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SHORT COMMUNICATION

Genetic characterization of *Raffaelea quercivora* isolates collected from areas of oak wilt in Japan

Yosuke Matsuda · Kyoko Kimura · Shin-ichiro Ito

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Abstract This study was undertaken to improve understanding of the phylogenetic position of pathogenic fungi implicated in the oak wilt in Japan. Sequences were obtained from three regions of partial nuclear ribosomal DNA of 25 isolates of *Raffaelea quercivora* including an ex-type strain, all of which were collected from seven areas of disease outbreak. All the isolates formed one clear clade with high bootstrap values, distinctly delimited from the closest species, *R. montetyi*. These results indicate that the *R. quercivora* is phylogenetically a well-defined taxon.

Keywords Ophiostomatoid fungi · *Quercus* · rDNA

Oak trees have been dying in Japan since the 1980s, and incidences of oak wilt have become gradually more conspicuous (Ito and Yamada 1998; Ito 2000). Dead trees are always found to be infested by the ambrosia beetle *Platypus quercivorus* (Murayama). *Raffaelea quercivora* Kubono & Shin. Ito has repeatedly been isolated from the mycangium, body surface, and galleries of the beetle (Ito 2000; Kinuura and Kobayashi 2006). The inoculation of the fungus caused wilting and/or death of some species of oak seedlings (Murata et al. 2005, 2007) and thus would be a pathogenic fungus. Another species of *Raffaelea*, *R. lauricola* T.C. Harr., Fraedrich & Aghayeva, which is carried by the ambrosia beetle *Xyleborus glabratus* Eichhoff, has been reported to cause a vascular wilting disease in trees of the Lauraceae (Fraedrich et al. 2008; Harrington et al. 2008). Tree diseases caused by these fungi could lead to loss of species diversity and reduced quality of timber products.

The genus *Raffaelea* is known to be intimately associated with ambrosia beetles (Batra 1967; Funk 1970; Scott and du Toit 1970; Morelet 1998). *Raffaelea* species are identified from the size and shape of the conidia and conidiophores (Batra 1967), so morphological traits are crucial to identification (Gebhardt and Oberwinkler 2005). Eleven species of *Raffaelea* were recognized as of 2002 (Kubono and Ito 2002), and two new additional species have been described since then: *R. lauricola* (Harrington et al. 2008) and *R. scolytodis* M. Kolařík (Kolařík and Hulcr 2009). Although the morphology of *R. lauricola* was suggested to be similar to that of *R. quercivora* (Harrington et al. 2008), the phylogenetic relationship between the species has not been clarified.

The genetic characterization of fungal ribosomal DNA (rDNA) has become commonly used for the identification and placement of the ambrosia fungi, *Ambrosiella* and *Raffaelea*, and other ophiostomatoid fungi (Cassar and Blackwell 1996; Jones and Blackwell 1998; Farrell et al. 2001; Ohtaka et al. 2006). The internal transcribed spacer (ITS) region of rDNA has been used for species discrimination because its basal sequences are highly variable between species but low within species (Bruns et al. 1991; Lee and Taylor 1992; Nilsson et al. 2008). However, analyses of the partial large subunit (LSU) and small subunit (SSU) rDNA are considered to be more highly conserved within species than that of the ITS region, so these regions are also analyzed to discriminate phylogenetic relationships at the genus and family levels (Masuya et al. 2004; Kolařík and Hulcr 2009; Massoumi Alamouti et al. 2009).

The aim of this study was to place *R. quercivora* within the *Raffaelea* genus and in relation to other species in the

Y. Matsuda · K. Kimura · S. Ito (✉)
Laboratory of Forest Pathology and Mycology, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya-cho, Tsu, Mie 514-8507, Japan
e-mail: ito-s@bio.mie-u.ac.jp

Ophiostomatales. To achieve this aim, we analyzed the ITS, LSU, and SSU regions of fungal rDNA.

We examined 25 isolates of *R. quercivora* including both the ex-type strain, MAFF410918, and four strains, MAFF410919–MAFF410922, morphologically examined by Kubono and Ito (2002). Twenty other isolates were newly obtained from dead or declining trees in seven geographically separate areas experiencing areas of oak wilt (Table 1). The isolates were maintained on potato dextrose agar (PDA). We also included one *R. montetyi* M. Morelet isolate (MPFN308) obtained from an individual of *Platypus cylindrus* (Fabr.) collected from an infested *Quercus suber* L. in Var, France, in 1993.

