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Development of Duplex PCR Assay for Detection and Differentiation of Typical and Atypical *Melissococcus plutonius* strains

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ABSTRACT. *Melissococcus plutonius* is the causative agent of an important honeybee disease, European foulbrood (EFB). In addition to *M. plutonius* strains with typical characteristics (typical *M. plutonius*), we recently reported the presence of atypical *M. plutonius*, which are phenotypically and genetically distinguished from typical *M. plutonius*. Because typical and atypical *M. plutonius* may have different pathogenic mechanisms, differentiation of these two types is very important for diagnosis and more effective control of EFB. In this study, therefore, a duplex PCR assay was developed to detect and differentiate typical and atypical *M. plutonius* rapidly and easily. On the basis of the results of comparative genomic analyses, we selected Na^+/H^+ antiporter gene and Fur family transcriptional regulator gene as targets for detection of typical and atypical strains, respectively, by PCR. Under optimized conditions, the duplex PCR system using the designed primers successfully detected and differentiated all typical and atypical *M. plutonius* strain/isolates tested, while no product was generated from any other bacterial strains/isolates used in this study, including those isolated from healthy honeybee larval guts. Detection limits of the PCR were 50 copies of chromosome/reaction for both types, and it could detect typical and atypical *M. plutonius* directly from diseased honeybee larvae. Moreover, the duplex PCR diagnosed mixed infections with both *M. plutonius* types more precisely than standard culture methods. These results indicate that the duplex PCR assay developed in this study is extremely useful for precise diagnosis and epidemiological study of EFB.

KEY WORDS: atypical strain, duplex PCR, European foulbrood, *Melissococcus plutonius*, typical strain.

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European foulbrood (EFB) is an important bacterial disease of honeybee larvae. It affects mainly unsealed larvae and kills them at the age of 4 to 5 days. The causative agent of EFB is a Gram-positive lanceolate coccus, *Melissococcus plutonius*. *M. plutonius* is a fastidious organism, requiring microaerophilic to anaerobic conditions and carbon dioxide for growth. In addition, the Na/K ratio required for its growth is described to be 1 or less [6], and thus, the addition of KH_2PO_4 to culture medium is required for the growth of typical *M. plutonius* strains.

This species had been thought to be remarkably homogeneous based on morphological, physiological, immunological and genetic studies [1, 7, 9]. However, Arai *et al.* [2] recently reported the presence and prevalence of atypical *M. plutonius*, which are phenotypically and genetically distinguished from typical *M. plutonius* strains, in Japan. The atypical *M. plutonius* was not fastidious, and the addition of KH_2PO_4 was not required for its normal growth [2, 26]. Moreover, unlike typical *M. plutonius*, it was positive for β-glucosidase activity, hydrolyzed esculin and produced acid from L-arabinose, D-cellobiose and salicin [2]. Interestingly, although typical *M. plutonius* is known to lose its virulence quickly when subcultured *in vitro* [2, 3, 18], atypical *M. plutonius* can maintain virulence even after repeated subculture [2]. Because these results imply that typical and atypical *M. plutonius* may have different mechanisms to regulate their virulence and have different impacts on apiculture, precise diagnosis of EFB including differentiation of the type of causative strains and accumulation of epidemiological information are needed for more comprehensive understanding and control of EFB.

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Although EFB is usually diagnosed in the field on the basis of the visual inspection of brood-combs and detection of diseased larvae, symptoms of EFB may easily be confused with other diseases or abnormalities in the larvae. In addition, some larvae affected by EFB may die after the cell is capped and show sunken capping, which resembles the symptoms of American foulbrood (AFB). Therefore, for reliable diagnosis of EFB, verification of the presence of *M. plutonius* is required in many cases. However, because isolation and identification of causative agents by standard culture methods are time-consuming and labor-intensive, in particular for fastidious bacteria like *M. plutonius*, many PCR assays are widely utilized at present as rapid and easy methods for detecting and identifying the causative agents of infectious diseases. For diagnosis of EFB, regular [13] and hemi-nested [8, 19] PCR assays targeting the 16S rRNA gene have been developed and used for the identification and detection of *M. plutonius*. However, as shown in this paper (see Results) and previously [2], these assays can detect both typical and atypical strains, but cannot differentiate them.

