The dehydrated materials were freeze-dried using a *t*-butyl alcohol freeze dryer (model VFD-21S; Vacuum Device, Ibaraki, Japan), and coated with gold using an Ion Sputter (E-1010; Hitachi, Tokyo, Japan). The coated materials were observed and photographed with an SEM (Hitachi S-4000) operating at 15 kV.

DNA analyses

To extract DNA, a small amount of each of the 14 isolates was scraped from the surface of mycelial colonies using

Table 1 Origins and conidiogenesis of 15 isolates of *Raffaelea quercivora*

Isolate number	Origin in Japan (prefecture)	Host tree or vector insect	Year of isolation	Conidiogenesis[a]	DDBJ/EMBL/GenBank accession no.[b]
MAFF410918[c]	Yamagata	*Quercus crispula*	1998	Sympodial, SPFAP, annellidic	–[d]
RA1490	Akita	*Q. crispula*	2008	Sympodial, SPFAP, annellidic	AB626738
RA1258	Niigata	*Q. crispula*	2004	Annellidic	–[e]
RA1026	Fukushima	*Castanea crenata*	2003	Sympodial, SPFAP, annellidic	AB626739
RA0085	Ishikawa	*Q. crispula*	1997	SPFAP, annellidic	AB626740
RA0129	Gifu	*Platypus quercivorus*	1999	Sympodial, SPFAP, annellidic	AB626741
RA0033	Fukui	*P. quercivorus*	1994	Annellidic	–[e]
RA0003	Shiga	*Q. serrata*	1989	Sympodial, SPFAP, Annellidic	AB626742
RA1385	Mie	*Q. phillyraeoides*	2007	Sympodial, SPFAP, annellidic	–[e]
RA1430	Wakayama	*Castanopsis* sp.	2008	SPFAP, annellidic	AB626743
RA1087	Hyogo	*Q. crispula*	2003	Sympodial, SPFAP, annellidic	AB626744
RA1091	Tottori	*Q. crispula*	2003	Sympodial, SPFAP, annellidic	AB626745
RA1100	Shimane	*Q. serrata*	2003	Sympodial, SPFAP, annellidic	AB626746
RA1384	Yamaguchi	*Castanopsis cuspidata* var. *sieboldii*	2007	Sympodial, SPFAP, annellidic	AB626747
RA1183	Kagoshima	*Q. crispula*	2003	Sympodial, SPFAP, annellidic	AB626748

[a] SPFAP indicates sympodial proliferation following annellidic proliferation

[b] All the accession numbers were originated from the 28S large subunit rDNA (D1/D2) regions

[c] The ex-holotype strain of *R. quercivora* (Kubono and Ito 2002)

[d] Not applied for sequences

[e] Not successfully sequenced

sterilized wood sticks and transferred into 1.5-ml centrifuge tubes. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were conducted for the 28S large subunit (LSU) rDNA (D1/D2) regions using the NL1/NL4 primer pair (O'Donnell 1993) and using MightyAmp DNA Polymerase (Takara, Otsu, Japan) according to the manufacturer's recommendations. Cycling conditions were an initial denaturing step at 98°C for 2 min, followed by 35 cycles at 98°C for 10 s, 60°C for 15 s, and 68°C for 1 min. When only one DNA band was detected after gel electrophoresis, PCR products were prepared for sequence analyses (i.e., gels with multiple bands were not analyzed). PCR products were purified using GFX PCR-DNA and the Gel Band Purification Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) following the manufacturer's protocols. They were then sequenced bidirectionally using the same primers for PCR using an ABI PRISM 3100 automatic sequencer at the Life Science Research Center, Center for Molecular Biology and Genetics, Mie University, Mie, Japan. Sequences were contiged and assembled using the GENETYX-WIN software (Software Development, Tokyo, Japan). Successfully sequenced isolates were deposited in GenBank under accession numbers AB626738 to AB626748 (Table 1).