The fungi were precultured on ½-strength PDA plates and then subcultured onto the new medium plates covered with a sheet of cellophane and grown at 25°C in the dark for 4–7 days. Approximately 250 mg of fresh mycelial mat of each isolate was transferred to a 1.5-ml centrifuge tube for DNA extraction. Total DNA was extracted and amplified by polymerase chain reaction (PCR) as described by Matsuda and Hijii (1999). We used nested PCR to obtain the ITS and D1/D2 (LSU) regions of the fungal nuclear rDNA using LA *Taq* polymerase (Takara, Otsu, Japan) according to the manufacturer's recommendations. The first PCR for both regions was conducted with the ITS5 primer pair (White et al. 1990) and NL4 (O'Donnell 1993). The second PCR used ITS1/ITSp3 (Kusaba and Tsuge 1995) for the ITS region and ITS3/NL4 for the D1/D2 region. To infer phylogenetic relationships between *R. quercivora* and other species of *Raffaelea*, we also analyzed the SSU rDNA, which has commonly been used for the phylogenetic placement of ophiostomatoid fungi (Jones and Blackwell 1998; Farrell et al. 2001; Gebhardt et al. 2005). The SSU rDNA region of three isolates of *R. quercivora* and one isolate of *R. montetyi* was amplified using the primer pairs NS1/NS4 and NS3/NS6 (White et al. 1990). When only one DNA band was present in a sample after amplification, PCR products were sequenced.

The products were cleaned with a JET-SORB Gel Extraction Kit (Genomed, Bad Oeynhausen, Germany) and sequenced with a CEQ 2000 Dye Terminator Cycle Sequencing With Quick Start Kit (Beckman Coulter, Fullerton, CA). The sequence reaction was conducted with one of ITS1, ITS2, ITS3, ITS4, or ITS5 primer for the ITS region; either NL1 or NL4 for the LSU region; and one of NS1, NS3, NS4, or NS6 for the SSU region. The obtained sequence data were manually adjusted with GENETYX v. 4.0 software (Software Development Co., Ltd.). The data that were successfully sequenced were submitted to the DNA Data Bank of Japan (DDBJ) as accessions AB496428–AB496476, including the *R. montetyi* (MPFN308) sequences of SSU rDNA (AB496431), ITS rDNA (AB496432), and LSU rDNA (AB496453).

Sequences from the different isolates were aligned by means of MAFFT v. 6 software (Katoh and Toh 2008; http://align.bmr.kyushuu.ac.jp/mafft/online/server/), using the L-INS-i option (slow; iterative refinement method) with default settings. No further manual corrections were made, to ensure that the results obtained were reproducible. Phylogenetic analyses were performed using MEGA v. 4 software (Tamura et al. 2007). The maximum-parsimony tree was obtained by using the close-neighbor-interchange algorithm (Nei and Kumar 2000) with search level 7, in which the initial trees were obtained with the random addition of sequences (ten replicates). Values of more than 60% of replicate trees in which the associated taxa clustered together in the bootstrap (BS) test (1000 replicates) were shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). We incorporated fungal sequences obtained from several ophiostomatoid fungi and other Ascomycetes (Cassar and Blackwell 1996; Jones and Blackwell 1998; Rollins et al. 2001; Gebhardt et al. 2004, 2005) as ingroups. *Grosmannia penicillata* (Grosmann) Goid. (AM943882) was used as the outgroup for ITS rDNA, *Taphrina wiesneri* (Ráthay) Mix (AF492075) and *Aspergillus fumigatus* Fresen. (U28463) for LSU rDNA, and *T. wiesneri* (AY548293) and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (J01353) for SSU rDNA.