In this study, therefore, in order to detect and differentiate typical and atypical *M. plutonius* rapidly and easily, we developed a novel duplex PCR method by comparing reported genome sequences and designing primers for genes specific to each type. The developed duplex PCR was highly specific, sufficiently sensitive and available to detect each *M. plutonius* type directly from diseased larvae, so it was considered to be very useful for precise diagnosis and epidemiological study of EFB.

MATERIALS AND METHODS

Bacterial strains and isolates: A total of 50 *M. plutonius* strain/isolates (24 typical and 26 atypical strain/isolates) and 36 other bacterial strains/isolates were used in this study (Table 1). Thirty-three *M. plutonius* strain/isolates, including the type strain ATCC 35311, were described previously [2], and the other *M. plutonius* isolates were isolated from diseased larvae of European honeybees (*Apis mellifera*) with clinical signs of EFB at Saitama Prefectural Chuo Livestock Hygiene Service Center as described below. Thirteen non-*M. plutonius* bacterial isolates were isolated from healthy larvae of European and Japanese (*Apis cerana japonica*) honeybees as described below at National Institute of Animal Health and NARO Institute of Livestock and Grassland Science. The other strains/isolates were from our laboratory collection.

Isolation and identification of M. plutonius from diseased honeybee larvae: Homogenized whole diseased larvae of European honeybees with clinical signs of EFB were streaked on KSBHI agar [brain heart infusion (BHI; Becton Dickinson, Sparks, MD, U.S.A.)-based medium supplemented with 0.15 M KH_2PO_4, 1% soluble starch and 1.5% agar] [2] and incubated at 37°C under anaerobic conditions using the Anaero Pack System (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) for 3 or 4 days. Bacterial colonies considered to be *M. plutonius* were subcultured on KSBHI agar and identified as *M. plutonius* according to morphological, cultural and biochemical characteristics of typical and atypical strains [2] and by regular *M. plutonius*-specific PCR assay [13].

Isolation and identification of bacteria from healthy honeybee larvae: Healthy European and Japanese honeybee larvae were collected from bee colonies in a disease-free apiary at NARO Institute of Livestock and Grassland Science. More than 10 larvae were surface-sterilized with 70% ethanol and then washed with sterile distilled water. The larval guts were removed by dissecting the larvae aseptically with sterilized forceps and homogenized with a plastic pestle in 100–500 μl of phosphate-buffered saline or BHI broth. The homogenates were streaked on BHI agar (Becton Dickinson), BHI agar with 5% horse blood, Lactobacilli MRS agar (Becton Dickinson) and/or GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% egg yolk saline solution (egg yolk diluted with an equal volume of sterile saline). BHI agar, BHI agar with 5% horse blood and the other agar plates were incubated at 35–37°C under aerobic, air plus 5% CO_2 and anaerobic conditions, respectively. Bacterial colonies grown on the agar plates were subcultured several times and characterized by Gram staining, catalase test and oxidase test. Genomic DNA of well-isolated bacteria was then extracted as described below, and 16S rRNA gene sequences of the isolates were determined as described previously [2]. Species, genus, family or class of the isolates was identified by analyzing the 16S rRNA gene sequences using EzTaxon-e (http://eztaxon-e.ezbiocloud.net) [16].

Bacterial DNA extraction: Genomic DNAs of bacterial strains/isolates cultured under appropriate conditions were extracted by a method described previously [2] (for determination of the sensitivity of PCR assays) and/or using InstaGene Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) according to the manufacturer's instructions (for the other experiments).

