Metarhizium bibionidarum and M. purpureogenum: new species from Japan

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Title

Metarhizium bibionidarum and *M. purpureogenum* present new species from Japan

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Key words

Green muscardine, entomopathogenic fungi, phylogenetic analysis, pigments

Abstract

Two new species of *Metarhizium*, *M. bibionidarum* and *M. purpureogenum* are newly described from Japan. *Metarhizium bibionidarum* is the phylogenetic sister species of *M. pemphigi* and a member of the *M. flavoviride* species complex. It is distinguished morphologically from *M. pemphigi* by its larger conidia. The species is based on a collection of an infected March fly larva (Diptera: Bibionidae) but is also known to occur on fruit beetle (Coleoptera: Scarabaeidae) encountered in France. *Metarhizium purpureogenum* was isolated from soil by plating and insect baiting methods and representes a unique phylogenetic lineage placed outside of the *M. anisopliae* and *M. flavoviride* species complexes. Three isolates of *M. purpureogenum* excreted a distinctive red-purple pigment into agar medium when co-cultured with *M. robertsii* or *Aspergillus oryza*e.

Fig. 1

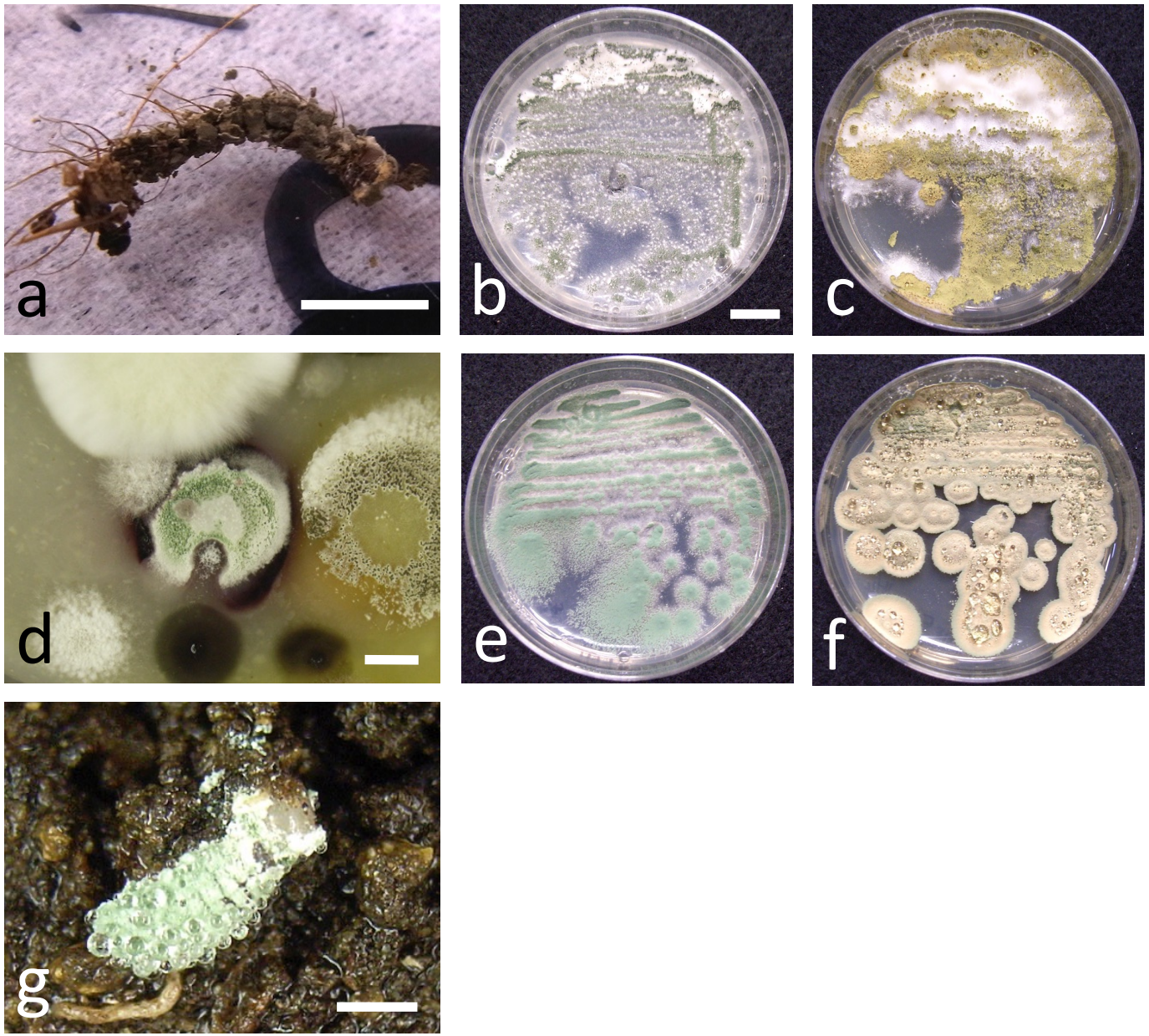

Fig. 2

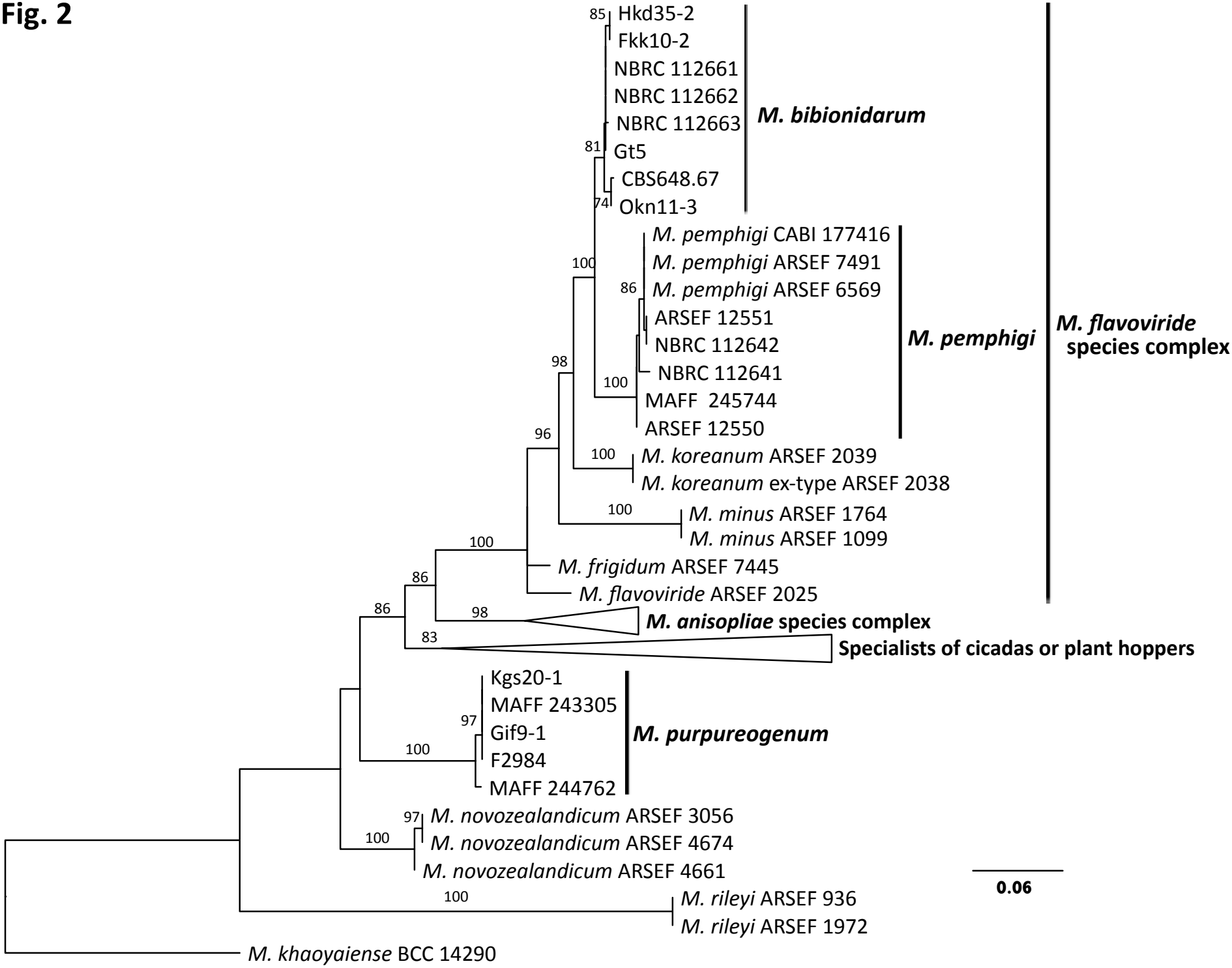
Hkd35-2
Fkk10-2
NBRC 112661
NBRC 112662
NBRC 112663
M. bibionidarum
Gt5
CBS648.67
Okn11-3
M. pemphigi CABI 177416
M. pemphigi ARSEF 7491
M. pemphigi ARSEF 6569
ARSEF 12551
NBRC 112642
NBRC 112641
MAFF 245744
ARSEF 12550
M. pemphigi
M. flavoviride species complex
M. koreanum ARSEF 2039
M. koreanum ex-type ARSEF 2038
M. minus ARSEF 1764
M. minus ARSEF 1099
M. frigidum ARSEF 7445
M. flavoviride ARSEF 2025
M. anisopliae species complex
Specialists of cicadas or plant hoppers
Kgs20-1
MAFF 243305
Gif9-1
F2984
MAFF 244762
M. purpureogenum
M. novozealandicum ARSEF 3056
M. novozealandicum ARSEF 4674
M. novozealandicum ARSEF 4661
M. rileyi ARSEF 936
M. rileyi ARSEF 1972
M. khaoyaiense BCC 14290
0.06