PCR amplification was successful for all the isolates. Partial sequences of the LSU rDNA region were successfully obtained for 23 isolates including the ex-type strain MAFF410918. One of 1490 equally most-parsimonious trees, consisting of 631 steps, was derived from analysis of the 158 phylogenetically informative positions (Fig. 1). The consistency index was 0.472, the retention index was 0.806, and the composite index was 0.381. The representative tree supported one of two clades, either Ophiostomatales or Microascales, with 88 and 79% BS values, respectively. All the *Raffaelea* species together with the *Grosmannia* species formed one clade with a rather low BS value (66%), which was clearly separated from a clade composed of *Ophiostoma* and *Ambrosiella* species. Within the *Raffaelea* clade, 23 isolates of *R. quercivora* and 1 isolate of *R. montetyi* had 10–12-bp changes forming one sub-clade with a BS value of 99%, and the *R. quercivora* isolates were solely grouped with a 97% BS value.

For the SSU sequences, 1 of 130 equally most-parsimonious trees of 378 steps was derived from analysis of the 114 phylogenetically informative positions (Fig. 2). The consistency index was 0.525, the retention index was 0.803, and the composite index was 0.422. As with the LSU analysis, the SSU sequence analysis placed the three analyzed isolates of *R. quercivora* and one isolate of *R. montetyi* into the Ophiostomatales clade that was

Table 1 The origins of 25 isolates of *Raffaelea quercivora* obtained from sites of oak wilt in Japan and one isolate of *R. montetyi*

Species	Isolates	Origin (no.)	Host trees	Year of first isolation	DDBJ accession number		
					SSU rDNA	ITS rDNA	LSU rDNA
Raffaelea quercivora	MAFF410918[a]	Higashitagawagun, Yamagata (1)	*Quercus crispula*	1998	AB496428	AB496433	AB496454
	MAFF410919	Higashitagawagun, Yamagata (1)	*Q. crispula*	1998	AB496429	AB496434	AB496455
	MAFF410920	Nanjyogun, Fukui (3)	*Q. crispula*	1995		AB496435	AB496456
	MAFF410921	Takashimashi, Shiga (3)	*Q. crispula*	1997		AB496436	AB496457
	MAFF410922	Iwamigun, Tottori (4)	*Q. crispula*	1995	AB496430	AB496437	
	N76	Higashitagawagun, Yamagata (1)	*Q. crispula*	1995			AB496458
	N77	Higashitagawagun, Yamagata (1)	*Q. crispula*	1995		AB496439	AB496459
	N97	Higashitagawagun, Yamagata (1)	*Q. crispula*	1998		AB496440	
	N21	Ojiyashi, Niigata (2)	Unknown	1992		AB496441	AB496463
	R26	Yamagun, Fukushima (2)	*Castanea crenata*	2003		AB496442	AB496464
	R27	Yamagun, Fukushima (2)	*Q. crispula*	2003		AB496443	AB496465
	N27	Nanjyogun, Fukui (3)	Unknown	1991		AB496444	AB496467
	N6	Nanjyogun, Fukui (3)	Unknown	1991			AB496469
	N50	Nanjyogun, Fukui (3)	*Q. crispula*	1995			AB496468
	N114	Maizurushi, Kyoto (4)	*Q. crispula*	1999		AB496445	AB496466
	N139	Maizurushi, Kyoto (4)	*Q. crispula*	2000		AB496446	AB496471
	N142	Maizurushi, Kyoto (4)	*Q. crispula*	2000		AB496447	AB496472
	N75	Iwamigun, Shimane (5)	*Q. serrata*	1995		AB496438	AB496470
	N157	Masudashi, Shimane (5)	Unknown	2000		AB496448	AB496473
	R59	Hamadashi, Shimane (5)	*Q. crispula*	2002		AB496449	AB496474
	N132	Minamimurogun, Mie (6)	*Q. phillyraeoides*	1999		AB496450	AB496460
	N134	Minamimurogun, Mie (6)	*Q. serrata*	1999			AB496461
	N118	Higashimurogun, Wakayama (6)	*Q. phillyraeoides*	1999		AB496451	AB496475
	N67	Kimotsukigun, Kagoshima (7)	*Pasania edulis*	1994			AB496462
	M6	Kimotsukigun, Kagoshima (7)	*P. edulis*	1996		AB496452	AB496476
R. montetyi	MPFN308	Var, France	*Q. suber*	1993	AB496431	AB496432	AB496453

The numeral in parentheses indicates the geographical area of oak wilt in Japan defined by Ito and Yamada (1998). *1* Yamagata-northern Niigata, *2* central to southern Niigata-Fukushima, *3* Shiga-Fukui-Ishikawa-Toyama-Gifu, *4* Kyoto-Hyogo-Tottori, *5* Shimane, *6* Mie-Nara-Wakayama, *7* Miyazaki-Kagoshima

[a] The ex-type strain was the same as reported by Kubono and Ito (2002)

supported with a BS value of 90%. Within this clade, the isolates of *R. quercivora* were grouped with *R. montetyi* with a BS value of 71%, and there were 1–2-bp differences between two species.

