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Incongruence between EF-1α Phylogeny and Morphology of *Metarhizium majus* and *Metarhizium guizhouense* in Japan

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In the phylogenetic analysis of *Metarhizium anisopliae* complex by BISCHOFF et al. (2009), the topology of the phylogeny obtained by the elongation factor 1 alpha gene (EF-1α) sequence had congruence with conidial size: One clade had the largest conidia followed by its sister clade. The two clades were defined as *M. majus* and *M. guizhouense*. However, the other locus used in their analysis showed disagreement with the topology. In the current study, a phylogenetic analysis was carried out with the 57 isolates of *Metarhizium* spp. from soil in Japan using the sequence of 5' end of EF-1α. Four and 3 isolates were classified as *M. guizhouense* and *M. majus*, respectively. The 7 isolates had relatively large conidia among all isolates, as suggested by BISCHOFF et al. (2009). However, one *M. majus* isolate had almost the same conidial dimensions as two *M. guizhouense* isolates and one *M. guizhouense* isolate had larger conidia than two *M. guizhouense* isolates and relatively close to two *M. majus* isolates. These results indicate that conidial morphology is incongruent with the sequence of the 5' end of EF-1α in MGT clade.

Key Words: *Metarhizium*, *M. majus*, *M. guizhouense*, Phylogeny, Elongation factor 1 alpha, Conidial morphology

NTRODUCTION

Metarhizium anisopliae is an asexual entomopathogenic fungus, which has been recorded to naturally infect over 200 insect species, including economically significant insect pests (HAJEK and ST. LEGER, 1994). This species has a global distribution and has a wide range of host insects (ROBERTS and ST. LEGER, 2004). It is generally present in the soil of various environments (ZIMMERMANN, 2007). *M. anisopliae* has been used commercially in many countries as a biological control agent (ZIMMERMAN, 2007).

Conidial morphology has been used to characterize *M. anisopliae* at variety level. There are two varieties in *M. anisopliae*, var. *anisopliae* with smaller conidia and var. *majus*, with larger conidia (TULLOCH, 1976). While isolates of *M. anisopliae* var. *anisopliae* have a rather wide host spectrum, *M. anisopliae* var. *majus* isolates preferentially attack coleopteran insects, especially larvae of *Oryctes* (FERRON et al., 1972).

Phylogenetic analyses using DNA sequencing have been done for *Metarhizium* spp. and the two varieties of *M. anisopliae* have been taxonomically redefined. DRIVER et al. (2000) analyzed sequences of the nuclear ribosomal internal transcribed spacer (ITS). They revealed that there were a number of evolutionally distinct lineages in *M. anisopliae* var. *anisopliae* and among them they defined the most major lineage distributed worldwide as *M. anisopliae* var. *anisopliae*. In their study, the three isolates of *M. anisopliae* var. *majus* composed a monophyletic group. Thus, the taxon name was retained. The two redefined varieties were closely related and could not be separated clearly by sequence information of ITS (BISCHOFF et al., 2009; NISHI et al., 2009).

BISCHOFF et al. (2009) did multi-locus phylogenetic analysis of an *M. anisopliae* complex. There were two large monophyletic groups, a PARB clade and an MGT clade, within the *M. anisopliae* complex and all of the isolates with relatively large conidia were included in the MGT clade. In the MGT clade, two species, *M. majus* and *M. guizhouense*, were defined based on the sequence of the elongation factor 1 alpha gene (EF-1α). This classification had congruence with conidial morphology: *M. majus* had the largest conidia (rarely below 10 μm long) and *M. guizhouense* had the second largest conidia (rarely exceeding 9 μm long).

However, the two species became non-monophyletic when the sequence loci other than EF-1α were used, indicating the congruence between phylogeny and morphology was still in doubt. Thus, a phylogenetic study with additional sequence loci and increased taxon sampling is needed to evaluate the phylogenetic relation between *M. guizhouense* and *M. majus*.

In Japan, the two morphological varieties of *M. anisopliae* have been isolated from soil (KAWAKAMI and NAKA, 1979; YAGINUMA, 1990; NISHI et al, 2009) but their phylogenetic locations in the taxonomy of BISCHOFF et al. (2009) have not been studied. In this study, we did phylogenetic analysis of the 5' end of EF-1α (5' EF-1α) region of *Metarhizium* spp. isolated from soil in Japan and examined the congruence of the

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result with conidial morphology.