Fig. 3

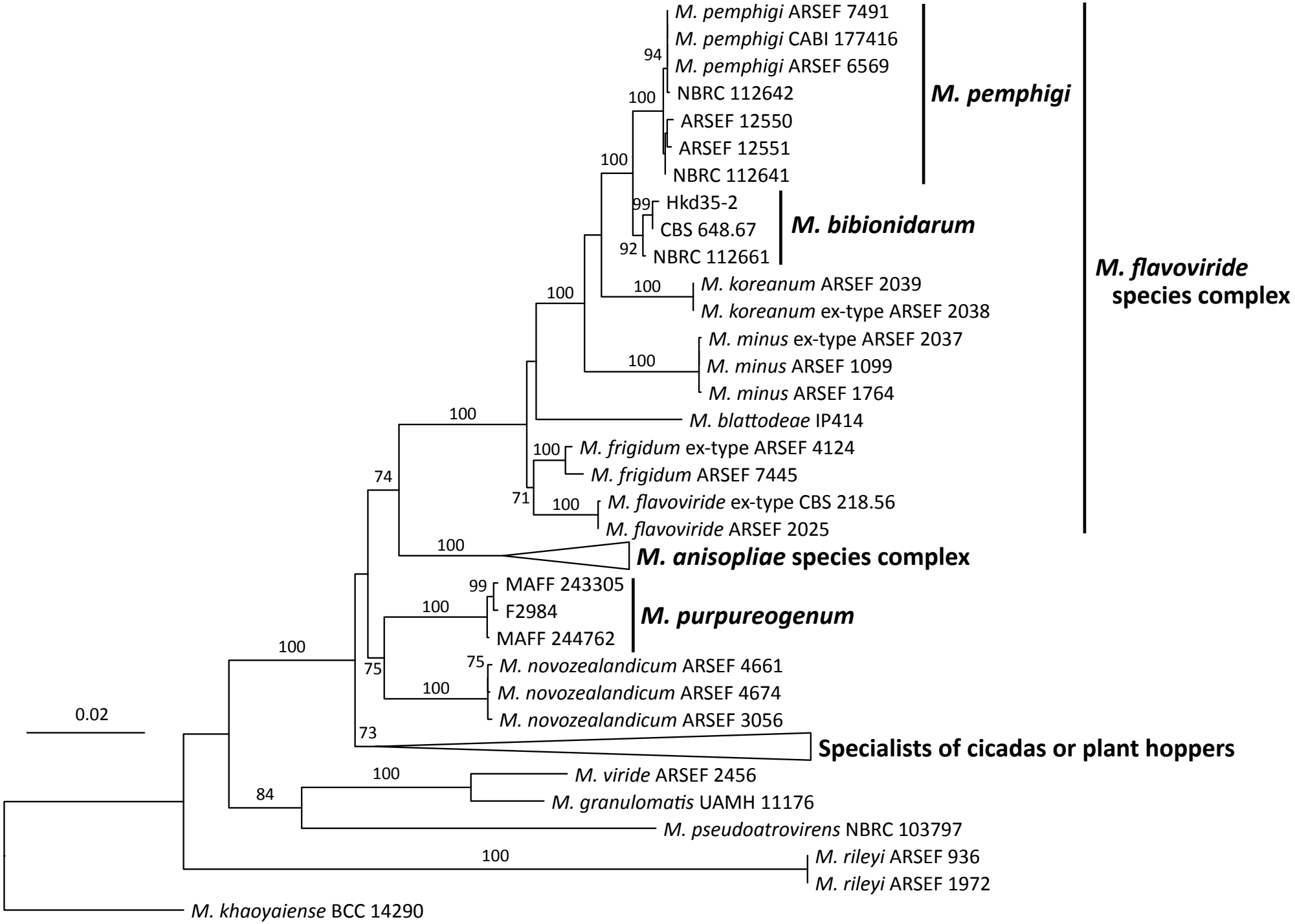
M. pemphigi ARSEF 7491
M. pemphigi CABI 177416
M. pemphigi ARSEF 6569
NBRC 112642
ARSEF 12550
ARSEF 12551
NBRC 112641
M. pemphigi
Hkd35-2
CBS 648.67
NBRC 112661
M. bibionidarum
M. flavoviride species complex
M. koreanum ARSEF 2039
M. koreanum ex-type ARSEF 2038
M. minus ex-type ARSEF 2037
M. minus ARSEF 1099
M. minus ARSEF 1764
M. blattodeae IP414
M. frigidum ex-type ARSEF 4124
M. frigidum ARSEF 7445
M. flavoviride ex-type CBS 218.56
M. flavoviride ARSEF 2025
M. anisopliae species complex
MAFF 243305
F2984
MAFF 244762
M. purpureogenum
M. novozealandicum ARSEF 4661
M. novozealandicum ARSEF 4674
M. novozealandicum ARSEF 3056
0.02
Specialists of cicadas or plant hoppers
M. viride ARSEF 2456
M. granulomatis UAMH 11176
M. pseudoatrovirens NBRC 103797
M. rileyi ARSEF 936
M. rileyi ARSEF 1972
M. khaoyaiense BCC 14290

Fig. 4

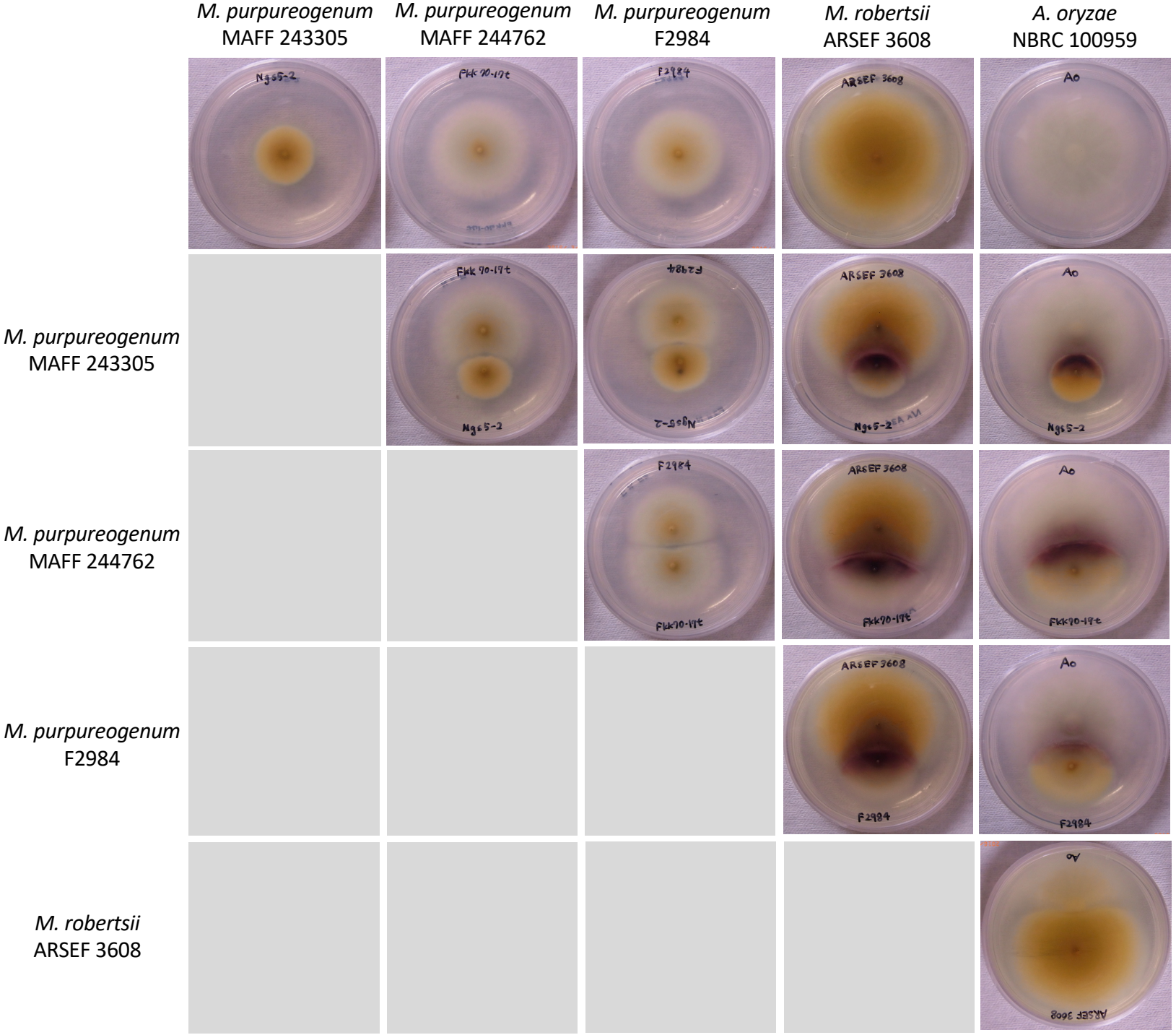
M. purpureogenum MAFF 243305
M. purpureogenum MAFF 244762
M. purpureogenum F2984
M. robertsii ARSEF 3608
A. oryzae NBRC 100959
M. purpureogenum MAFF 243305
M. purpureogenum MAFF 244762
M. purpureogenum F2984
M. robertsii ARSEF 3608