Extraction of chromosomal regions unique to each type of M. plutonius and primer design for duplex PCR assay: Chromosomal regions unique to typical and atypical *M. plutonius* were extracted by comparing the genome sequences of *M. plutonius* ATCC 35111 (typical strain: DDBJ/EMBL/GenBank accession no. AP012200) and DAT561 (atypical strain: accession no. AP012282) using *in silico* MolecularCloning Genomics Edition (in silico biology, inc., Yokohama, Japan). On the basis of the results, two primer sets were designed for duplex PCR to give typical and atypical *M. plutonius* strain-specific PCR products of easily distinguishable sizes (Table 2). The specificity of the primers against DNA sequences of related bacteria available in the GenBank database was assessed by BLAST search (http://blast.ncbi.nlm.nih.gov).

Specificity and sensitivity of M. plutonius-specific

Table 1. Bacterial strains/isolates used in this study and results of *M. plutonius*-specific PCR developed in this study and reported previously

Bacterial species/strain	No. of strains/ isolates tested	Result of [a]				Origin/reference	Accession no. of 16S rRNA gene sequence
		Duplex PCR		Regular PCR[b]	Hemi-nested PCR[c]		
		Typical strain	Atypical strain				
Melissococcus plutonius typical strain	24[d]	+	–	+[e]	+[e], NS (0.4 kbp)	Diseased European honeybee larvae [2]	
Melissococcus plutonius atypical strain	26	–	+	+[e]	+[e], NS (0.4 kbp)	Diseased European honeybee larvae [2]	
Paenibacillus larvae	2	–	–	–	–	Diseased European honeybee larvae	
Paenibacillus alvei	1[d]	–	–	NS (1.3 kbp)	–	Foulbrood in bees	
Brevibacillus laterosporus	1[d]	–	–	–	–	Foulbrood in bees	
Enterococcus faecalis	3[d]	–	–	NS (1.5 kbp)	NS (0.28 kbp)	Diseased European honeybee larvae, healthy chicken, [24] (type strain)	
Staphylococcus aureus	1	–	–	–	–	Diseased pig	
Bacillus cereus	2	–	–	–	NS (0.28 kbp)	Diseased cow	
Streptococcus suis	1	–	–	–	–	Diseased pig	
Lactococcus lactis	1	–	–	–	–	[11] (strain MG1363)	
Enterococcus faecium	1	–	–	–	–	Diseased European honeybee larvae	
Clostridium perfringens	1	–	–	–	–	Diseased cow	
Erysipelothrix rhusiopathiae	1	–	–	–	–	Diseased pig	
Escherichia coli	1	–	–	–	–	Diseased pig	
Salmonella Typhimurium	1	–	–	–	–	Diseased cow	
Klebsiella pneumoniae	1	–	–	–	–	Diseased cow	
Pseudomonas aeruginosa	1	–	–	–	–	Diseased chicken	
Campylobacter jejuni	1	–	–	–	–	Healthy chicken	
Arcanobacterium (*Trueperella*) *pyogenes*	1	–	–	–	–	Diseased pig	
Mycobacterium avium ssp. *paratuberculosis*	1	–	–	–	–	Diseased cow	
Bifidobacterium longum ssp. *longum*	1[d]	–	–	–	–	Intestine of adult	
Bifidobacterium sp.	1	–	–	–	–	Healthy Japanese honeybee larva	AB777200
Lactobacillus sp.	2	–	–	–	–	Healthy Japanese honeybee larva	AB777201, AB777202
Staphylococcus hominis	1	–	–	–	NS (0.5 and 0.3 kbp)	Healthy Japanese honeybee larva	AB777203
Bifidobacterium asteroides	1	–	–	–	–	Healthy Japanese honeybee larva	AB777204
Streptomyces sp.	1	–	–	–	–	Healthy Japanese honeybee larva	AB777205
Acetobacteraceae bacterium	2	–	–	NS (1.5 kbp)	–	Healthy European honeybee larva	AB777206, AB777207
Dermacoccus sp.	1	–	–	–	–	Healthy European honeybee larva	AB777208
Fructobacillus fructosus	1	–	–	–	–	Healthy European honeybee larva	AB777209
Lactobacillus kunkeei	1	–	–	–	NS (0.2 kbp>)	Healthy European honeybee larva	AB777210
Lactobacillus sp.	1	–	–	NS (0.8 kbp)	–	Healthy European honeybee larva	AB777211
Gamma proteobacterium[f]	1	–	–	–	–	Healthy European honeybee larva	AB777212