For 20 isolates of *R. quercivora*, sequence analyses were successful only for the partial ITS region, 5.8S-ITS2 region. None of the primers used were successful in reproducing sequences of the ITS1 region from the samples examined. The GC content in the ITS2 region of isolates was relatively high (79.3–80.5%, data not shown); thus, the ITS1 region might also have had a high enough GC content to hamper PCR amplification (H. Masuya personal communication; Mullineux and Hausner 2009). Therefore, only the sequences from the 5.8S-ITS2 region were applied for phylogenetic analyses. One of 12842 equally most-parsimonious trees, consisting of 99 steps, was derived from analysis of the 19 phylogenetically informative positions (Fig. 3). The consistency index was 0.880, the retention

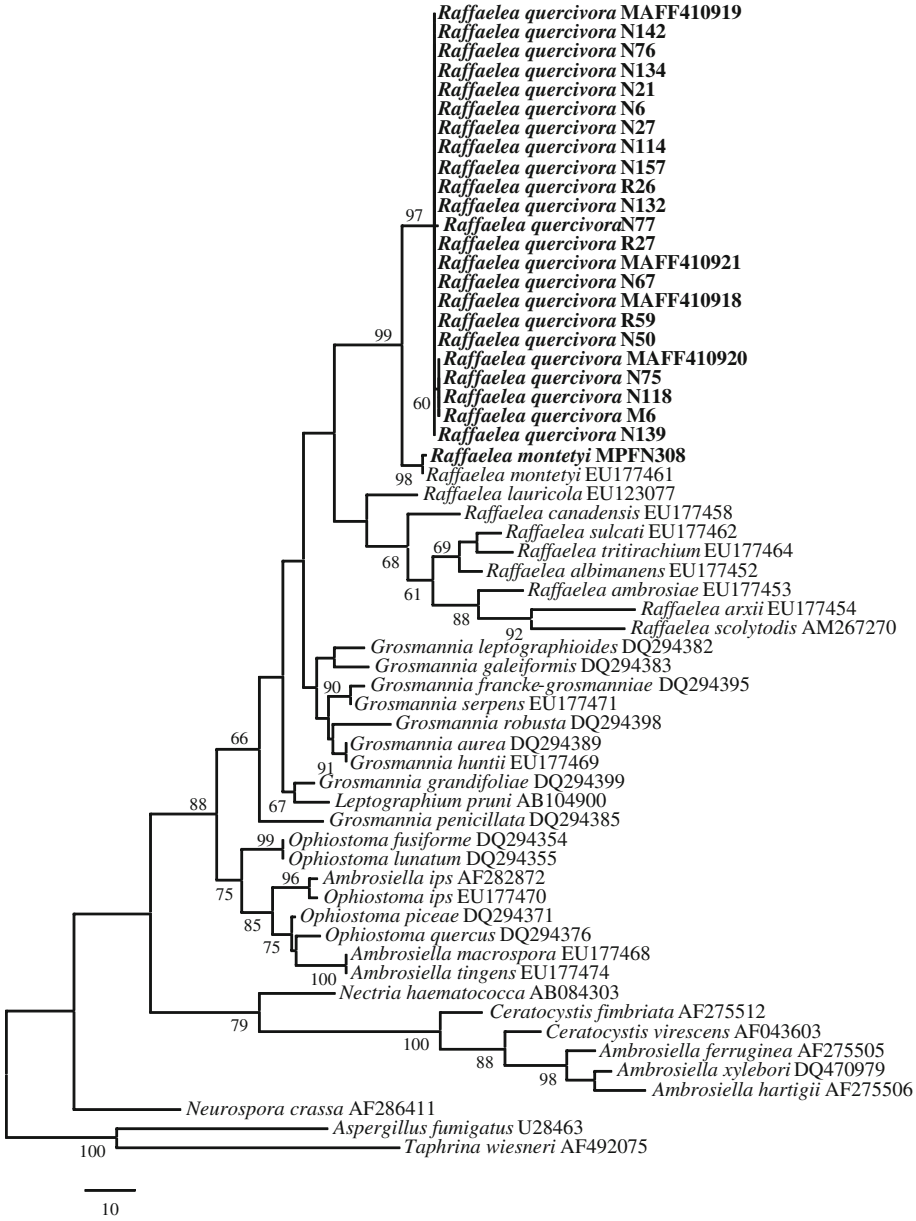

Fig. 1 One of 1490 maximum-parsimony trees showing phylogenetic relationships among different species of ophiostomatoid and other ascomycetous fungi. The tree was generated from the analysis of the partial sequence of large subunit (LSU) ribosomal DNA. Bootstrap values (1000 replicates) greater than 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

index was 0.864, and the composite index was 0.760. *Raffaelea quercivora* isolates, including *R. montetyi*, were placed in one clade, with a BS value of 99%, and 41–42-bp differences were detected between species. The isolates of *R. quercivora* were further nested within the clade supported with a 99% BS value.

The phylogenetic trees based on the LSU and SSU rDNA sequences clearly showed that *R. quercivora* formed a monophyletic group with *R. montetyi* in the Ophiostomatales, but was separated from *Ophiostoma* and *Grosmannia*. Within the trees constructed from ITS and LSU rDNA, *R. quercivora* was obviously delimited from *R. montetyi* with high BS values, whereas using SSU rDNA the relationship was ambiguous. In addition, *R. quercivora* was placed at some distance from *R. lauricola*, a vascular wilting pathogen (Fraedrich et al. 2008; Harrington et al. 2008), as well as from other *Raffaelea* species based on our SSU and LSU rDNA trees. This was also inferred by a tree constructed with multi-gene sequences in that *R. lauricola* and *R. montetyi* were positioned at a different clade from other *Raffaelea* species examined (Massoumi Alamouti et al. 2009). These results suggest that *R. quercivora* is phylogenetically different from other known *Raffaelea* species.

The position of the *Raffaelea* species in the constructed trees was consistent in that they were placed in the Ophiostomatales clade. This agrees with previous studies (Cassar and Blackwell 1996; Farrell et al. 2001; Rollins et al. 2001; Gebhardt et al. 2005). A recent multigene phylogeny provided a more comprehensive picture of the