Fig. 5

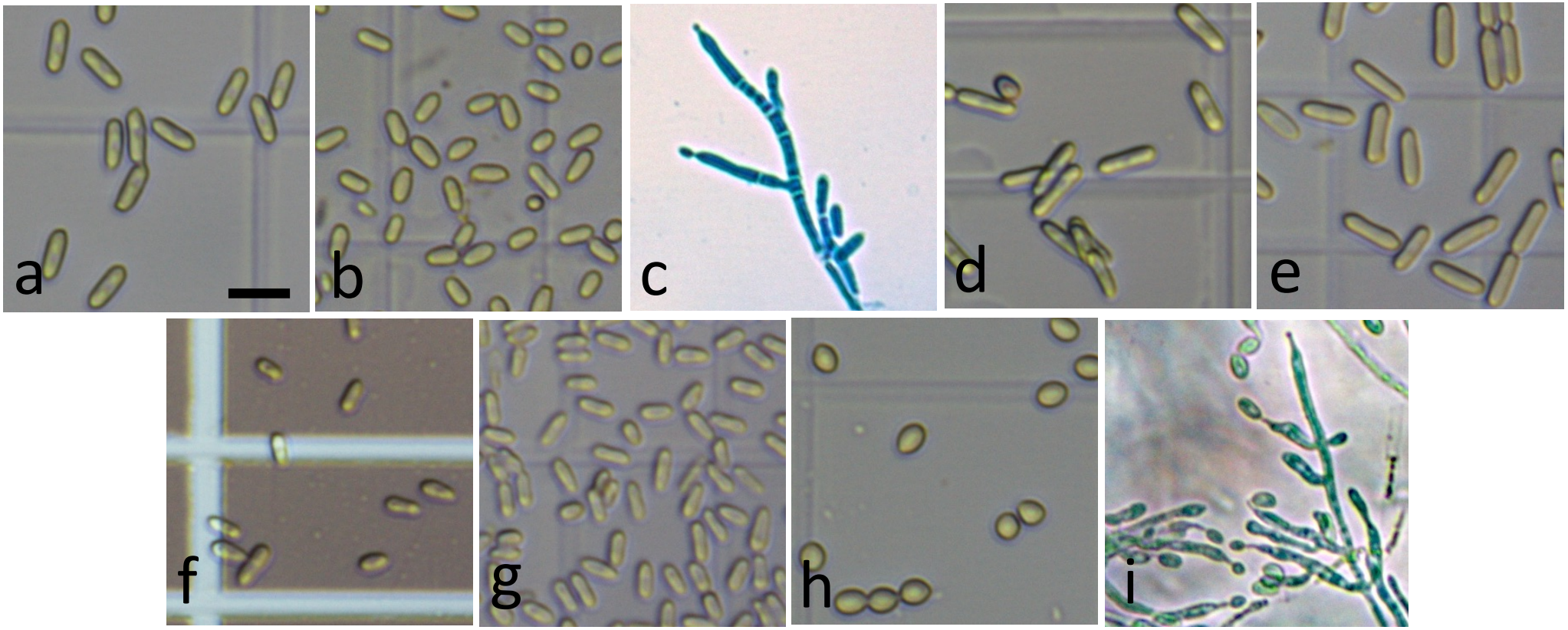

Table 1. Number of soil samples from which *Metarhizium* spp. were recovered in different prefectures in Japan.

Prefectures	**Number of soil samples from which *Metarhizium* spp. were recovered [a]**							
	Plating method				**Bait method with termites**			
	Total	***Metarhizium* spp.**	***M. bibionidarum***	***M. purpureogenum***	**Total**	***Metarhizium* spp.**	***M. bibionidarum***	***M. purpureogenum***
Hokkaido	28	15	1		0			
Aomori	2	1			0			
Miyagi	2	1			0			
Chiba	2	2			0			
Tokyo	2	1			0			
Nara	2	1			0			
Gifu	3	3		1	0			
Hiroshima	1	1			0			
Tottori	3	2			2	1		
Yamaguchi	2	2			1	1		
Shimane	4	3			3	3		
Okayama	3	2			0			
Fukuoka (Mt. Tachibana)	82	65		10	19	16		1
Fukuoka (others)	29	14	1	4	15	10		1
Saga	14	10			0			
Oita	16	10			0			

Nagasaki	11	8		1	0			
Kumamoto	14	8			0			
Miyazaki	14	8			0			
Kagoshima	28	24		4	11	7		1
Yakushima island	10	9			0			
Kikaijima island	4	2			0			
Okinawa	9	7	1		9	5		
Total	285	199	3	20	60	43	0	3

[a] Gt5 and F2984 are not included here because the two strains were isolated in the context of other studies.

[b] Species identification of nineteen of the 23 isolates of *M. purpureogenum* were based on conidial shape and production of a red-purple pigment.

Table 2. Isolates used in this study.

Species	Isolate code	Isolate Host/Substrate (habitat type of soil samples)	Location	Genbank Accession Numbers				
				5TEF	3TEF	BTUB	RPB1	RPB2
M. bibionidarum	NBRC 112661 [T]	Diptera: Bibionidae	Japan, Tokyo	LC187676	LC126076	LC126066	LC125908	LC125924
	NBRC 112662	Diptera: Bibionidae	Japan, Tokyo	LC187677	–	–	–	–
	NBRC 112663	Diptera: Bibionidae	Japan, Tokyo	LC187678	–	–	–	–
	CBS648.67	Coleoptera: Scarabaeidae: *Cetonia aurata*	France	AB807450	LC126075	LC126065	LC125907	LC125923
	Hkd35-2	Soil (forest)	Japan, Hokkaido	AB807804	LC126077	LC126067	LC125912	LC125925
	Okn11-3	Soil (forest)	Japan, Okinawa	AB807452	–	–	–	–
	Fkk10-2	Soil (forest)	Japan, Fukuoka	AB808474	–	–	–	–
	Gt5	Soil (habitat type is not clear)	Japan, Gifu	AB807451	–	–	–	–
M. purpureogenum	MAFF 243305 [T] (ARSEF 12571)	Soil (lawn)	Japan, Nagasaki	LC187674	LC126078	LC126068	LC125913	LC125920
	MAFF 244762	Soil (forest), with a	Japan, Fukuoka	LC187671	LC126079	LC126070	LC125911	LC125922

	(ARSEF 12570)	termite as a bait						
	Gif9-1	Soil (forest)	Japan, Gifu,	LC187672	–	–	–	–
	F2984	Soil (forest)	Japan, Kumamoto	LC187675	LC200452	LC126069	LC125910	LC125921
	Kgs20-1	Soil (forest)	Japan, Kagoshima	LC187673	–	–	–	–
M. pemphigi	ARSEF 6569	Hemiptera: Apididae	Britain	KJ398813	KJ398813	KJ398586	DQ468363	DQ468378
	NBRC 112641	Hymenoptara: Tenthredinidae	Japan	LC188955	LC126071	LC126061	LC125914	LC200451
	NBRC 112642	Hymenoptara: Formicidae	Japan	LC188954	LC126072	LC126062	LC125909	LC125919
	MAFF 245744	Hymenoptara: Vespidae	Japan, Ibaraki	LC188994	–	–	–	–
	ARSEF 12551 (Yks3-2)	Soil (forest)	Japan, Yakushima	AB807454	LC126074	LC126064	LC125916	LC125917
	ARSEF 12550 (Hkd27-1)	Soil (forest)	Japan, Hokkaido	AB807457	LC126073	LC126063	LC125915	LC125918
M. anisopliae	ARSEF 549	not clear	Brazil	–	–	–	–	–
M. brunneum	ARSEF 3297	Acari: Ixodidae	Mexico	–	–	–	–	–
M. robertsii	ARSEF 3608	Coleoptera: Curculionidae	USA	–	–	–	–	–

Aspergillus oryzae	NBRC 100959	broad bean	Japan, Kyoto	–	–	–	–	–

[T] ex-type strain

Table 3 Species clade support values for *M. bibionidarum* and *M. purpureogenum* from analyses of each of the four gene regions (BTUB, RPB1, RPB2, and 3TEF) sequenced.

Locus	**Support values (ML BS[a], BI PP[b])**	
	M. bibionidarum	***M. purpureogenum***
BTUB	98, 0.99	100, 1.00
RPB1	66, 0.20	100, 1.00
RPB2	55, 0.47	100, 1.00
3TEF	81, 0.98	98, 0.99

[a] Proportions of bootstrap replicates (n=1000) in the ML analysis

[b] Posterior probabilities calculated by the Bayesian analysis

Table 4. Comparisons of the sizes of conidia of *M. bibionidarum* and *M. pemphigi*.

Species	**Strains**	**qSDAY**		**PDA**		**Difference of conidial length between conidia produced on qSDAY and PDA** [b]
		Length (±S.D.) [a]	Width (±S.D.)	Length (±S.D.) [a]	Width (±S.D.)	
M. bibionidarum	CBS 648.67	9.7±1.0 [A]	2.7±0.2	9.7±0.8 [A]	2.5±0.2	n.s. ($p = 0.926$)
	NBRC 112661	5.2±0.5 [C]	2.6±0.2	7.6±0.7 [B]	2.7±0.2	significant ($p < 0.001$)
	Hkd35-2	9.1±2.0 [A]	2.4±0.2	9.0±0.8 [A]	2.6±0.3	n.s. ($p = 0.680$)
	Okn11-3	6.8±0.5 [B]	2.8±0.2	8.8±1.0 [A]	2.7±0.3	significant ($p < 0.001$)
M. pemphigi	ARSEF 6569	5.2±0.6 [C]	2.2±0.2	5.4±0.8 [C]	2.4±0.2	n.s. ($p = 0.324$)
	NBRC 112641	5.7±0.6 [C]	2.4±0.2	5.6±0.5 [C]	2.3±0.2	n.s. ($p = 0.745$)
	NBRC 112642	5.6±0.6 [C]	2.1±0.3	5.9±0.6 [C]	2.3±0.2	n.s. ($p = 0.094$)
	MAFF 245744	5.3±0.6 [C]	2.2±0.2	5.7±0.5 [C]	2.0±0.2	n.s. ($p = 0.023$)

[a] Lengths of conidia among the same column followed by the same letters are not significantly different ($p > 0.01$) after adjustment for multiple comparison test using Holm's method.

[b] Conidial length produced on qSDAY and PDA were compared by Welch's t test (n=20).

61 Introduction

62 The genus *Metarhizium* (Ascomycota, Hypocreales, Clavicipitaceae) is largely composed of
63 entomopathogenic fungi that mostly produce green conidia on the corpses of their arthropod hosts and is
64 known as “green muscardine fungus” (Kepler et al. 2014). *Metarhizium* spp. are generally found as
65 anamorphs on more than 200 host species that belong to 17 families of Insecta and Acari (Roberts and St.
66 Leger 2004; Zimmermann 2007). Several species have been isolated from soil where they often exhibit a
67 close association with plant roots (Hu and St. Leger 2002; Nishi et al. 2011; Wyrebeck et al. 2011). Species
68 in this genus are used as biological control agents against various pests in agriculture and forestry and also
69 human disease vectors (e.g., Zimmermann 1993; Milner and Pereire 2000; Lomer et al. 2001). Of the
70 biopesticides that are made up of entomopathogenic fungi, 33.9% are based on strains of *Metarhizium*
71 (Faria and Wraight 2007). Species of *Metarhizium* are also used for soil bioremediation or as producers of
72 bioactive compounds (Lee et al. 2005; Kikuchi et al. 2009; Kozone et al. 2009; Rhee et al. 2012).