MATERIALS AND METHODS

Fungal isolates and DNA extraction: The 57 isolates used in this study were obtained from soil samples collected in Japan from April 2008 to April 2009 by applying a semi-selective medium (60 g/l oatmeal, 12.5 g/l agar, 0.3 g/l chloramphenicol, 1 g/l cycloheximide), a modified medium of YAGINUMA and TAKAGI (1986). All of the isolates were microscopically confirmed to have conidia and conidiogenesis characteristics for *Metarhizium anisopliae*. The 41 isolates were phylogenetically analyzed using ITS sequences and classified into *M. anisopliae* var. *anisopliae* or *M. anisopliae* var. *majus* based on the taxonomy of DRIVER et al. (2000) (NISHI et al., 2009). The other isolates were also confirmed to belong to the same varieties by the same method.

For each fungal isolate, about 50 mg of mycelium was homogenized in DNA extraction buffer (4 mmol/l Tris-HCl, 250 mmol/l NaCl, 25 mmol/l EDTA, 1% SDS) and extracted with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) for 10 min on a vortex mixer. The solution was then centrifuged at 17,000 *g* to separate the phases and the upper aqueous phase was transferred to a 1.5 ml tube. The DNA was precipitated from the solution by addition of about 0.8 volumes of iso-propanol, washed with 70% ethanol, dried under a vacuum, resuspended in RNase solution (10 µmol/ml pH8.0 Tris-HCl, 1 µmol/ml pH8.0 EDTA, 10 g/l RNase A), and stored at 4 ˚C before use.

PCR amplification and nucleotide sequencing: Using aliquots of extracted DNA, the region of 5' EF-1α was amplified by polymerase chain reaction (PCR). This is the most informative region to use in routine species identification within the genus of *Metarhizium* (BISCHOFF et al., 2009). Primer sequences used were 5' -CGTCAGGACACACTGCAAATCTC-3' (EF1a-f597) and 5'-GCAGTCAGCCTGGGAAGTAC-3' (EF1a-r1397). The PCR reactions were performed in 20 µl reaction volumes containing 2-20 ng of genomic DNA, 1x reaction buffer, 0.2 mmol/l dNTPs, 1.5 mmol/l $MgCl_2$, 0.5 µmol/l of each primer, and 1 U Taq DNA polymerase (Applied Biosystems, Japan). The conditions of amplification were 5 min at 94 ˚C followed by 35 cycles for 30 second at 94 ˚C, 30 seconds at 58 ˚C, 1 min at 72 ˚C, and a final step for 10 min at 72 ˚C. The amplified DNA was purified with 30% PEG 8000/30 mmol/l $MgCl_2$ solution. The nucleotide sequences of the PCR products were determined on both strands using a BigDye Terminator Cycle sequencing kit (Applied Biosystems) and an ABI 377 automated sequencer (Applied Biosystems). The sequences of both strands were confirmed to be the same nucleotide pattern using the DNASIS program (version 3.6; Hitachi Software Engineering Co. Ltd.) and the sequence between the primers was determined.

Sequence alignment and phylogenetic analysis: The sequences of 5' EF-1α were aligned using CLUSTAL W (THOMPSON et al., 1994) within Mega 4.0 (TAMURA et al., 2007) at default settings. The phylogeny of *Metarhizium* was estimated by the sequence data set of EF-1α for 40 isolates and the 37 data of BISCHOFF et al. (2009) from Genbank. *M. lepidiotae* ARSEF 7488 was used as an outgroup because this strain was placed as a sister to the monophyly composed of the MGT clade and PARB clade in the phylogenetic analysis of BISCHOFF et al. (2009).

Bayesian analysis was conducted using MrBayes 3.1.2 (RONQUIST and HUELSENBECK, 2003). MrBayes was run with two times with each run including 4 mcmc chains for 1,000,000 generations, sampling every 100 generations for a total of 20,002 trees. The first 25% of the resulting trees (5,002 trees) were discarded (burn in), and a 50% majority rule consensus tree was then calculated from the remaining trees (Fig. 1). Neighbor-joining (NJ) analysis with a Maximum Composite Likelihood option (TAMURA et al., 2004) was also done on the same alignment (tree not shown) using Mega 4.0 (TAMURA et al., 2007) and the branch support was estimated by bootstrap analysis (FELSENSTEIN, 1985) based on 1000 bootstrap replicates.