a) +, amplification positive; –, amplification negative; NS, nonspecific product was amplified in several independent experiments. Approximate size of the nonspecific products is shown in parentheses. b) Regular *M. plutonius*-specific PCR [13]. c) Hemi-nested *M. plutonius*-specific PCR [8, 19]. d) Including type strain. e) PCR products from typical and atypical *M. plutonius* strains showed the same size on agarose gels and could not be distinguished from each other. f) The 16S rRNA gene sequence showed 98.3% homology with that of *Gilliamella apicola* proposed as a novel species in a novel genus in 2013 [17].

Table 2. *M. plutonius*-specific PCR primers designed in this study and reported previously

Target bacteria	Target gene	Primer	Oligonucleotide sequence (5'-3')	PCR product size	Source or reference
Typical *M. plutonius*	Na^+/H^+ antiporter gene, *napA* (MPTP_0420 in ATCC 35311)	Mp-T-F	TGGTAGCTTAGGCGGAAAAC	187 bp	This study
		Mp-T-R	TGGAGCGATTAGAGTCGTTAGA		
Atypical *M. plutonius*	Fur family transcriptional regulator gene (MPD5_0863 in DAT561)	Mp-A-F	GAGAACGATTCGGTACAAGC	424 bp	
		Mp-A-R	CCTTTTCTTCACATTCTGGACAT		
M. plutonius	16S rRNA gene	Primer 1	GAAGAGGAGTTAAAAGGCGC	832 bp[a)]	[13]
		Primer 2	TTATCTCTAAGGCGTTCAAAGG		
M. plutonius	16S rRNA gene	MP1	CTTTGAACGCCTTAGAGA	485 bp[b)]/ 276 bp[c)]	[8]
		MP2	ATCATCTGTCCCACCTTA		
		MP3	TTAACCTCGCGGTCTTGCGTCTCTC		

a) Although PCR product size was reported as 831 bp by Govan *et al.* [13], the size was considered to be 832 bp according to the 16S rRNA gene sequences determined in our previous study [2]. b) First PCR product size amplified by MP1 and MP2. Although PCR product size was reported as 486 bp by Djordjevic *et al.* [8], the size was considered to be 485 bp according to the 16S rRNA gene sequences determined in our previous study [2]. c) Second PCR product size amplified by MP1 and MP3.

PCR assays: To investigate the specificity of the duplex and reported *M. plutonius*-specific PCR assays, genomic DNAs extracted from a broad range of bacterial species (Table 1), including those closely related to *M. plutonius* and commonly found in bee larvae, were used as templates. Duplex PCR assay was carried out using Multiplex PCR Assay Kit (Takara Bio, Otsu, Japan) in a final reaction volume of 25 μl containing 12.5 μl of Multiplex PCR Mix 2, 0.2 μM of each primer, 0.125 μl of Multiplex PCR Mix 1 and 10 *n*g of template DNA. The cycling conditions consisted of an initial denaturation step at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 90 sec and extension at 72°C for 90 sec and a final extension step at 72°C for 10 min. Reported *M. plutonius*-specific PCR assays were carried out according to previously reported protocols with the following modifications. The regular PCR assay reported by Govan *et al.* [13] was performed in a final reaction volume of 50 μl containing 1 U *Ex* Taq polymerase (Takara Bio), 1× *Ex* Taq PCR buffer (Mg^{2+} free), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 10 *n*g of template DNA. The hemi-nested PCR assay reported by Djordjevic *et al.* [8] and McKee *et al.* [19] was carried out in a final reaction volume of 50 μl containing 1 U *Ex* Taq polymerase (Takara Bio), 1× *Ex* Taq PCR buffer (Mg^{2+} free), 3 mM (first PCR) or 1.5 mM (second PCR) $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 10 *n*g of template DNA (first PCR) or 1 μl of first PCR product (second PCR).