73 According to the latest taxonomic revision of the genus by Kepler et al. (2014), some
74 *Metarhizium* species can be pathogenic to reptiles. The genus is phylogenetically closely related to
75 pathogens of nematodes and rotifers. Including the here described, at least 35 species can currently be
76 identified on the basis of DNA sequence information. Species belonging to the PARB clade defined by
77 Bischoff et al. (2009) (*M. pingshaense*, *M. anisopliae*, *M. robertsii*, and *M. brunneum*) have a wide host
78 range and a global distribution and often comprise majority groups among *Metarhizium* spp. isolated from
79 soil in several areas (e.g., Nishi et al. 2011; Wyrebek et al. 2011; Rocha et al. 2013; Steinwender et al. 2014).
80 Other species are less routinely encountered and their host associations are insufficiently known.

Comparative genomics and phylogenetic analyses suggested that the generalist pathogens in the PARB clade have evolved relatively recently from specialist lineages, and this process was accompanied by emergence of species with intermediate host range (Hu et al. 2014).

Improving our understanding of *Metarhizium* species diversity by investigating new isolates may be beneficial for pest management. *Metarhizium* in East Asia seems to be particularly phylogenetically diverse. All described teleomorphs were encountered in this region, and the species number of *Metarhizium* soil isolates previously identified in Japan was larger than that in Brazil, Canada, and Denmark (Nishi et al. 2011; Wyrebek et al. 2011; Kepler et al. 2012; Rocha et al. 2013; Steinwender et al. 2014). Here, we investigated new isolates of *Metarhizium* in Japan to reveal an extensive diversity of this genus and discovered two new species, *M. bibionidarum* and *M. purpureogenum*. This article describes their phylogenetic affinities within the genus and their morphological and cultural traits.

Materials and Methods

Fungal strains

The fungal strains used in this study are listed in Table 1. Isolates used in the study are deposited in culture collections such as ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA), MAFF (Genebank of the National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan), and NBRC (Biological Resource Center of National Institute of

101 Technology and Evaluation, Kisarazu, Chiba, Japan).

102 Strains were isolated by using soil plating or bait methods with subterranean worker termites
103 (*Reticulitermes speratus*). A total of 297 soil samples were collected from 20 prefectures in Japan, which
104 ranged from 26 to 45°N (Table 2). The plating method was applied to 285 of the samples with an oatmeal-
105 based semi-selective agar medium (6% fine oat flour, 1.25% agar, 0.03% chloramphenicol, 0.1%
106 cycloheximide) (Nishi et al. 2011). The bait method was applied to 60 samples, 48 of which were also used
107 for the plating method. Twenty-four workers were used for one soil sample. Termites collected in Fukuoka,
108 Japan, were maintained in plastic boxes on half-dried wood in a dark chamber at 25˚C; selected worker
109 termites were placed into plastic boxes with moistened filter paper and maintained in a dark chamber at
110 25˚C for one to three weeks. Twenty-four aliquots of a soil sample were taken and placed into wells of a
111 24-well cell culture plate up to approximately 3 mm in depth. A single termite was released into each well
112 and the plates were maintained at 25˚C in the dark. The soil sub-sample in each well was moistened with
113 sterile distilled water every other day. Mortality was checked daily for 14 days; cadavers were inspected
114 daily for the presence of aerial mycelia until aerial mycelia was observed or cadavers became brown and
115 melted and all potential fungal pathogens were observed microscopically. Conidia or mycelia of these
116 isolates were transferred to potato dextrose agar medium (PDA, 2.1% dextrose, 1.4% agar, 0.4% potato
117 extract, 0.03% chloramphenicol) and morphologically identified as *Metarhizium* species. To investigate
118 fungi that the termites originally possessed, control experiments were performed where termites were
119 incubated on small pieces of sterilized filter paper (approximately 7×7 mm, two pieces per well) or on soil
120 sterilized through autoclaving (121˚C, 20 min). No aerial mycelia were observed on termites used in the

121 control experiments.

122

123 DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

124 DNA extraction was conducted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany)
125 or the protocol of Nishi et al. (2011). Partial regions of the following genes were used in this study and
126 amplified and sequenced with primers described in Supplementary Table S1: beta-tubulin (BTUB), RNA
127 polymerase II largest subunit (RPB1), RNA polymerase II second largest subunit (RPB2), a 3′ partial region
128 of elongation factor 1-alpha (3TEF), and a 5′ partial region of elongation factor 1-alpha (5TEF). 5TEF was
129 analyzed for species identification and discrimination. The other four loci were analyzed for the
130 clarification of phylogenetic placements of new species. New primers used for the PCR amplification of
131 these loci were designed with the aid of ApE-A plasmid Editor v1.17
132 (http://biologylabs.utah.edu/jorgensen/wayned/ape/) and Amplify 3 for MacOS X v3.1.4
133 (http://engels.genetics.wisc.edu/amplify/) to obtain difficult to amplify product.

134 PCR was performed in 10–20-μl reaction volumes comprising approximately 0.1–20 ng of
135 genomic DNA, 1× PCR buffer (KOD FX Neo, Toyobo, Osaka, Japan), 0.2 mM dNTPs, 20 μM of each
136 primer, and 0.02 U/μl DNA polymerase (KOD FX Neo). The amplification conditions were initial
137 denaturation for 2 min at 94°C, followed by 35–38 cycles for 10 s at 98°C, annealing for 30 s at 50–54°C,
138 elongation for 50–130 s at 68°C, and a final holding step for 7 min at 68°C (for locus specific conditions,
139 see Supplementary Table S2).

140 The primers were removed from the amplified DNA solutions by the polyethylene glycol

141 precipitation method (Nakayama and Nishikata, 1995). The nucleotide sequences of the PCR products were
142 determined using a BigDye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA)
143 and an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Edited sequences were
144 deposited under accession numbers listed in Table 1.
145
146 Phylogenetic analyses
147 The following phylogenetic analyses were conducted in accordance with Bischoff et al. (2009)
148 and Kepler et al. (2014). 5TEF was used as a general DNA barcode for species identifications and to infer
149 currently undescribed taxa. The dataset of 5TEF consisted of 54 isolates, including 43 reference isolates.
150 The datasets of the other four loci consisted of 55 isolates, including 45 reference isolates. Sequences of
151 reference isolates (Supplementary Table S3) were from Bischoff et al. (2006, 2009), Kepler et al. (2012,
152 2014), Nishi et al. (2011, 2013), and Montalva et al. (2016). The four loci were first analyzed separately to
153 test congruency of single gene based genealogies and then analyzed as combined dataset. The sequence
154 data of the reference isolates were selected from the core *Metarhizium* clade described by Kepler et al.
155 (2014). *Metarhizium khaoyaiense* BCC 14290 was designated as an outgroup of the core *Metarhizium* clade.
156 Sequence alignment of the two datasets was conducted using the default settings in MUSCLE (Edgar 2004)
157 as implemented in MEGA6.0 (Tamura et al. 2013). The alignment length of the 5TEF dataset was 772 bp
158 and included exon regions and introns. Data used aligned to bp positions 57 and 716 of the 1852 bp long
159 TEF of *M. robertsii* (GenBank: ADNJ02000003.1, MAA_03797). For analyzing the other four loci,
160 identified intron regions were removed from the alignments. Alignment lengths for BTUB, RPB1, RPB2,

and 3TEF were 656, 569, 1029, and 756 bp, respectively. Data used aligned to bp positions 596 and 1251 of the 1767 bp long BTUB (*M. robertsii*, ADNJ02000001.1, MAA_02081), 340 and 357 and 430 and 980 of the 5408 bp long RPB1 (ADNJ02000007.1, MAA_00658), 1366 and 2394 of the 3898 bp long RPB2 (ADNJ02000003.1, MAA_00336), and 837 and 1592 of the 1852 bp long TEF (ADNJ02000003.1, MAA_03797). The total alignment length was 3,010 bp. The DNA sequences of the four loci were combined with SequenceMatrix–MacOSX–1.7.8.1 (Vaidya et al. 2011). The most appropriate partitioning schemes and substitution models for the two datasets were determined using PartitionFinder program using the greedy search option (Lanfear et al. 2012). Three data blocks were defined for exons of each of the loci analyzed. They covered the first, second, or, respectively, third codon positions. A fourth datablock was defined for 5TEF that comprised intron regions. The Bayesian information criterion was used to evaluate the partition scheme and the model choice. The selected partition schemes and models are presented in Supplementary Tables S4 and S5.

Maximum likelihood analyses were conducted for 5TEF and the combined dataset using RAxML 7.4.4 program (Stamatakis 2006). The GTR model with a gamma distributed rate variation among sites was used for all defined partitions in accordance with recommendations in the RAxML manual against the use of invariant sites (Supplementary Table S4). The support values for nodes were evaluated by 1,000 bootstrap replicates (Felsenstein 1985).

Bayesian phylogenetic reconstructions were conducted using MrBayes 3.2 program (Ronquist et al. 2012). MrBayes was run twice, and each run included four Markov chain Monte Carlo chains. Trees were sampled every 100 generations until the average standard deviation of split frequencies dropped below

181 0.01. The first 25% of the resulting trees were discarded (burn-in), and posterior probabilities for the
182 branches were calculated from the remaining trees. A 50% majority rule consensus tree was calculated from
183 the remaining trees. The numbers of generations and sampled trees for the individual analyses are presented
184 in Supplementary Table S6.

185 Trees from the two analyses were edited using Figtree (ver. 1.4.2, Rambaut A., Institute of
186 Evolutionary Biology, University of Edinburgh, http://tree.bio.ed.ac.uk/software/figtree/). The results of
187 the multiple sequence alignment and the phylogenetic trees generated for each of the five loci were
188 deposited in TreeBASE (http://treebase.org/treebase-web/home.html) under study number S19952.