Conidial measurements: Conidia of each isolate were grown on PDA media at 25 ˚C in the dark for about 2 weeks. Morphological observations of cultured isolates were made with 7 isolates of the MGT clade. Conidia of each isolate were mounted in a drop of 0.05% Tween 80 solution and observed using a Nikon Microscope Optiphot-2 equipped with a Nikon COOLPIX 4500 digital camera. Twenty randomly selected conidia were measured in each specimen and the mean values were calculated. The minimum and maximum values are given in parentheses (Table 1).

RESULTS

Phylogenetic analysis

The sequences of 5' EF-1α were determined for 57 *Metarhizium* isolates from soil in Japan. From the results of Bayesian analysis, 7 isolates were grouped in the MGT clade with 4 and 3 isolates classified as *M. guizhouense* and *M. majus*, respectively, based on the taxonomy of BISCHOFF et al. (2009) (Fig. 1). All of the other isolates were included in the PARB clade. Regarding these points, the Bayesian tree showed no conflict with the topology of the tree obtained using the NJ method.

Table 1. Isolates classified into the MGT clade in this study.

Taxon	Isolate	Location	Size of conidia (μm) Length x Width
M. guizhouense	Hkd25-2	Hokkaido	12.2 (9.8-15.1) x 4.0 (3.6-4.7)
	Kgs15-1	Kagoshima	9.0 (6.7-10.8) x 3.5 (3.1-4.0)
	Myg2-1	Miyagi	7.0 (6.0- 7.8) x 2.7 (2.4-3.0)
	Sag6-1	Saga	9.2 (6.5-10.4) x 3.3 (2.8-4.5)
M. majus	Kkj2-1	Kagoshima, Kikai island	9.2 (7.2-10.2) x 3.3 (2.9-3.6)
	Oit8-3	Oita	12.4 (8.9-15.1) x 3.1 (2.7-3.6)
	Sag14-1	Saga	12.7 (9.3-15.8) x 2.8 (2.0-3.7)

Morphological observation

The conidia of the 7 MGT clade isolates were observed and measured (Table 1) using a light microscope. Based on the average size of conidia, Kgs15-1, Kkj2-1 and Sag6-1 were nearly identical sizes, and so were Oit8-3 and Sag14-1. Hkd25-2 was the largest in width of conidia. The size of conidia from Myg2-1 was the smallest. The conidia of MGT clade except Myg2-1 were obviously larger than those of other isolates.

DISCUSSION

In the phylogenetic analysis of the *M. anisopliae* complex of Bischoff et al. (2009), all of the isolates with relatively large conidia were grouped into the MGT clade. In this clade, two species were defined based on the sequence analysis of the EF-1α region, which was congruent with conidial morphology: *M. majus* was the largest and *M. guizhouense* was the second largest in terms of conidial size. Although sequence analysis of RNA polymerase II second largest subunit gene and the beta tubulin gene contradicted the monophyly of the two taxa, they were accepted as a species because of the congruence of the morphology and EF-1α phylogeny. In this study, we did phylogenetic analysis of the 5' EF-1α region of *Metarhizium* spp. isolated from soil in Japan. As in the above study, our result showed that 6 isolates with obviously large conidia were included in MGT clade. However, *M. majus* Kkj2-1 had almost the same conidial dimensions as two *M. guizhouense* isolates (Kgs15-1 and Sag6-1). In addition, the conidia of *M. guizhouense* Hkd25-2 were larger than that of the two *M. guizhouense* isolates and were relatively close to two *M. majus* isolates (Oit8-3 and Sag14-1) in conidial length. These results indicated that conidial morphology was incongruent with sequence of 5' EF-1α. As mentioned in Bischoff et al. (2009), more detailed phylogenetic study of the MGT clade is needed to further evaluate the status of *M. guizhouense* and *M. majus*.