Phylogenetic analysis

Sequences were used in BLAST searches (Altschul et al. 1997) to infer the closest taxon within the sequences deposited in the DDBJ/GenBank/EMBL database. To place the isolates with respect to known *Raffaelea* species, phylogenetic trees were constructed incorporating related species and two outgroup species (Table 2). We aligned the sequence data of 42 taxa, consisting of 30 taxa from the downloaded alignments, the 11 *Raffaelea* isolates obtained from this study for which we were able to obtain a sequence (of the 15 total isolated), and a previously deposited sequence of the ex-holotype of *R. quercivora*. Alignment was performed with version 6 of the MAFFT software (Katoh and Toh 2008), using the L-INS-i option (a slow, iterative-refinement method) with default settings and adjusting the scoring matrix as "1PAM/$\kappa = 2$". No further manual corrections were required to obtain reproducible results. The aligned data set was then analyzed using the parsimony methods in the MEGA4 software (Tamura et al. 2007). The alignment gaps and missing data were eliminated from the dataset using the "complete deletion" option. The phylogenetic trees were constructed using the maximum-parsimony method based on the close-neighbor-interchange algorithm (Nei and Kumar 2000), with search level 7; the initial trees were obtained with the

Table 2 Source numbers for the 28S large subunit ribosomal DNA sequences included in this study and the corresponding accession numbers

Species ID	Source	DDBJ/EMBL/GenBank accession no.
Ambrosiella ferruginea	CBS 408.68	AF275505
A. hartigii	CBS 403.82	AF275506
A. xylebori	CBS 110.61	DQ470979
Aspergillus fumigatus (outgroup)	NRRL 166	U28463
Ceratocystis fimbriata	C685	AF275512
C. virescens	C74	AF043603
Grosmannia francke-grosmanniae	CMW 2975	DQ294395
G. grandifoliae	CMW 703	DQ294399
G. huntii	C583	EU177469
G. leptographioides	CMW 481	DQ294382
G. robusta	CMW 2805	DQ294398
G. serpens	CBS 141.36	EU177471
Hyalorhinocladiella macrospora (=*A. macrospora*)	C2231	EU177468
H. tingens (=*A. tingens*)	C2232	EU177474
Nectria haematococca	IFO 31094	AB084303
Ophiostoma ips	CBS 137.36	EU913644
O. lunatum	CMW 10564	DQ294355
O. quercus	CMW 3110	DQ294376
Raffaelea albimanens	CBS 271.70	EU984296
R. amasae (=*Dryadomyces amasae*)	CBS 116694	EU984295
R. ambrosiae	CBS 185.64	EU984297
R. arxii	C2372	EU177455
R. canadensis	CBS 168.66	EU984299
R. gnathotrichi	CBS 379.68	EU177460
R. lauricola	CBS 121567	EU177440
R. montetyi	CBS 451.94	EU177461
R. quercivora	MAFF 410918	AB496454
R. scolytodis	CCF 3572	AM267270
R. sulcati	CBS 806.70	EU177462
R. tritirachium	CBS 726.69	EU177464
Taphrina wiesneri (outgroup)	NRRL T-460	AF492075

CBS Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, *NRRL* ARS Culture Collection, Illinois, USA; IFO (=NBRC), NITE Biological Resource Center, Chiba, Japan, *CMW* Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, *C* Culture Collection of T.C. Harrington, Iowa, USA, *MAFF* Genebank, National Institute of Agrobiological Sciences, Tsukuba, Japan

random addition of sequences (10 replicates). Values greater than 60% in the replicate trees, in which the associated taxa clustered together in the bootstrap (BS) test (1,000 replicates), are shown next to the branches (Felsenstein 1985).

Results

Table 3 summarizes LM characterizations of the ex-holotype of *R. quercivora* and of the other 14 Japanese isolates, and Fig. 1 illustrates typical LM observations. Conidial shapes for both the ex-holotype and the other isolates were all obovoid to pyriform and were oblong. The conidial sizes of the ex-holotype were 3.9–8.4 × 1.5–3.1 μm. The length-to-width ratio of its conidia ranged from 1.6 to 3.5, and the conidiophore dimensions ranged between 13.7 and 45.8 × 1.1 and 3.1 μm. The conidial sizes of the other 14 isolates were 3.3–10.3 × 1.1–4.5 μm. Their length-to-width ratios ranged from 1.3 to 5.2, and the conidiophore dimensions were 13.6–69.5 × 0.9–2.8 μm.