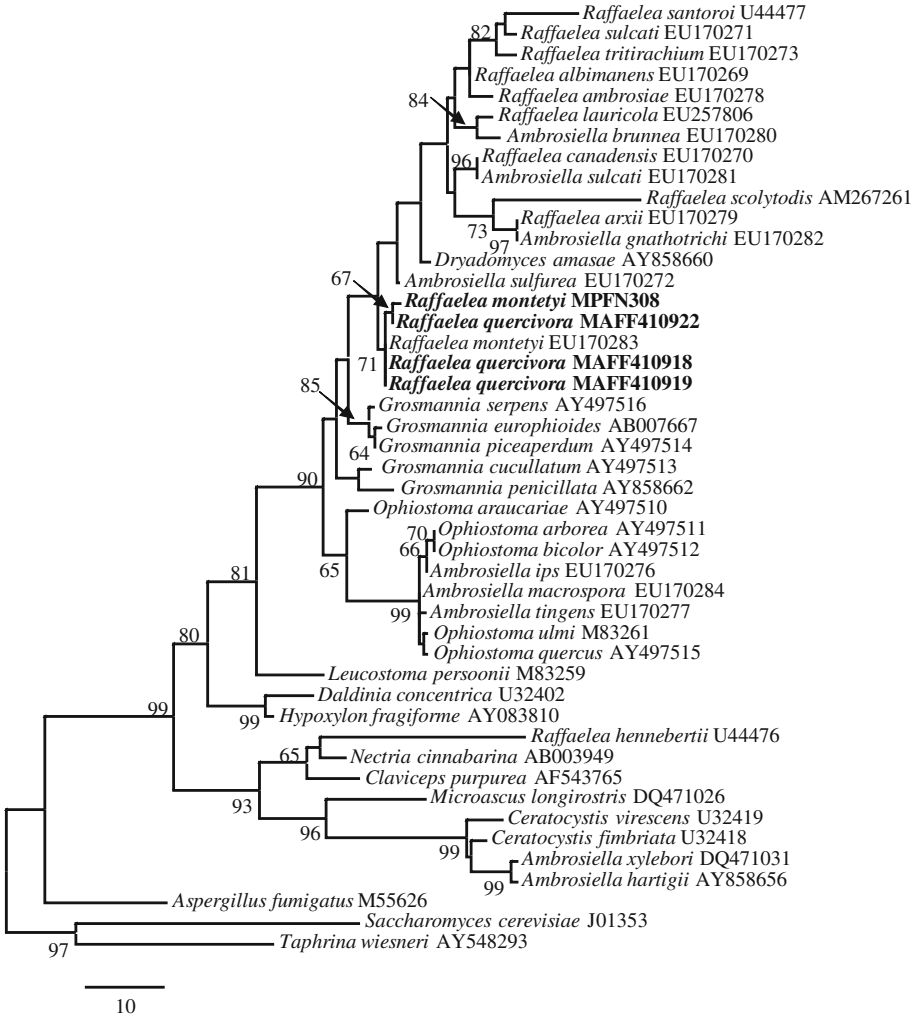

Fig. 2 One of 130 maximum-parsimony trees showing phylogenetic relationships among different species of ophiostomatoid and other ascomycetous fungi. The tree was generated from the analysis of the partial sequence of small subunit (SSU) ribosomal DNA. Bootstrap values (1000 replicates) greater than 60% are indicated at the branch nodes. Fungal taxa analyzed in this study are shown in *bold*. *Taphrina wiesneri* and *Saccharomyces cerevisiae* were used as outgroup taxa

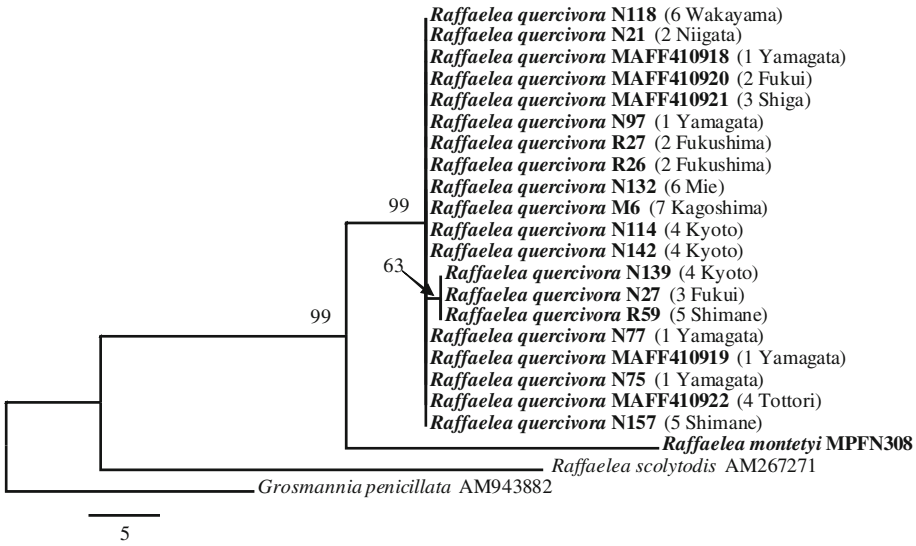

Fig. 3 One of 12842 maximum-parsimony trees showing phylogenetic relationships among the type-strain of *Raffaelea quercivora* and different isolates obtained from areas of oak wilt at seven sites. The tree was generated from the analysis of the partial sequence of internal transcribed spacer (5.8S-ITS2) region of ribosomal DNA. Bootstrap values (1000 replicates) greater than 60% are indicated at the branch nodes. Fungal taxa analyzed in this study are shown in *bold*. *Grosmannia penicillata* was used as an outgroup taxon

relationships between the ambrosia fungi *Dryadomyces*, *Raffaelea*, and *Ambrosiella* and the related genera *Grosmannia* and *Ophiostoma* (Massoumi Alamouti et al. 2009). Massoumi Alamouti et al. (2009) demonstrated that the *Raffaelea* members were placed into two different clades, and among their nine tested species of *Raffaelea*, *R.*

montetyi and *R. lauricola* were only placed in the same clade. In the present study, *Raffaelea montetyi* was placed phylogenetically adjacent to *R. quercivora* (Figs. 1, 2). To gain further insight into a relationship between phylogenetic and pathological traits of these fungal species, additional sequences of *R. quercivora* and other members of the genus *Raffaelea* should be analyzed. The β-tubulin gene would be a suitable candidate for next sequencing to achieve such discrimination (Zipfel et al. 2006; Massoumi Alamouti et al. 2009).