189

190 Morphological observations

191 The dimensions of conidia produced on PDA or quarter-strength Sabouraud's dextrose agar
192 medium with 1% yeast extract (2% agar, 1% dextrose, 1% yeast extract, 0.25% peptone, 0.03%
193 chloramphenicol) (qSDAY) (Bischoff et al. 2009) were measured for *M. bibionidarum, M. pemphigi,* and
194 *M. prupureogenum*. The dimensions of four isolates each from *M. bibionidarum* and *M. pemphigi* were
195 compared to confirm whether the two species are morphologically distinguishable. For the measurements,
196 the conidia of each isolate were grown on PDA or qSDAY at 25°C in the dark for 14–21 days. The conidia
197 collected from the agar plates were suspended in sterile distilled water containing 0.05% Tween 80, applied
198 to a slideglass and were observed and captured using a Olympus differential interference contrast
199 microscope (BX-60) equipped with a Cannon EOS KissX2 digital camera.

200 The dimensions of phialides produced on qSDAY were measured for the ex-type strains of *M.*

bibionidarum and *M. prupureogenum*. Conidia of each isolate were first grown on qSDAY for two to seven days for *M. bibionidarum* and two days for *M. prupureogenum*. Small amounts of the agar (approximately 1 mm^3) containing mycelium were picked up using sterile toothpicks, placed and pressed flat on a sterile slide glass, and incubated at highly humid conditions at 25°C for 24–48 h. The small agar blocks were stained and mounted with Mycoperm-Blue (Scientific Device Laboratory, Tokyo, Japan). Phialides were observed and captured using a Nikon differential interference contrast microscope (Optiphot-2) equipped with a Nikon COOLPIX 4500 digital camera.

Twenty randomly selected conidia and phialides from the images were measured for each specimen using ImageJ 1.48v (http://imagej.nih.gov/ij) and min, 95% CI, and max of the dimensions were calculated. Lengths of conidia were compared among the isolates of *M. bibionidarum* and *M. pemphigi* by multiple comparison Welch's t-test with Holm's adjustment. Lengths of conidia produced on PDA and qSDAY were also compared for the isolates by Welch's t-test.

In vitro pigment production by *M. purpureogenum* sp. nov.

During soil isolation of *Metarhizium* using semi-selective agar medium, a red-purple pigmentation was observed to accumulate in the medium below colonies of some isolates described here as *M. purpureogenum* sp. nov. that were in close proximity to colonies of other fungal species. To determine whether pigment production was a constitutive cultural characteristic of this species or a response to the proximity of other fungal species, we evaluated pigment production by *M. purpureogenum* in the absence

or presence of other fungi. For this experiment, *M. purpureogenum* isolates were co-cultivated with *M. robertsii* (ARSEF 3608) or *Aspergillus oryzae* (NBRC 100959). Each isolate was first purely cultured on PDA for 2 days at 25°C in the dark, and mycelia on the agar plates were transferred using sterile toothpicks to a new PDA plate (ø 6 cm). For the co-culture, mycelia of two isolates were inoculated on agar plates at a distance of 1 cm from each other. *Aspergillus oryzae* was inoculated by using 5µl of a conidial suspension (1.0×10^6 conidia/ml). After incubation for 3 weeks at 25°C in the dark, pigment production was evaluated.

Results

Phylogenetic analyses of 5TEF

Phylogenetic analyses by both maximum likelihood and Bayesian methods of 5TEF of *Metarhizium* spp. indicated that *M. bibionidarum* and *M. purpureogenum* were relatively distantly related to currently known species (Fig. 1, Supplementary. S1). The topologies from the two analyses were congruent in terms of the placement of the two new species and their close relative species. *Metarhizium bibionidarum* NBRC 112661 formed a species clade together with two Japanese isolates from March flies (NBRC 112662, 112663), CBS648.67 isolated from France, and four soil isolates from Japan. The clade composed of these isolates belonged to the *M. flavoviride* species complex and formed a strongly supported monophyletic group with *M. pemphigi*. *Metarhizium purpureogenum* MAFF 243305 formed a species clade with four soil isolates from Japan. The clade composed of these isolates was relatively distant from any

241 known *Metarhizium* species or species complex.

242

243 Phylogenetic analyses of BTUB, RPB1, RPB2, and 3TEF

244 Genealogies based on individual loci of BTUB, RPB1, RPB2, and 3TEF equally assigned
245 above mentioned isolates to species clades, although the clade corresponding to the *M. bibionidarum* clade
246 was relatively weakly supported in inferences based on RPB1 and RPB2 (Table 3). *Metarhizium*
247 *bibionidarum* was consistently inferred as a close relative of *M. pemphigi*. However, the phylogenetic
248 placement of *M. purpureogenum* varied among the analyses of the four loci. Based on the concatenated
249 dataset, the clade corresponding to the *M. bibionidarum* lineage received 90% bootstrap support and was
250 placed as a sister species to *M. pemphigi* in the *M. flavoviride* complex (Fig. 2). The *M. purpureogenum*
251 lineage received 100% bootstrap support and was marginally supported as a sister taxon of *M.*
252 *novazealandicum* (75%).

253

254 Isolation of the two new species from soil samples

255 Total *Metarhizium* spp. were recovered from the soil samples at the rate of 69.8 %. The rate of
256 *M. bibionidarum* was 1.1 and that of *M. purpureogenum* was 7.0 % by using the plating method. By using
257 the baiting method, rates were 71.7 % for *Metarhizium* spp. and 5.0 % for *M. purpureogenum* while *M.*
258 *bibionidarum* was not retrieved (Table 2). *Metarhizium bibionidarum* was recovered from three and *M.*

purpureogenum from four different prefectures. *Metarhizium purpureogenum* were relatively frequently recovered from soil samples collected at Mt. Tachibana (Fukuoka, Japan) (12.2 % by the plating method).

Morphology of conidia and phialides

The conidia of *M. bibionidarum* NBRC 112661 were cylindrical to ellipsoid (Fig. 3). Its phialides were cylindrical and had short narrow necks. Typically, similar length ranges of conidia were encountered when isolates were cultured on qSDAY and PDA. However, conidia of *M. bibionidarum* strains (NBRC 112661 and Okn11-3) were significantly longer when formed on PDA compared with conidia formed on qSDAY ($p < 0.01$) (Table 4).

Conidia of *M. bibionidarum* were larger than those of *M. pemphigi* on PDA (Fig. 3 a, f), whereas the difference was less pronounced on qSDAY. The differences in conidial length within species or clades were significant only for *M. bibionidarum* ($p < 0.01$). The shape of conidia was not clearly different in the two species.

The conidia of *M. purpureogenum* MAFF 243305 were ovoid to ellipsoid on both media. The phialides of the isolate were cylindrical with short narrow necks (<2.5 μm) or flask- shaped with long narrow necks (>2.5 μm).

In vitro pigment production by *M. purpureogenum*

No red pigment was observed in pure or dual culture systems of strains of *M. purpureogenum*

(F2984, MAFF 243305, MAFF 244762). A distinct red pigment was formed obviously by these strains in dual culture systems with *M. robertsii* and *A. oryzae*. The pigmentation was observed around the centre of *M. purpureogenum* colonies. The strongest pigmentation by *M. purpureogenum* occurred along the zone of contact with colonies of the co-cultured fungi (Fig. 4).

Taxonomy

Metarhizium bibionidarum O. Nishi, H. Sato, sp. nov. Fig. 3 a–e, Fig. 5 a–c

MycoBank: MB 819797

Etymology: The species epithet for this fungus refers to its original host, a larva of March fly (Bibionidae).

Phialides formed on qSDAY cylindrical, with a short narrow neck, (9.5) 14.5–17.5 (24.5) × (1.5) 2.0–2.0 (2.0) µm. **Conidia** on qSDAY cylindrical to ellipsoid, (4.5) 5.0–5.5 (6.0) × (2.0) 2.5–2.5 (3.0) µm.

Culture characteristics: Colonies on qSDAY at first fluffy and white because of developing mycelium, becoming brownish-yellow because of the colour of conidial masses produced within 10 d when incubated at 25˚C.

Typus: JAPAN (Tokyo). On cadaver of March fly larva, collected in woods on the Hongo Campus of the University of Tokyo, 1993 (HOLOTYPE TNS-F-53529 [dried cadaver]: NBRC 112661 ex-type).

298 Distribution: Known from France and forest environments of five prefectures in Japan, 26˚N
299 (Okinawa) to 43˚N (Hokkaido).
300 Notes: CBS 648.67, an isolate from France originating from the fruit beetle *Cetonia aurata*
301 (Coleoptera: Scarabaeidae), and soil isolates from Japan also belonged to this species. Conidia of CBS
302 648.67 are longer than those of NBRC 112661 when cultured on PDA or qSDAY (Table 4); the dimensions
303 of conidia produced on PDA and qSDAY were different for NBRC 112661 but not for CBS 648.67. Soil
304 isolates Okn11-3 and Hkd35-2 had similar conidial dimensions as NBRC 112661 and CBS 648.67 (Table
305 4).
306
307 ***Metarhizium purpureogenum*** O. Nishi, S. Shimizu, H. Sato, **sp. nov.** Fig. 3 h, i, Fig. 5 d–g
308 MycoBank: MB 819551
309 Etymology: The species epithet for this fungus refers to the nature of this species to produce a
310 red-purple pigment.
311 **Phialides** on qSDAY cylindrical, with a short narrow neck or flask-shaped with a long narrow
312 neck, (7.0) 10.5–13.0 (19.0) × (2.0) 2.0–2.5 (2.5) μm. **Conidia** on qSDAY ovoid to ellipsoid, (4.5) 5.0–5.0
313 (5.5) × (3.5) 3.5–4.0 (4.0) μm.
314 Culture characteristics: Colonies on qSDAY at first white because of developing mycelium,
315 becoming pale ocher or tan because of the colour of conidial masses produced within 10 d when incubated
316 at 25˚C.
317 Typus: Japan. Shimabara, Nagasaki, oatmeal agar culture isolated from a soil sample collected

in grass lawn, 2008 (holotype NIAES 20610, ex-type strain MAFF 243305, ARSEF 12571).