Table 1 summarizes the conidiogenesis characteristics of the 14 isolates and the ex-holotype of *R. quercivora* observed using an SEM. Of the 15 isolates, 11 (including the ex-holotype) showed three different conidiogenesis patterns: (1) sympodial proliferation observed on conidiogenous cells (Fig. 2c, d, h), (2) an annellidic-percurrent proliferation on conidiogenous cells, with tightly packed annellations (Fig. 2e, f), and an annellidic-percurrent proliferation that created the illusion of sympodial proliferation as a result of a remaining conidium hanging loosely along the sides of the conidiogenous cells after its secession (Fig. 2b), and (3) sympodial proliferation following annellidic-proliferation (SPFAP; Fig. 2a, g). Two isolates (RA0085 and RA1430)

Table 3 Morphological characterization of the conidia and conidiophores of 15 isolates of *Raffaelea quercivora* (the ex-type and 14 isolates)

Isolate	Conidia dimensions (μm)		Length-to-width ratio for the conidia	Conidiophore dimensions (μm)	
	Length	Width		Length	Width
MAFF410918[a]	3.9–8.4	1.5–3.1	1.6–3.5	13.7–45.8	1.1–3.1
RA1490	3.9–8.9	1.4–2.9	1.6–4.0	17.7–59.2	1.1–1.9
RA1258	4.4–9.5	1.9–3.4	1.8–3.5	16.2–55.6	0.9–2.1
RA1026	3.5–8.9	1.5–3.1	1.7–4.2	17.3–56.1	1.1–1.8
RA0085	4.2–9.1	1.8–3.0	1.6–4.1	17.2–48.6	1.1–2.4
RA0129	3.6–9.1	1.7–3.6	1.5–3.7	17.3–48.5	0.9–2.3
RA0033	3.8–8.4	1.7–3.3	2.0–3.5	13.6–37.5	1.1–2.6
RA0003	4.7–8.7	2.1–4.5	1.3–3.4	17.8–52.0	0.9–1.9
RA1385	3.6–8.5	1.8–2.9	1.7–3.7	16.2–50.1	1.1–2.1
RA1430	3.9–8.7	1.6–3.0	1.7–3.8	18.9–56.2	1.1–2.0
RA1087	3.5–9.4	1.5–2.8	1.7–5.2	17.9–69.5	1.0–2.0
RA1100	3.5–8.5	1.4–2.8	2.0–4.2	17.1–60.5	1.0–2.8
RA1091	3.7–8.6	1.5–2.9	1.6–3.9	16.7–51.8	1.1–2.8
RA1384	3.3–10.3	1.1–3.2	1.8–4.5	18.8–68.6	1.1–2.7
RA1183	3.3–8.4	1.6–2.9	1.7–4.3	14.1–41.3	1.0–2.3

For each item, values were recorded based on 50 measurements grown of strains on PDA for 3–4 days at 25°C. Conidial shapes among isolates were obovoid to pyriform, oblong

[a] The ex-holotype strain of *R. quercivora* reported by Kubono and Ito (2002)

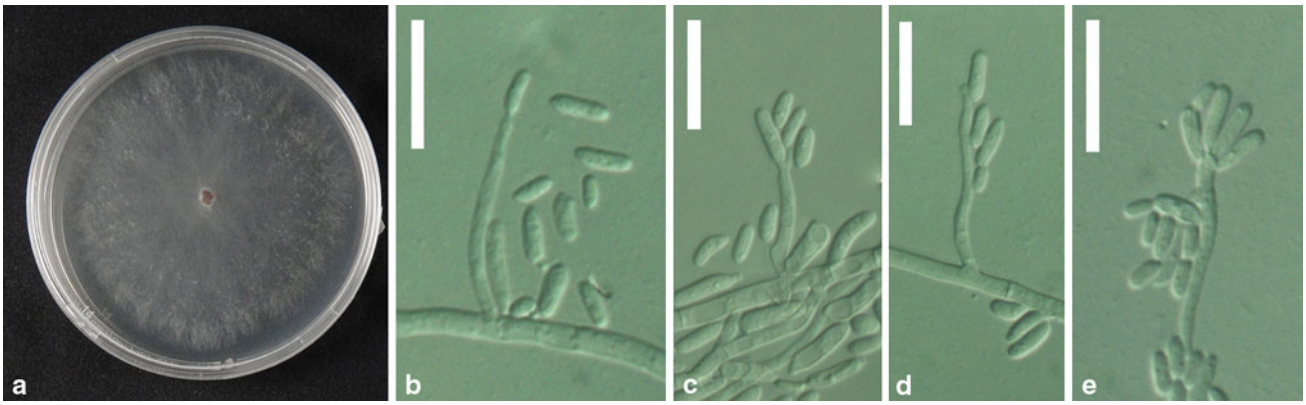

Fig. 1 Light microscopic images of *Raffaelea quercivora* (MAFF410918, ex-holotype). **a** Colonies after 14 days on a malt extract agar plate. **b–e** Morphological characteristics observed by light microscopy after 7 days on oatmeal agar plates. **b** Solitary conidium on a tapered conidiophore. **c** Sympodial proliferation of the conidiophore. **d** Conidium hanging loosely along the side of the conidiophore. **e** Polyblastically produced conidia. *Bars* **b–e** 20 μm