Among the examined isolates of *R. quercivora*, the sequences of the 5.8S-ITS2 region showed more than 98.6% similarity. However, they were clearly separated from *R. montetyi*, forming a distinct clade, as supported by the tree of the LSU region with high BS values. Although the fungal isolates were obtained from geographically distant areas, the DNA sequences indicate that they could well be considered as one species (Nilsson et al. 2008). In the field, however, hundreds of *P. quercivorus* ambrosia beetles are found in tree trunks (Hijii et al. 1991) at given localities, and thus thousands of the beetles would have been associated with the various oak wilt sites sampled. This suggests the potential for the existence of a greater diversity of fungal isolates than was sampled. Thus, the intimate association between *P. quercivorus* and *R. quercivora* could be considered proven, but the degree of genetic diversity within *R. quercivora* remains unanswered.

Although our results show the monophyletic grouping of *R. quercivora*, some ambiguity remains, because polymorphic conidia have been reported in the genus *Raffaelea* (Gebhardt et al. 2004; Gebhardt and Oberwinkler 2005). A recent study utilizing scanning electron microscopy showed that conidial development in three *Raffaelea* species, including the type species *R. ambrosiae* Arx & Hennebert, was achieved by annellidic percurrent proliferation (Gebhardt and Oberwinkler 2005). However, such a key of a developmental pattern has not been involved in the identification key for the genus *Raffaelea*, i.e., sympodial proliferation of the conidiogenous cells (Batra 1967). To accommodate this morphological variety of *Raffaelea*, Harrington et al. (2008) proposed a new comprehensive definition for the genus: "Conidiogenous cells proliferating percurrently or sympodially, leaving denticles, inconspicuous scars or annelations".

Raffaelea quercivora exhibits sympodial proliferation (Kubono and Ito 2002). Our fungal isolates were phylogenetically placed in a larger ophiostomatoid clade by both LSU and SSU rDNA sequences (Jones and Blackwell 1998). Within the clade, our samples and *R. montetyi* were nested in the sub-clade of *Grosmannia*, a genus that was recently reinstated by splitting from *Ophiostoma* (Zipfel et al. 2006). The members of the anamorphic genus, *Leptographium* and *Pesotum*, and *R. montetyi* form their conidia by annellidic proliferation (Wingfield et al. 1991; Gebhardt et al. 2004). In some cases, an anamorphic state of *G. clavigera* (R.C. Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. showed a continuum of conidial development from sympodial to annellidic with intermediate forms [*O. clavigerum* (R.C. Rob.-Jeffr. & R.W. Davidson) T.C. Harr. as in Tsuneda and Currah 2006]. It is likely that our *R. quercivora* isolates share the same morphological trait, and there is a need to re-examine its morphologies by scanning electron microscopy of *R. quercivora* and other *Raffaelea* species in future studies (Kubono and Ito 2002; Gebhardt and Oberwinkler 2005; Massoumi Alamouti et al. 2009).

In this study we examined the genetic characteristics of the type-strain of *R. quercivora* and isolates originating from widespread areas where oak trees died en masse. The DNA sequences were not clearly separated from each other, but were delimited from other related fungal species. This indicates that one species, *R. quercivora*, is intimately associated with the decline and death of oak trees in Japan. However, this taxonomic consistency of the disease-causing fungi does not necessarily mean that the pathogenicity is the same in all tree species involved. The oak species concerned come from geographically diverse areas and range from evergreen to deciduous broadleaf species, so the pathogenicity of the fungal isolates and each local strain of *R. quercivora* to each of the affected tree species needs to be clarified.

The authors declare that the experiments comply with the current laws of Japan.

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