Known distribution; Japan (five prefectures), from soil in forest environments.

Notes: *Metarhizium purpureogenum* isolates excrete a red-purple pigment into the agar in zones of contact with co-cultured other species of fungi (Fig. 4).

Four other soil isolates also belong to this species. MAFF 244762 was isolated from soil by using the insect baiting method with workers of *Reticulitermes speratus* and the others by using the plating method. These isolates are morphologically similar to the ex-type strain MAFF 243305. Conidia of MAFF 244762 and F2984 produced on qSDAY were both ovoid to ellipsoid, and their dimensions were (4.5) 5.0–5.5 (6.0) × (3.0) 3.5–4.0 (4.0) μm and (4.0) 4.5–5.0 (5.5) × (3.0) 3.5–3.5 (4.0) μm, respectively. *Metarhizium purpureogenum* was mostly isolated only sporadically but frequently from soil collected at Mt. Tachibana (Fukuoka, Japan).

Discussion

This study describes two new species of *Metarhizium*, *M. bibionidarum* and *M. purpureogenum*, on the basis of multilocus phylogenetic analyses and phenotypic characteristics that facilitate their distinction from other *Metarhizium* species. Multilocus phylogenetic analyses place *M. bibionidarum* in the *M. flavoviride* complex as the sister to *M. pemphigi*. In the individual phylogenetic analyses of the four loci, the species clade of *M. bibionidarum* clade was relatively weakly supported in the inferences based on RPB1 and RPB2. The lack of phylogenetically informative characters due to a short internodal

time between *M. bibioidarum* and *M. pemphigi* may contribute to the weak support of the species clade especially for RPB1; the number of variable sites in the multiple alignments composed of *M. bibionidarum* and *M. pemphigi* of BTUB, RPB1, RPB2, and 3TEF were 8, 1, 16, and 10, respectively.

The morphological characters support segregation of *M. bibionidarum* from its closest phylogenetic sister taxon because its conidia on PDA are consistently longer than those of *M. pemphigi* (Table 4). *Metarhizium bibionidarum* is, however, difficult to distinguish from other *Metarhizium* species such as *M. majus*, *M. guizhouense*, and *M. flavoviride* on the basis of morphological characteristics only (Bischoff et al. 2006, 2009). Probably therefore, *M. bibionidarum* CBS 648.67 was deposited in the Westerdijk institute erroneously as *M. anisopliae* var. *majus*. Nevertheless, the colouration of conidial masses of *M. bibionidarum* may distinguish this species from *M. majus*, *M. guishouense* and *M. flavoviride* that typically form olive or pale-green conidia (Bischoff et al. 2006, 2009). The phylogenetic analysis of the DNA sequence of 5TEF showed that this region contained enough DNA sequence variations to identify *M. bibionidarum* (Fig. 1, Supplementary Fig. 1).

Metarhizium bibionidarum and *M. pemphigi* may have an equally broad host range as *Metarhizium* species of the PARB clade (Bischoff et al. 2009) because *M. bibionidarum* was isolated from species of Diptera and Coleoptera and *M. pemphigi* from Homoptera (Driver et al. 2000), Coleoptera (Brownbridge et al. 2010), and Hymenoptera (Table 1). The two host insects of *M. bibionidarum* may have a similar ecological niche as their larval stages are litter detrivores (e.g., Frouz 1999; Stefanelli et al. 2014) what may allow *M. bibionidarum* to infect the two different insects. However, *M. bibionidarum* isolates encountered from Diptera and Coleoptera produced differently long conidia on PDA (Table 4). Further

358 research is required to test the hypothesis whether these differences could be interpreted as adaptations to
359 the different host taxa. Already St. Leger et al. (1992) suggested that different *in vitro* nutritional
360 requirements could associate host selection of *Metarhizium* spp.

361 *Metarhizium* spp. are distributed in the soil of various environments (reviewed by Zimmermann
362 2007). In eastern Canada, genetically distinguishable groups of *Metarhizium anisopliae* like taxa were
363 associated with different habitats (Bidochka et al. 2001). Growth temperature characteristics of one of these
364 groups, today classified as *M. brunneum* (Bischoff et al. 2009; Nishi et al. 2013), suggested that its
365 representatives could be adapted to a cooler environmental temperature. A similar ecological preference
366 could apply to *M. bibionidarum* and *M. pemphigi* because their conidia germinated relatively faster at cold
367 conditions (Nishi et al. 2013).

368 *Metarhizium purpureogenum* represents a unique lineage in *Metarhizium* with a weak affinity
369 to *M. novazealandicum*. Available isolates of this species were all recovered from soil and host associations
370 of this new species are unknown. However, successful recovery of *M. purpureogenum* by baiting soil
371 samples with termites, as well as experimental infection of silkworm larvae (unpubl. data) demonstrate its
372 potential as an entomopathogen.

373 *Metarhizium album*, *M. granulomatis*, and *M. minus* produce ovoid to ellipsoid conidia like
374 *M. purpureogenum* (Rombach et al. 1986, 1987; Sigler et al. 2010). *Metarhizium purpureogenum* is
375 distinguished from *M. album* and *M. minus* by its wider conidia because the width of conidia of the two
376 species are typically less than 3 µm. On the other hand, it is difficult to distinguish *M. purpureogenum* from
377 *M. granulomatis* by dimensions of conidia. The colouration of conidial masses may distinguish the two

species because *M. granulomatis* typically forms greenish gray conidia on PDA.

Recently, various metabolites from *Metarhizium* spp. have been identified without references to their insecticidal activity (e.g., Kikuchi et al. 2009; Kozone et al. 2009). This study demonstrated that *M. purpureogenum* produced a red-purple pigment into agar medium in dual cultures with *M. robertsii* and *Aspergillus oryzae*. The pigment could have antifungal properties. The pigment may be a virulence factor also against insects, just like oosporein, a red-purple pigment produced by *Beauveria* sp. (Feng et al. 2015), although *M. purpureogenum* did not visibly produce this pigment in virulence assays against silkworms (unpubl. data). Clarification of the bioactivities and conditions responsible for the production of this red-purple pigment may be helpful for a better understanding of the ecological traits of *M. purpureogenum*.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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499 Figure legends
500
501 Fig. 1 Maximum likelihood tree inferred from 5TEF molecular sequences. The support values were

obtained from 1,000 bootstrap replicates; values >70% are indicated above or below branches. *Metarhizium khaoyaiense* BCC 14290 was used as outgroup. The clades of the *M. anisopliae* species complex (*M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*) and specialists of cicadas or plant hoppers (*M. album*, *M. brasiliense, M. cylindrosporum*, *M. owariense*, and *M. viridulum*) were condensed.

Fig. 2 Maximum likelihood tree inferred from analyses of a concatenated dataset including aligned partial sequences of the BTUB, RPB1, RPB2, and 3TEF genes. The support values were obtained from 1,000 bootstrap replicates, and values >70% are indicated above or below branches. *Metarhizium khaoyaiense* BCC 14290 was used as outgroup. The clades of *M. anisopliae* species complex (*M. anisopliae*, *M. brunneum, M. guizhouense, M. indigoticum*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*) and specialists of cicadas or plant hoppers (*M. album*, *M. brasiliense*, *M. cylindrosporum*, *M. owariense*, and *M. viridulum*) were condensed.

Fig. 3 Micrographs of *Metarhizium bibionidarum* (a–e) *M. pemphigi* (f–g) and *M. purpureogenum* (h, i). a, d, f. Conidia produced on potato dextrose agar. b, e, g, h. Conidia produced on quarter-strength Sabouraud's dextrose agar medium supplemented with 1% yeast extract (qSDAY). c, i. Branched conidiophores and conidia produced on qSDAY. a–c, NBRC 112661; d–e, CBS 648.67; f, g, ARSEF 6569; h, i, MAFF 243305. Scale bar, 10 μm (a), applying to all.

Fig. 4 Illustration of pigments produced by *M. purpureogenum* in pure or dual culture experiments. Horizontally arranged species and isolate specifications refer to colonies in upper parts of dual cultures; vertically arranged specifications to colonies in lower parts of dual cultures. The purple pigment is formed when *M. purpureogenum* is cultured together with *Metarhizium robertsii* and *Aspergillus oryzae*.

Fig. 5 Macrographs of *M. bibionidarum* (a–c) and *M. purpureogenum* (d–g). a. Dried March fly larva (Diptera: Bibionidae) colonized by *M. bibionidarum* and source for isolate (NBRC 112661), b, e. Cultures on potato dextrose agar incubated for 21 d. c, f. Cultures on quarter-strength Sabouraud's dextrose agar medium supplemented with 1% yeast extract incubated for 21 d. d. Selective agar medium used in soil inventories, g. *Reticulitermes speratus* colonized by *M. purpureogenum* used as bait for the isolation of MAFF 244762. a–c. NBRC 112661; d–f, MAFF 243305; g, MAFF 244762. Scale bars, 5 mm (a, d), 1 cm (b, c, e, f), 1 mm (g).