To determine the sensitivity, genomic DNA of *M. plutonius* ATCC 35311 (typical strain) and DAT561 (atypical strain) was serially diluted to a final concentration of 10 fg/reaction (5 copies of chromosome/reaction) and used for the duplex and previously reported regular *M. plutonius*-specific PCR assays as described above. In both assays, PCR reactions were repeated for 35 cycles.

All PCR amplifications were performed in iCycler (Bio-Rad Laboratories) or Mx3000P QPCR system (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). Five microliters of amplification product was electrophoresed (100 V, 30 min) through a 1.5% agarose gel and visualized by staining the gel with ethidium bromide.

Duplex PCR assay and bacterial isolation using diseased larval samples: Forty-three diseased European honeybee larval samples collected from 26 colonies in 19 different apiaries in Saitama Prefecture, Japan, were used for bacterial isolation and duplex PCR assay. Forty-one and two samples showed clinical signs of EFB and AFB, respectively. Larval DNA was extracted from approximately 1 μl of homogenized whole larvae using InstaGene Matrix (Bio-Rad Laboratories) according to the manufacturer's instructions, and 5 μl of the extracted DNA was used as a template for the duplex PCR assay. The same homogenized samples were also used for the isolation of *M. plutonius* and *Paenibacillus larvae* (the causative agent of AFB). *M. plutonius* was isolated as described above and classified into typical and atypical isolates by the duplex PCR. *P. larvae* was isolated by culturing the homogenized samples on Colombia agar (Becton Dickinson) with 5% sheep blood at 37°C under air plus 5% CO_2 conditions and identified by morphological and biochemical characteristics and *P. larvae*-specific PCR assay [12].

Nucleotide sequence accession numbers: The 16S rRNA gene sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers listed in Table 1.

RESULTS

Target genes and primers for duplex PCR assay: Chromosomal regions unique to typical *M. plutonius* strain ATCC 35311 and atypical strain DAT561 extracted by *in silico* MolecularCloning Genomics Edition included 89 and 27 entire/partial genes, respectively (data not shown). Among these genes, we selected Na^+/H^+ antiporter gene (MPTP_0420 in ATCC 35311, *napA* [26]) and Fur family transcriptional regulator gene (MPD5_0863 in DAT561) as targets for typical and atypical *M. plutonius*, respectively, and designed two primer sets for the duplex PCR assay to give typical and atypical strain-specific PCR products of easily distinguishable sizes (187 bp and 424 bp in size, respectively) (Table 2).