demonstrated only two different conidiogenesis patterns: (1) SPFAP and (2) an annellidic-percurrent proliferation on conidiogenous cells with tightly packed annellations. The other two isolates (RA1258 and RA0033) exhibited only one type of conidiogenesis, an annellidic-percurrent proliferation on conidiogenous cells with tightly packed annellations. As shown in Fig. 2, these conidiogenesis patterns were clearly observed by means of SEM, although they could not be seen in LM images (Fig. 1b–e).

PCR amplification succeeded for all the isolates. However, sequences were only successfully obtained for 11 of the 14 isolates, excluding isolates RA1258, RA0033, and RA1385 (Table 1). Based on LSU sequence analysis, all the isolates, including the ex-holotype, belonged to genus *Raffaelea*, within the Ophiostomatales. The LSU sequence alignment contained 42 taxa, including two outgroups, and had a total length of 322 characters, of which 109 (34%) were parsimony informative. Figure 3 shows 1 of the 752 most parsimonious trees derived from this analysis, which had a total length of 374 steps, a consistency index of 0.558, a retention index of 0.814, and a composite index of 0.455. An ophiostomatoid clade was clearly formed with a 85% BS value, and the *Grosmannia* sequences that did not form one clade were placed intermediate between

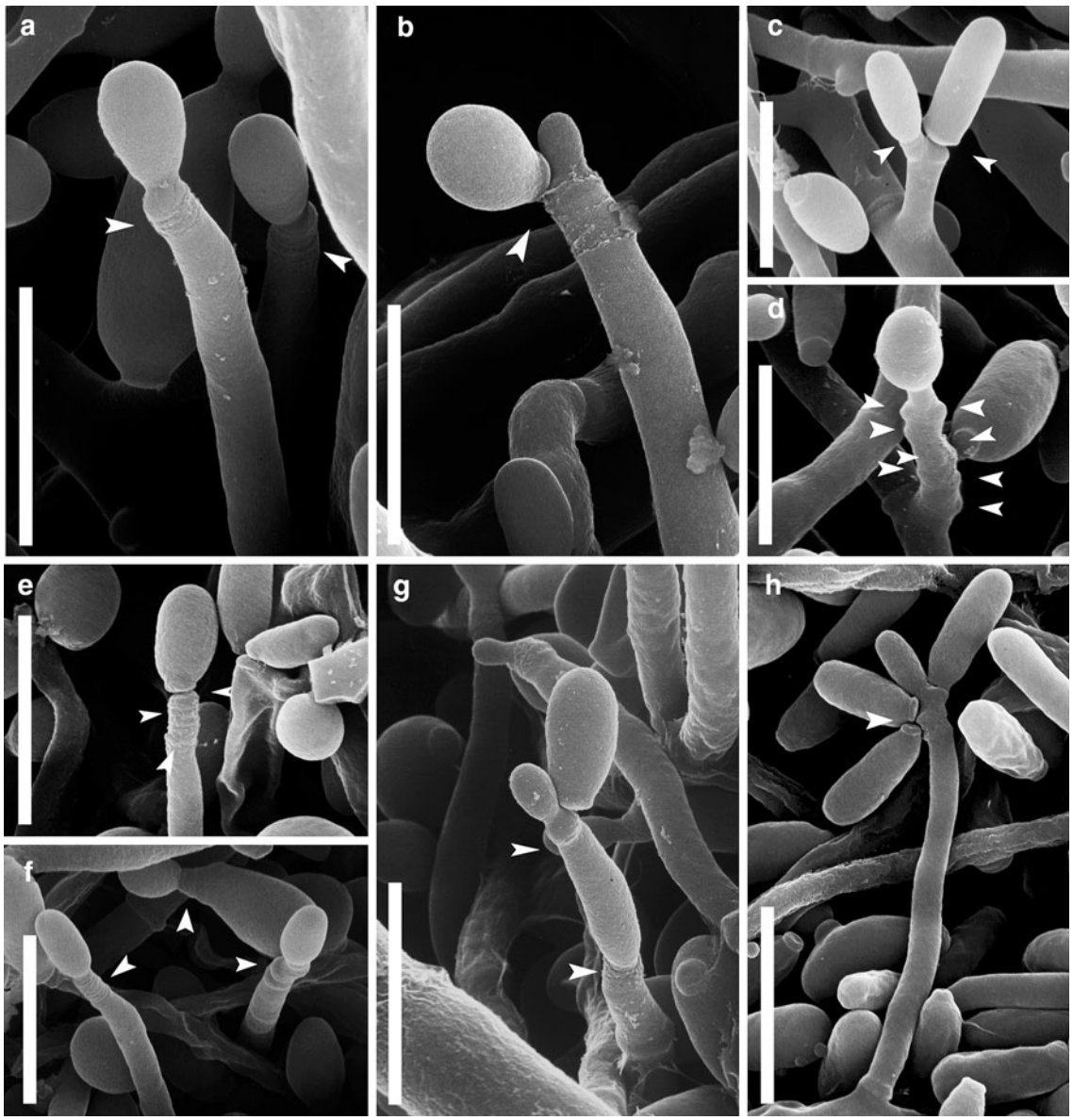