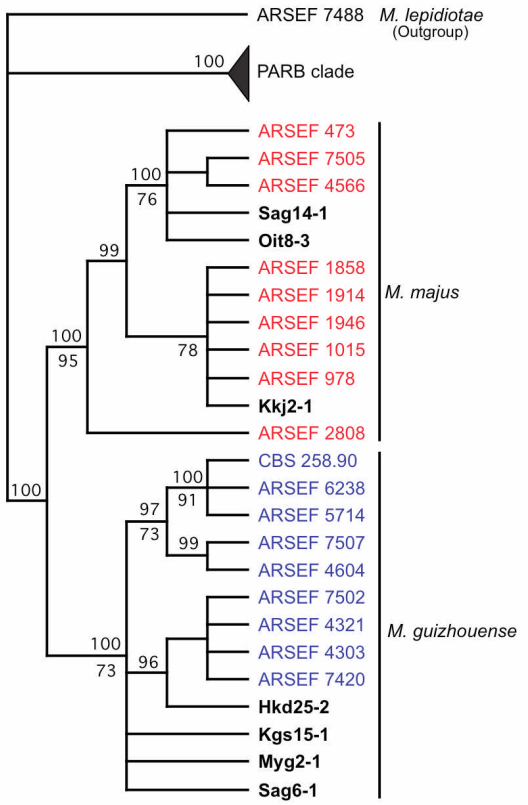

Fig. 1. Majority rule consensus phylogeny of the genus *Metarhizium anisopliae* complex constructed from the Bayesian analysis of the 5' end of EF-1α for 57 isolates from soil in Japan and 37 reference strains from Genbank. ARSEF 7488 (*M. lepidiotae*) is an outgroup. The PARB clade is compressed. Taxa of the reference strains classified into *M. guizhouense* and *M. majus* in Bischoff et al. (2009) are shown in blue and red, respectively. Taxa of the isolates in this study are shown in bold font. The numbers above and below the branch represent the posterior probability (percent) of bayesian analysis (values greater than 95% are shown) and the proportion (percent) of 1,000 bootstrap replicates calculated by the neighbor-joining method (values greater than 70% are shown) for each branch, respectively.

Further research may prove the congruence between phylogeny and conidial size, but it is possible that the morphology is indifferent to phylogeny because the

conidial size of fungi sometimes changes due to polyploidy. It has been reported for some fungal species that diploidization causes large conidia. As for *Verticillium dahliae*, a species of phytopathogenic fungus, the majority of long spored isolates possessed nearly double the nuclear DNA content of short-spored isolates and was referred to as near-diploid, allodiploid or amphidiploid (Karapapa et al., 1997; Collins et al., 2003). Most of the isolates in the MGT clade in Bischoff et al. (2009) were morphologically classified as *M. anisopliae* var. *majus* according to Tulloch (1976), which was a large spored variety of *M. anisopliae* that was implied to be diploid in some studies (St. Leger et al., 1992; Pipe et al., 1995). In addition, Magoon and Al-Aidroos (1984) reported that the conidia of forced diploids of *M. anisopliae* var. *anisopliae* produced in a laboratory were larger than those of the parent strains. These studies support the idea that the large conidia of the isolates in the MGT clade originate from diploidization. However, St. Leger et al. (1992) found that a forced diploid of *M. anisopliae* var. *anisopliae* and its parent strain was indistinguishable by the size of conidia. In any case, it is necessary to directly examine the polyploidy of *Metarhizium* to gain further understanding of the relation between morphology and phylogeny in the MGT clade.

M. anisopliae var. *majus* is characterized by its long conidia and has long been accepted as a specialist of coleopteran insect. In the study of Bischoff et al. (2009), all of the isolates with relatively large conidia belonged to the MGT clade and all of the *M. majus* isolates were originated from coleopteran insect (or soil) other than ARSEF 1015, an isolate from the silkworm, *Bombyx mori*. These results imply that there are lineages of specialist pathogens of coleopteran insects in the MGT clade. *M. anisopliae* var. *majus* has been studied by pathogenic assay in a laboratory and exhibited obviously low pathogenicity toward non-coleopteran insects compared to *M. anisopliae* var. *anisopliae*, such as *Nilaparvata lugens* (Samuels et al., 1988) and *Bombyx mori* (Kawakami and Naka, 1979). From these results, laboratory pathogenic assay may be effective to identify specialist and generalist lineages in the MGT clade. It is important to clarify the evolutionary location of host specific strains to understand the evolution of host specificity and the factors associated with host range. Although *M. acridum* was a phylogenetically well-resolved specialist species (Bischoff et al., 2009; Driver et al., 2000) and was used as a specialist pathogen compared to *M. anisopliae* var. *anisopliae* as a generalist pathogen in a comparative study of virulence gene using microarray (Wang et al., 2009), a comparison of pathogenicity with evolutionarily closer strains with different host ranges will show more detailed differences. The MGT clade possibly includes desirable strains for such experiment and further evolutionary study within the clade is needed.

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