Fig. S1

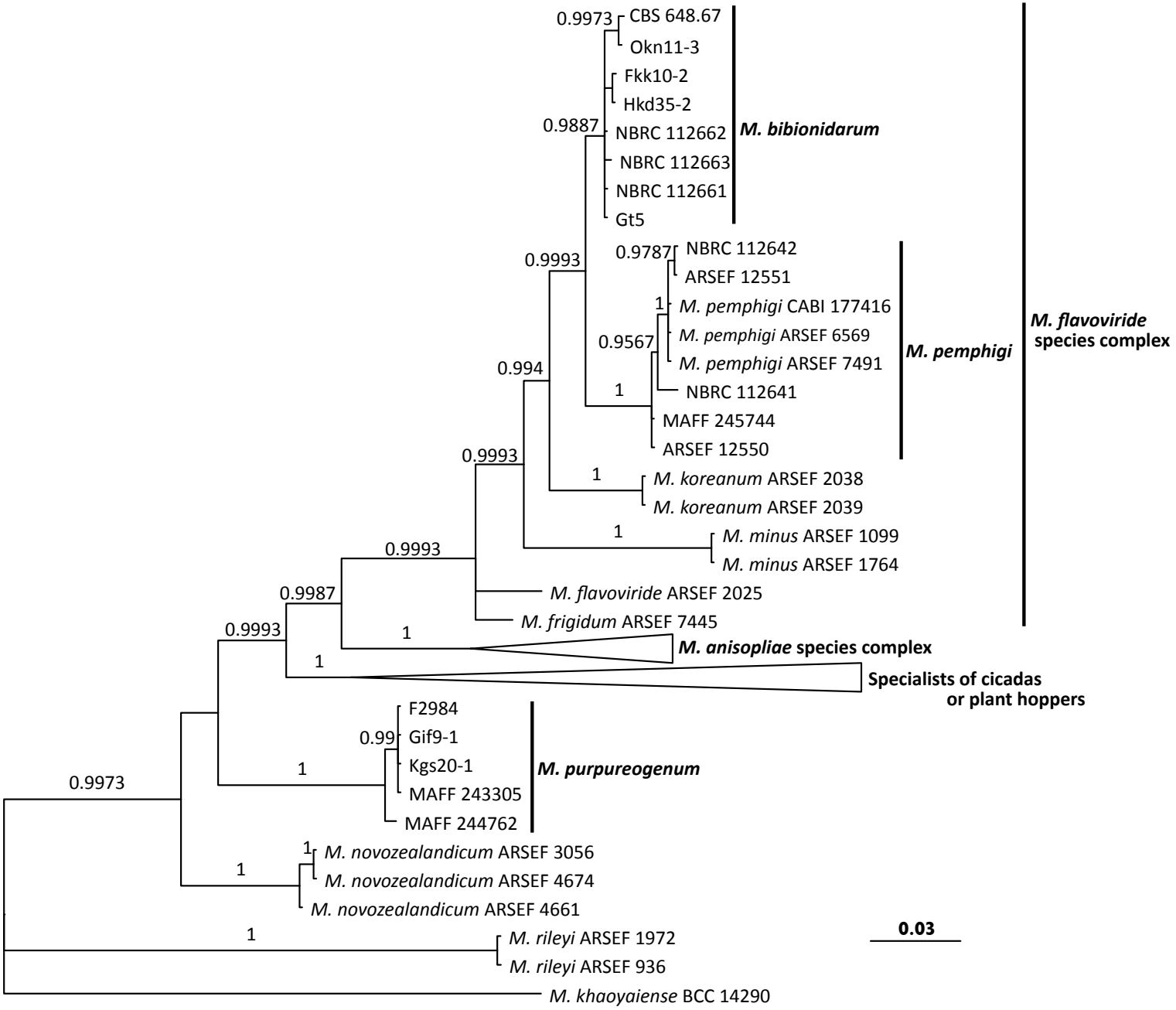

Fig. S1 A 50% consensus phylogenetic tree of 5TEF of *Metarhizium* spp. inferred by a Bayesian analysis. Posterior probabilities of each branch are indicated above branches. *M. khaoyaiense* BCC 14290 was designated as an outgroup. The clades of *M. anisopliae* species complex (*M. anisopliae, M. brunneum, M. guizhouense, M. lepidiotae, M. majus, M. pingshaense, and M. robertsii*) and specialists of cicadas or plant hoppers (*M. album*, *M. brasiliense, M. cylindrosporum*, *M. owariense*, and *M. viridulum*) were condensed.

Fig. S2

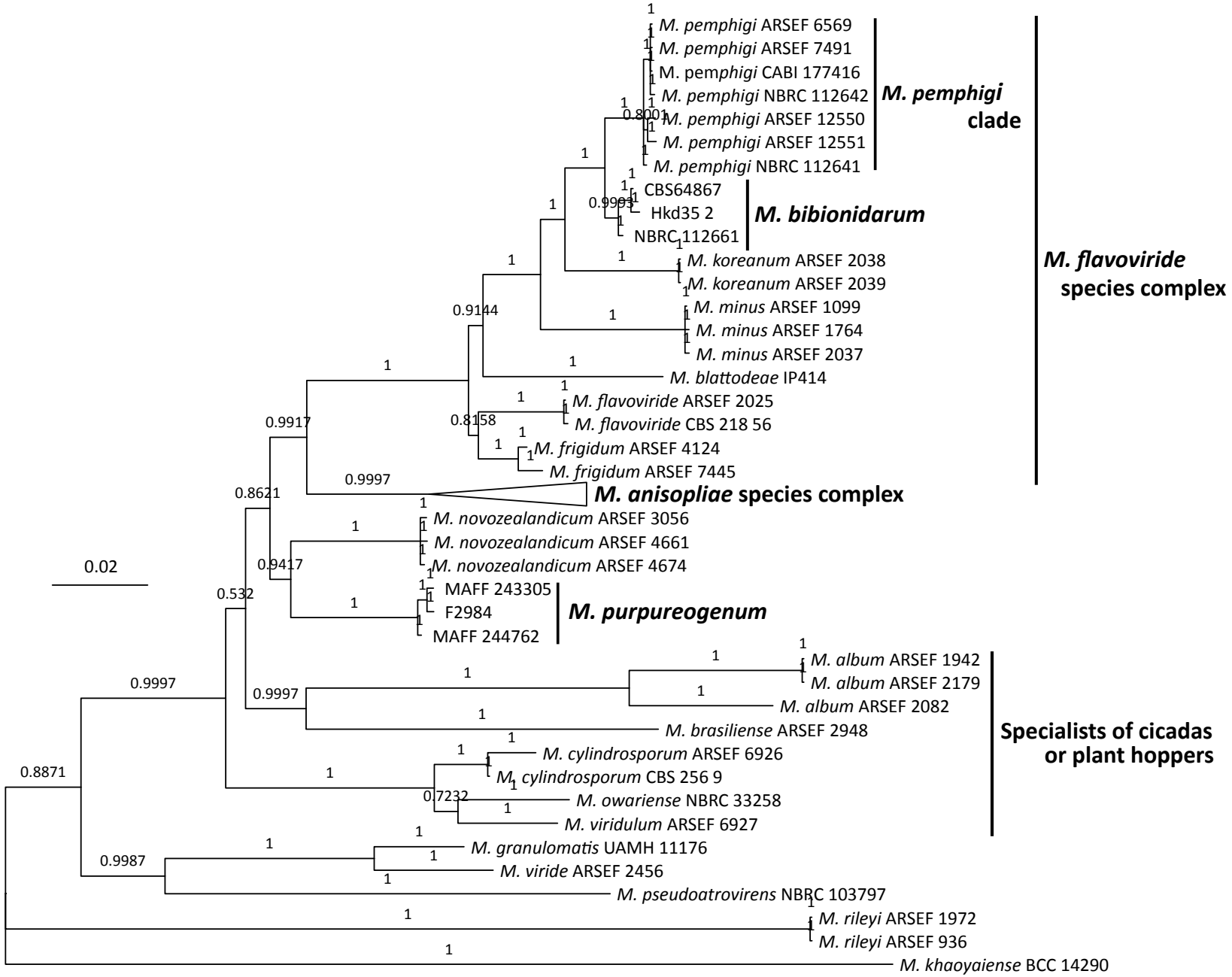

Fig. S2 A 50% consensus phylogenetic tree of the combined dataset of *Metarhizium* spp. inferred by a Bayesian analysis. Posterior probabilities of each branch are indicated above branches. *M. khaoyaiense* BCC 14290 was designated as an outgroup. The clades of *M. anisopliae* species complex (*M. anisopliae, M. brunneum, M. guizhouense, M. indigoticum, M. lepidiotae, M. majus, M. pingshaense, and M. robertsii*) was condensed.

Table S1. Primers used in this study

Targets	Primer	Sequences (5'-3')	References
BTUB	Bt_claviF (PCR amplification and sequencing)	CAACATGCGTGAGATTGTGAG	This study
	Bt_claviR (PCR amplification and sequencing)	TCTTGTTCTGCACGTTACGCATC	This study
RPB1	RPB1_F1 (PCR amplification and sequencing)	GCCAAGCCCGTGTACCATCC	This study
	RPB1_R900 (PCR amplification and sequencing)	CAGAGAAATCCACACGCTTG	This study
RPB2	RPB2_F1 (PCR amplification and sequencing)	CAAACCGAAGGCAGCGAGAC	This study
	RPB2_F76 (PCR amplification and sequencing)	CCTGATGACCGTGATCACTT	This study
	RPB2_R1400 (sequencing)	AGCAGGCAATAGCGACGATG	This study
	RPB2_R2200 (PCR amplification and sequencing)	CTGTGCTCTCAGCTTCTTGC	This study
3TEF	EF983F (PCR amplification and sequencing)	GCYCCYGGHCAYCGTGAYTTYAT	Rehner and Buckley (2005)
	EF1577F (sequencing)	CARGAYGTBTACAAGATYGGTGG	Bischoff et al. (2009)
	EF1567R (sequencing)	ACHGTRCCRATACCACCRAT	Bischoff et al. (2009)
	EF2212R (sequencing)	CCRAACRGCRACRGTYYGTCTCAT	Bischoff et al. (2009)
	EF2218R (PCR amplification)	ATGACACCRACRGCRACRGTYTG	Bischoff et al. (2009)
5TEF	EF1T (PCR amplification and sequencing)	TTTGCGAAGATGCGTTGAAG	Rehner and Buckley (2005)
	EF2T (PCR amplification and sequencing)	CCGCTGCTTACCTGATTGTG	Bischoff et al. (2006)
	5EF_F4_b (PCR amplification and sequencing)	GGGTAAGGAGGACAAGACTCACAT	This study
	5EF_R2s_b (PCR amplification and sequencing)	CTGGGAAGTACCAGTAATCATGTT	This study

Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97: 84–98.