Fig. 2 Images of conidiogenesis of *Raffaelea quercivora* by scanning electron microscopy (SEM; 15 kV). **a**–**d** An ex-holotype strain, MAFF410918. **e**–**h** Isolates collected in Japan: RA0033 (**e**); RA1087 (**f**); RA1384 (**g**); RA0129 (**h**). **a**, **e**, **f** Annellidic proliferations. **b**, **g** Percurrent proliferations. **c**, **d**, **h** Sympodial proliferation. **a**, **g** Sympodial proliferations following annellidic proliferations of conidiogenous cells. **a**, **e**, **f** *Arrowheads*, tightly packed annellations at the apices of gradually tapered conidiophores. **b** *Arrowhead*, previously formed conidium hanging loosely along the side of a conidiophore after secession. **c**, **d**, **h** *Arrowheads*, series of flattened loci on the shoulder caused by sympodial proliferation. *Bars* 5 μm

Ophiostoma and *Raffaelea* clades. Within the *Raffaelea* clade that was no higher BS support, all the isolates of *R. quercivora* formed a sub-clade with an 87% BS value that discriminated it from the closest clade, that for *R. montetyi*, also at an 87% BS value.

Discussion

DNA sequences have been previously applied to classify the fungal symbionts of ambrosia beetles (Cassar and Blackwell 1996; Jones and Blackwell 1998; Rollins et al. 2001). In the present study, phylogenetic trees created using the LSU D1/D2 region showed that members of the genus *Raffaelea* were clearly separated from the other ophiostomatoid fungi sampled. However, all the isolates of *R. quercivora* were nested in a single clade, with a higher BS value (87%) differentiating them from other known *Raffaelea* species sampled (Fig. 3). These results were consistent with those from other studies based on phylogenetic analyses using the internal transcribed spacer (ITS), 18S and 28S rDNA, and β-tubulin regions, although only