Specificity of PCR assays: Under optimized conditions

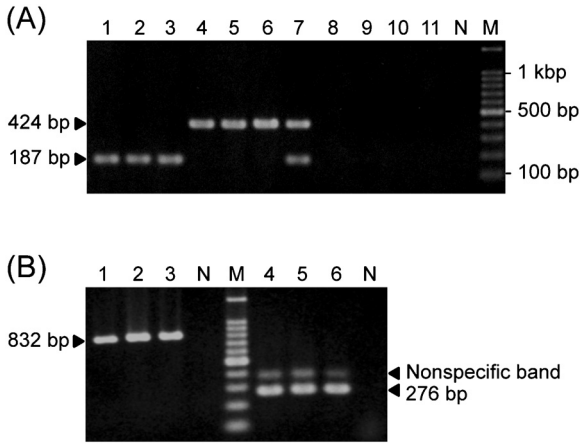

Fig. 1. Results of *M. plutonius*-specific PCR assays developed in this study and reported previously. DNA samples extracted from bacterial cultures were used as templates for the PCR. Five microliters of PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. (A) Results of duplex PCR developed in this study. Lanes 1–3, typical *M. plutonius* strains; Lanes 4–6, atypical *M. plutonius* strains; Lane 7, a DNA sample, mixing DNA from typical and atypical *M. plutonius*; Lane 8, *P. larvae*; Lane 9, *P. alvei*; Lane 10, *B. laterosporus*; Lane 11, *E. faecalis*; N, no template control; M, molecular size marker (100 bp DNA Ladder). PCR products from typical and atypical *M. plutonius* strains were distinguishable from each other. (B) Results of regular (Lanes 1–3) [13] and hemi-nested (Lanes 4–6) [8, 19] *M. plutonius*-specific PCR reported previously. Lanes 1 and 4, typical *M. plutonius* strains; Lanes 2 and 5, atypical *M. plutonius* strains; Lanes 3 and 6, DNA samples, mixing DNA from typical and atypical *M. plutonius*; N, no template control; M, molecular size marker (100 bp DNA Ladder). PCR products from typical and atypical *M. plutonius* strains could not be distinguished from each other.

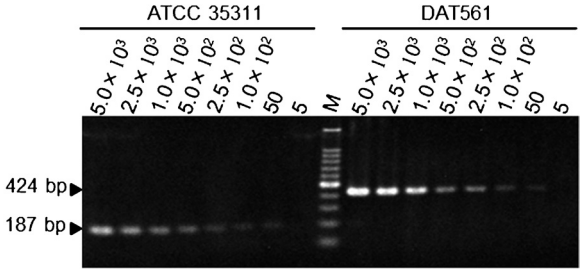

Fig. 2. Sensitivity of the duplex PCR assay. Serial dilutions of DNA extracted from *M. plutonius* ATCC 35311 (typical strain) and DAT561 (atypical strain) were used to investigate the sensitivity of the duplex PCR. The copy number of chromosome used as template DNA for each reaction is indicated above each lane. Five microliters of PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. M, molecular size marker (100 bp DNA Ladder).

described in Materials and Methods, the duplex PCR developed in this study yielded specific PCR products of expected size from all 24 typical and 26 atypical *M. plutonis* strain/isolates, while no products were generated from any other bacterial strains/isolates tested in this study (Table 1 and Fig. 1A). The differently sized specific products from typical and atypical *M. plutonius* were easily distinguishable from each other on agarose gels (Fig. 1A). On the other hand, although both regular [13] and hemi-nested [8, 19] *M. plutonius*-specific PCR assays reported previously also yielded specific PCR products from both typical and atypical *M. plutonius*, the products showed the same size on agarose gels and could not be distinguished from each other (Table 1 and Fig. 1B). In addition, the reported PCR assays generated nonspecific PCR products in *M. plutonius* and some non-*M. plutonius* bacterial strains/isolates (Table 1 and Fig. 1B). In particular, nonspecific hemi-nested PCR products were always amplified from *Enterococcus faecalis* and *Bacillus cereus*, and the size of the products was indistinguishable from that of specific products from *M. plutonius* on agarose gels (data not shown).

Sensitivity of duplex PCR assay: The duplex PCR detected both typical and atypical *M. plutonius* from 50 copies of chromosome (Fig. 2). The results were reproducible in three independent experiments. This sensitivity was equal to that of the regular *M. plutonius*-specific PCR assay reported by Govan *et al.* [13] (data not shown).

Comparison of the culture methods and duplex PCR assay for detection of typical and atypical M. plutonius in diseased larval samples: By the culture method described in Materials and Methods, *M. plutonius* was isolated from 35 of the 43 diseased larval samples tested. All *M. plutonius* isolates were classified into either typical or atypical type by the duplex PCR, and there was no isolate that yielded both typical and atypical strain-specific products. From 11, 14 and 10 of the 35 samples, both typical and atypical, only typical and only atypical *M. plutonius* were isolated, respectively. From two of the other eight samples, *P. larvae* was isolated, and the two samples showed clinical signs of AFB in the field. On the other hand, neither *M. plutonius* nor *P. larvae* was isolated from the remaining six samples (Table 3).