Bischoff JF, Rehner SA, Humber RA (2006) *Metarhizium frigidum* sp. nov.: A cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. Mycologia 98(5):737–745

Bischoff JF, Rehner SA, Humber RA (2009) A multilocus phylogeny of the *M. anisopliae* lineage. Mycologia 101(4): 512–530.

Table S2 Condition of PCR amplifications

Targets	**Primer pairs (forward, reverse)**	**Annealing temperature (˚C)**	**Elongation time (s)**
BTUB	Bt_claviF, Bt_claviR	54	80
RPB1	RPB1_F1, RPB1_R900	54	60
RPB2 of *M. pemphigi* and *M. bibionidarum*	RPB2_F1, RPB2_R2200	52	130
RPB2 of *M. purpureogenum*	RPB2_F76, RPB2_R2200	52	130
3TEF	EF983F, EF2212R	50–54	60
5TEF of *M. pemphigi* and *M. bibionidarum*	EF1T, EF2T	50–54	50
5TEF of *M. purpureogenum*	5EF_F4_b, 5EF_R2s_b	50	50

Table S3 Reference isolates for phylogenetic analysis

Species	**Isolates**	**Isolation source**	**Location**	**BTUB**	**RPB1**	**RPB2a**	**3TEF**	**5TEF**
M. acridum	ARSEF 324	Orthoptera	Australia	EU248812	EU248896	EU248924	EU248844	EU248844
	ARSEF 7486	Orthoptera	Niger	EU248813	EU248897	EU248925	EU248845	EU248845
M. album	ARSEF 2082	Hemiptera	Indonesia	KJ398579	KJ398617	KJ398715	DQ522352	-
	ARSEF 2179	Hemiptera	Pilippines	KJ398580	KJ398618	KJ398716	KJ398807	KJ398807
	ARSEF 1942	Hemiptera	Pilippines	KJ398572	KJ398611	KJ398709	KJ398802	KJ398802
M. anisopliae	ARSEF 7450	Coleoptera	Australia	EU248823	EU248904	EU248932	EU248852	EU248852
	ARSEF 7487	Orthoptera	Ethiopia	EU248822	DQ468355	DQ468370	DQ463996	DQ463996
M. blattodeae	IP 414	Blattodea	Brazil	KU182914	KU182918	KU182916	KU182917	-
M. brasiliense	ARSEF 2948	Hemiptera	Brazil	KJ398582	KJ398620	KJ398718	KJ398809	KJ398809
M. brunneum	ARSEF 2107	Coleoptera	USA	EU248826	EU248907	EU248935	EU248855	EU248855
	ARSEF 4179	soil	Australia	EU248825	EU248906	EU248934	EU248854	EU248854
M. cylindrosporum	ARSEF 6926	Hemiptera	China	KJ398587	KJ398625	KJ398723	KJ398814	KJ398814
	CBS 256.90	Hemiptera	Taiwan	KJ398543	KJ398594	KJ398691	KJ398783	KJ398783
M. flavoviride	ARSEF 2025	soil	Germany	KJ398575	KJ398614	KJ398712	KJ398804	KJ398804
	CBS 218.56	Coleoptera	Czech Republic	KJ398555	KJ398598	KJ398694	KJ398787	-
M. frigidum	ARSEF 7445	Isoptera	Australia	KJ398590	KJ398628	KJ398727	KJ398818	KJ398818
	ARSEF 4124	Coleoptera	Australia	EU248828	DQ468361	DQ468376	DQ464002	DQ463978
M. granulomatis	UAMH 11176	Chamaeleo	Denmark	KJ398541	KJ398593	KJ398689	KJ398782	-

		calyptratus						
M. guizhouense	CBS 258.90	Lepidoptera	China	EU248834	EU248914	EU248942	EU248862	EU248862
	ARSEF 6238	Lepidoptera	China	EU248830	EU248909	EU248937	EU248857	EU248857
M. indigoticum	TNS F18553	Lepidoptera	Japan	KJ398569	JN049886	JF415992	JF416010	-
	NBRC 100684	Lepidoptera	Japan	KJ398544	KJ398595	KJ398692	KJ398784	-
M. khaoyaiense	BCC 14290	Lepidoptera	Thailand	KJ398565	JN049888	KJ398704	KJ398797	KJ398797
M. koreanum	ARSEF 2039	Hemiptera	Korea	KJ398578	KJ398616	KJ398714	KJ398806	KJ398806
	ARSEF 2038	Hemiptera	Korea	KJ398577	KJ398615	KJ398713	KJ398805	KJ398805
M. majus	ARSEF 1946	Coleoptera	Pilippines	EU248839	EU248919	EU248947	EU248867	EU248867
	ARSEF 1914	Coleoptera	Pilippines	KJ398571	KJ398610	KJ398708	KJ398801	KJ398801
M. minus	ARSEF 1764	Hemiptera	Solomon Island	KJ398570	KJ398609	KJ398707	KJ398800	KJ398800
	ARSEF 2037	Hemiptera	Pilippines	KJ398576	DQ522400	DQ522454	DQ522353	-
	ARSEF 1099	Hemiptera	Pilippines	KJ398567	KJ398608	KJ398706	KJ398799	KJ398799
M. novazealandicum	ARSEF 3056	Coleoptera	New Zealand	KJ398583	KJ398621	KJ398719	KJ398810	KJ398810
	ARSEF 4661	soil	Australia	KJ398584	KJ398622	KJ398720	KJ398811	KJ398811
	ARSEF 4674	soil	Australia	KJ398585	KJ398623	KJ398721	KJ398812	KJ398812
M. owariense f. *viridescens*	NBRC 33258	Hemiptera	Japan	KJ398545	KJ398596	JF415996	JF416017	-
M. pemphigi	CABI 177416	Hemiptera	United Kingdom	KJ398592	KJ398630	KJ398729	KJ398820	KJ398820
	ARSEF 6569	Hemiptera	United	KJ398586	KJ398624	KJ398722	KJ398813	KJ398813

			Kingdom					
	ARSEF 7491	Hemiptera	United Kingdom	KJ398591	KJ398629	KJ398728	KJ398819	KJ398819
M. pingshaense	ARSEF 4342	Coleoptera	Solomon Island	EU248821	EU248903	EU248931	EU248851	EU248851
M. pseudoatrovirens	NBRC 103797	Coleoptera	Japan	KJ398546	JN049893	JF415997	KJ398785	-
M. rileyi	ARSEF 936	Hemiptera	Brazil	KJ398566	KJ398607	KJ398705	KJ398798	KJ398798
	ARSEF 1972	Hemiptera	Brazil	KJ398574	KJ398613	KJ398711	KJ398803	KJ398803
M. robertsii	ARSEF 7501	Coleoptera	Australia	EU248818	EU248901	EU248929	EU248849	EU248849
	ARSEF 727	Orthoptera	Brazil	EU248816	DQ468353	DQ468368	DQ463994	DQ463994
M. viride	ARSEF 2456	Chamaeleo lateralis		KJ398581	KJ398619	KJ398717	KJ398808	-
M. viridulum	ARSEF 6927	Hemiptera	Taiwan	KJ398588	KJ398626	KJ398724	KJ398815	KJ398815

Table S4 Selected partition schemes and models for the five loci by PartitionFinder program.

Analysis	Loci	Partitions	Posisions	Selected models
Maximum likelihood with RaxML	BTUB	1	codon 1, codon 2	GTR+I+G [a]
		2	codon 3	GTR+G
	RPB1	1	codon 1, codon 2	GTR+G
		2	codon 3	GTR+G
	RPB2	1	codon 1	GTR+G
		2	codon 2	GTR+I+G
		3	codon 3	GTR+G
	3TEF	1	codon 1, codon 2	GTR+I+G
		2	codon 3	GTR+G
	5TEF	1	intron	GTR+G
		2	codon 3	GTR+G
		3	codon 1, codon 2	GTR+G
Bayesian inference with Mrbayes	BTUB	1	codon 1	K80+I
		2	codon 2	JC
		3	codon 3	GTR+G
	RPB1	1	codon 1	K80+I
		2	codon 2	HKY+G
	RPB2	1	codon 1	GTR+I

		2	codon 2	F81+I
		3	codon 3	HKY+G
	3TEF	1	codon 1	F81+I+G
		2	codon 2	JC+I
		3	codon 3	GTR+G
	5TEF	1	intron	HKY+G
		2	codon 3	HKY+G
		3	codon 1, codon 2	JC

[a] In the maximum likelihood analyzes with RaxML, the GTR+G was used for the all defined partitions including partitions where GTR+G+I was selected, in accordance with recommendations in the RAxML manual against the use of invariant sites.

Table S5 Results of PartitionFinder for the combined data set of the four loci.

Analysis	Partitions	Positions	Selected model
Maximum likelihood with RaxML	1	3TEF codon1	GTR+I+G [a]
	2	3TEF codon2, BTUB codon2, RPB1 codon2, RPB2 codon2	GTR+I+G
	3	3TEF codon3, BTUB codon3	GTR+G
	4	BTUB codon1, RPB1 codon1, RPB2 codon1	GTR+G
	5	RPB1 codon3, RPB2 codon3	GTR+G
Bayesian inference with Mrbayes	1	3TEF_pos1	F81+I+G
	2	3TEF_pos2, BTUB_pos2, RPB2a_pos2	F81+I
	3	3TEF_pos3, BTUB_pos3	GTR+G
	4	BTUB_pos1, RPB1a_pos2	K80+I
	5	RPB1a_pos1, RPB2a_pos1	GTR+I
	6	RPB1a_pos3, RPB2a_pos3	HKY+G

[a] In the maximum likelihood analyzes with RaxML, the GTR+G was used for the all defined partitions including partitions where GTR+G+I was selected, in accordance with recommendations in the RAxML manual against the use of invariant sites.

Table S6 Summaries of the phylogenetic analyses by Bayesian inferences.

Loci	**Generations per run**	**Sampled trees per run**	**Trees used for the calculation of consensus trees and posterior probabilities**
BTUB	300000	3002	4502
RPB1	800000	8002	12002
RPB2	400000	4002	6002
3TEF	600000	6002	9002
5TEF	100000	1001	1502
Combined data of the four loci	200000	2002	3002