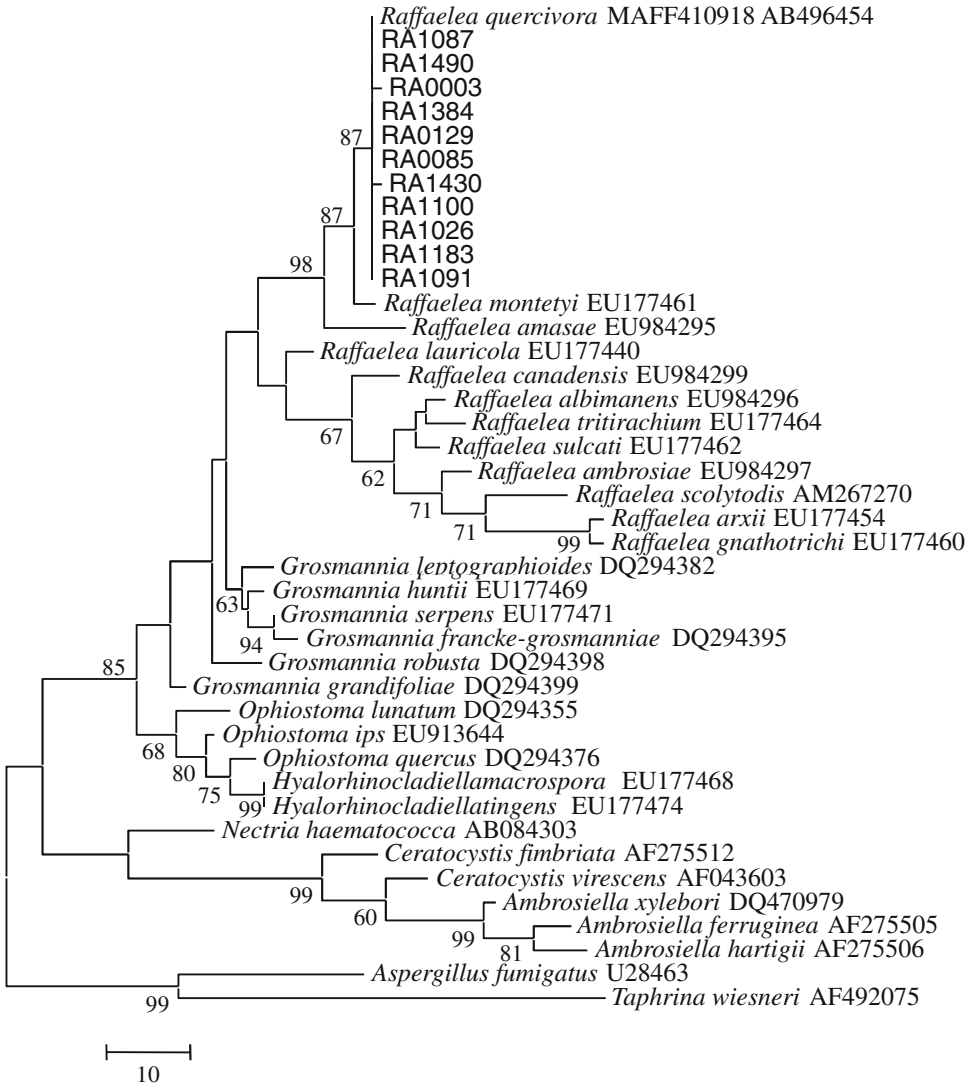

Fig. 3 One of the 752 most parsimonious trees, showing the phylogenetic relationships among the different species of ophiostomatoid fungi. The tree was generated by analysis of the partial sequence of large subunit (LSU) ribosomal DNA. Bootstrap values (1,000 replicates) greater than 60% are indicated at the branch *nodes*. Fungal taxa collected and analyzed in this study are shown in *bold*. *Taphrina wiesneri* and *Aspergillus fumigatus* were used as outgroup taxa

322 characters were used in the current study (Gebhardt et al. 2004; Kim et al. 2009; Kolařík and Hulcr 2009; Massoumi Alamouti et al. 2009; Harrington et al. 2010; Matsuda et al. 2010). Moreover, although Harrington et al. (2010) mentioned that sequences of *R. quercivora* are similar with *R. montetyi*, the present study and Matsuda et al. (2010) showed that these two species were clearly separated with higher BS supports. Thus, the phylogenetic placement of the isolates examined based on rather limited numbers of nucleotide sequences on the single gene suggests their genetic identity to be among the previously known *Raffaelea* species and further confirms their affiliation within the *R. quercivora* clade.

In this study, we examined 15 *R. quercivora* isolates, including an ex-holotype strain, from morphological and phylogenetic aspects. From the results of our LM observations, the ex-holotype strain and the 14 Japanese isolates showed a wider range of conidial and conidiophore sizes than that of its protologue, as described by Kubono and Ito (2002). Moreover, the present observations using SEM revealed that conidiogenesis of the ex-holotype strain of *R. quercivora* follows a pattern of sympodial or annellidic-percurrent proliferation with delayed secession, creating the illusion of sympodial proliferation (Fig. 2), as was shown by van Wyk et al. (1988) for *Leptographium* spp. Van Wyk et al. (1988) demonstrated that illusionary sympodial development can be produced by incomplete dehiscence of conidia from percurrently proliferating conidiogenous cells. That is, a delayed secession stage overlaps the onset of proliferation, likely leaving conidia hanging along the sides of the conidiogenous cells (Benade et al. 1995). These events were sometimes observed in the *R. quercivora* isolates (see Fig. 2b). Considering these facts, the conidiogenesis descriptions of *R. quercivora* should be revised to

include various conidiogenesis patterns, which include sympodial and annellidic-percurrent proliferation, as Gebhardt and Oberwinkler (2005) reported.