The results of detection of *M. plutonius* by the duplex PCR assay were almost consistent with those of the culture methods. Typical and/or atypical *M. plutonius*-specific PCR products were obtained from all 35 diseased larval samples, from which *M. plutonius* was isolated. On the other hand, no PCR product was amplified from eight larval samples, from which *M. plutonius* was not isolated, including those infected with *P. larvae*. In addition, specific PCR products of both typical and atypical *M. plutonius* were generated from all 11 samples, from which both types of *M. plutonius* were isolated, and only typical *M. plutonius*-specific PCR product was amplified from all 14 samples, from which only typical *M. plutonius* was isolated (Table 3 and Fig. 3). However, although only atypical *M. plutonius*-specific PCR product was generated from two of the 10 larval samples, from which only atypical *M. plutonius* was isolated (Table 3 and Fig. 3), both typical and atypical *M. plutonius*-specific PCR products were obtained from the other eight samples (Table 3), suggesting that these eight samples were infected with both types of *M. plutonius* in the field, but such mixed infection

Table 3. Results of culture method and duplex PCR assay for detection of typical and atypical *M. plutonius* in 43 diseased larval samples

Isolation by culture method (typical strain/atypical strain[a)])	Detection by duplex PCR (typical strain/atypical strain[a)])				Total
	+/+	+/–	–/+	–/–	
+/+	11[b)]	0	0	0	11
+/–	0	14	0	0	14
–/+	8	0	2	0	10
–/–	0	0	0	8[c)]	8[c)]
Total	19	14	2	8[c)]	43

a) +, isolation or PCR amplification positive; –, isolation or PCR amplification negative. b) No. of samples. Each sample contains several larvae from the same colony. c) *P. larvae* was isolated from two of the eight samples.

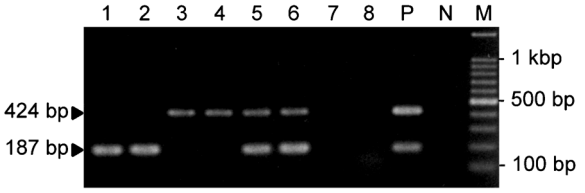

Fig. 3. Direct detection of *M. plutonius* from diseased larval samples using duplex PCR assay. Lanes 1–2, samples, from which only typical *M. plutonius* was isolated; Lanes 3–4, samples, from which only atypical *M. plutonius* was isolated; Lanes 5–6, samples, from which both typical and atypical *M. plutonius* were isolated; Lane 7, a sample, from which *P. larvae* was isolated; Lane 8, a sample, from which neither *M. plutonius* nor *P. larvae* was isolated; P, positive control (a DNA sample, mixing DNA from typical and atypical *M. plutonius*); N, no template control; M, molecular size marker (100 bp DNA Ladder). Five microliters of PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

could not be detected, at least by the culture methods performed in this study. These results indicate that the duplex PCR assay developed in this study is a powerful tool for accurate understanding of the infection status of *M. plutonius* in diseased colonies.

DISCUSSION

Honeybees are not only valuable for the honey and bee products they produce, but are also vital pollinators of agricultural crops; therefore, precise diagnosis and detailed epidemiological study of honeybee diseases including EFB are very important to control the diseases and reduce damage to agriculture. However, due to the fastidious growth requirements of *M. plutonius*, the relative complexity of the culture procedure and secondary bacteria, such as *E. faecalis* and *Paenibacilllus alvei*, verification of EFB by isolation of *M. plutonius* is difficult and labor-intensive [8, 15, 19]. In addition, because the growth of *M. plutonius* is relatively slow, isolation of *M. plutonius* and characterization of the isolates take a lot of time.