SEM examinations revealed that the conidiogenesis patterns of four *Raffaelea* species (*R. albimanens*, *R. ambrosiae*, *R. arxii*, and *R. montetyi*) exhibited both sympodial and annellidic-percurrent proliferation and were identical to the pattern of *Ophiostoma* synanamorphs belonging to the Ophiostomataceae (e.g., *Leptographium*, *Hyalorhinocladiella*, and *Pesotum*) (Wingfield et al. 1991; Benade et al. 1996; Gebhardt et al. 2004; Gebhardt and Oberwinkler 2005). The anamorphic states of *O. clavigerum* (R.C. Rob.-Jeffr. & R.W. Davidson) T.C. Harr. showed a continuum of conidial development, from sympodial to annellidic-proliferation and with intermediate forms (Tsuneda and Currah 2006). Thus, the conidial development of *Raffaelea* species, including *R. quercivora*, appears to be more plastic than previously recognized, based mostly on LM examinations (von Arx and Hennebert 1965; Batra 1967; Funk 1970).

According to Kim et al. (2009), a newly described *Raffaelea* fungus (*R. quercus-mongolicae* K.H. Kim, Y.J. Choi & H.D. Shin) was phylogenetically closely related to *R. quercivora*. On the other hand, they demonstrated that these species were easily distinguished based on morphological characteristics: the conidia of *R. quercus-mongolicae* were larger (4.8–8.3 × 2.6–3.6 μm) than those of *R. quercivora* (3.1–4.7 × 2.0–2.4 μm) and smaller than those of *R. montetyi* (6.6–13 × 3–6.6 μm), which is another closely related *Raffaelea* species. In the present study, the size of the conidia of the ex-holotype and the other strains of *R. quercivora* was 3.9–8.4 × 1.5–3.1 and 3.3–10.3 × 1.1–4.5 μm, respectively. Because the range of conidial sizes of *R. quercus-mongolicae* encompassed that of *R. quercivora*, the conidial size alone does not appear to be a tenable solution for distinguishing these species. Regarding the phylogenetic relationship among the *Raffaelea* species, Kim et al. (2009) demonstrated that *R. quercivora* and *R. quercus-mongolicae* were distinguishable based on the rDNA ITS, 18S, and β-tubulin regions. In our preliminary study (data not shown), Korean isolates that were suggested to be *R. quercus-mongolicae* based on a partial 28S rDNA sequences were genetically independent species from *R. quercivora*, even though the morphological characteristics of the two species were indistinguishable. Thus, additional studies will be necessary to reliably distinguish between the two species, based on analyses of many isolates, including both ex-holotype cultures.

These results showed that *R. quercivora* isolates vary greatly in their phenotypic characters. On the basis of these results, we propose the following taxonomic revisions of *R. quercivora*.

Emended description: *Raffaelea quercivora* Kubono & Shin. Ito, Mycoscience 43: 256, 2002. Colonies on PDA at 20°–25°C effuse, spreading rapidly, reaching 80 mm diameter in 5 days with an indistinct white margin, appearing water-soaked and mucilaginous; aerial mycelium abundant, floccose, composed of branched, septate, hyaline, smooth hyphae, arranged in fascicles and simulating coremia, reaching 1 cm high; color diffusing and turning pale olive to olive-brown after 2 weeks; odor fragrant, resembling that of ethyl alcohol. Sporodochia of several fascicles confluent and mucilaginous. Conidiophores macronematous, mononematous, formed in sporodochia or produced separately, simple or branched, straight, hyaline, septate, smooth, 13.5–70 × 1–3 μm; conidiogenous cells gradually narrowed toward the apex, proliferating sympodially and/or annellidic-percurrently, indeterminate, discrete or integrated, terminal or intercalary, hyaline, smooth, with a series of flat, inconspicuously protruding scars on the shoulders caused by sympodial proliferation or tightly packed annellations or both. Conidia obovoid to pyriform, oblong, slimy, produced in acropetal order, hyaline, aseptate, straight, smooth, obovoid to pyriform, tapered markedly toward the base, apex rounded, base truncated and/or protruding, often producing sprout cells in droplets, 3–10 × 1–4.5 μm.

Sequences of ex-holotype culture (MAFF410918): AB496428 for 18S rDNA, AB661322 for ITS rDNA, and AB496454 for 28S rDNA.

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