As rapid and easy methods for the identification and detection of *M. plutonius*, specific PCR assays were reported previously [8, 13, 19]; however, as shown in this study and previously [2], the assays cannot distinguish typical strains from atypical strains. In addition to these PCR assays, other molecular and immunological techniques including a real-time PCR assay targeting the manganese-dependent superoxide dismutase gene (*sodA*) [22], a gold nanoparticles (AuNPs)-based assay targeting the cell wall-associated protease gene [23] and a lateral flow device using *M. plutonius*-specific antibody [27] have been developed for identification and detection of *M. plutonius*. However, according to the whole genome sequences [20, 21], the target sequences in the *sodA* and cell wall-associated protease genes of typical *M. plutonius* are identical to those of the corresponding genes of atypical *M. plutonius*; therefore, primers and probes for the real-time PCR and AuNPs-based assays are considered to be unable to differentiate the two types (Takamatsu D., unpublished observations). Moreover, the *M. plutonius*-specific antibody used for the lateral device was unsuitable to detect atypical strains [25].

In this study, we successfully developed a novel *M. plutonius*-specific PCR, which can detect typical and atypical *M. plutonius* directly from diseased larvae without cumbersome steps to isolate the causative agents. In addition, the specificity of the PCR was extremely high, and the two types of *M. plutonius* strain were differentiated very precisely. Moreover, the duplex PCR showed high sensitivity and could detect *M. plutonius* from 50 copies of chromosome. Because Forsgren *et al.* [10] reported that the detection level of PCR decreased 10-fold when a mixed DNA template, i.e. purified *M. plutonius* DNA plus purified DNA from healthy larvae, was used, the sensitivity of our PCR assay may also decrease when using DNA from larvae samples directly. However, this assay could detect *M. plutonius* from all diseased larval samples, from which *M. plutonius* was isolated. Furthermore, the duplex PCR revealed the presence of eight mixed infection samples, which could not be detected by the culture methods. Therefore, the developed PCR assay is thought to be sufficiently practical and will be a very powerful and easy tool for the precise diagnosis and detailed epidemiological study of EFB. Of note, in most mixed infection cases, typical *M. plutonius* could not be isolated by the culture methods. Because atypical *M. plutonius* grows more rapidly and forms larger colonies than typical *M. plutonius* [2], typical *M. plutonius* in mixed infections might be missed due to its small colony size or the growth of atypical strains over the small colonies of typical strains.

In Japan, atypical *M. plutonius* has been isolated in various regions [2]. Although *M. plutonius* strains that are phenotypically identical to Japanese atypical strains have not been reported in other countries, several unusual or non-fastidious putative *M. plutonius* strain/isolates were isolated from samples in England, Brazil and India [1, 4, 5, 7]. In particular, the Brazilian isolates were phenotypically similar to Japanese atypical strains [1, 2, 5]. Recently, Haynes *et al.* [14] reported a modified multi-locus sequencing typing (MLST) scheme for *M. plutonius* and analyzed *M. plutonius*

strains/isolates isolated in various countries. Intriguingly, the analyzed strains/isolates were also divided into typical and atypical groups by the scheme, and the latter included not only the Japanese atypical strain (DAT561) but also five isolates from the U.K., the U.S.A. (2 isolates), Brazil and the Netherlands [14], suggesting that so-called atypical strains are widely distributed in the world. Therefore, although the specificity needs to be verified using non-Japanese isolates, our duplex PCR may be a globally useful tool for future EFB studies.

Interestingly, EFB of most clinical samples investigated in this study was caused by mixed infections with typical and atypical *M. plutonius* (19 samples, 54.3%) or single infection with typical *M. plutonius* (14 samples, 40%), whereas EFB of only two samples (5.7%) resulted from single infection with atypical *M. plutonius*. In the previous study [2], atypical strains showed strong virulence to artificially reared European honeybee larvae. Therefore, it is conceivable that larvae infected with a high dose of atypical strains developed EFB earlier than those of mixed infection or single infection with typical strains and thus were ejected from the colony completely by nurse bees before bee inspectors noticed the disease. Because the presence of atypical *M. plutonius* strains was recognized very recently, information about their ecology and impact on apiculture remains limited. A further epidemiological study using a range of geographically diverse, international honeybee samples by the duplex PCR assay will give us a better understanding of the evolution and pathogenesis of this important honeybee pathogen.

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