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Simultaneous Detection and Differentiation of *Vairimorpha* spp. and *Nosema* spp. by Multiplex Polymerase Chain Reaction

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Abstract A multiplex polymerase chain reaction (PCR) was developed for the simultaneous detection and differentiation among *Vairimorpha* spp. and *Nosema* spp. and identification of *Vairimorpha necatrix* from Lepidoptera insects. Three sets of primers were selected from different genomic sequences to specifically amplify an 831 bp amplicon within the SSU rRNA gene, specific for both *Vairimorpha* spp. and *Nosema* spp. (MSSR primer); a 542 bp amplicon within the SSU rRNA gene, specific for *Vairimorpha* spp. (VSSU primer); and a 476 bp amplicon within the actin gene, specific for *Vairimorpha necatrix* (VNAG primer). Using the primers in conjunction with multiplex PCR, it was possible to detect *Vairimorpha* spp. and *Nosema* spp. and to differentiate between them. The sensitivity of this PCR assay was approximately 10 spores per milliliter. It is proposed that the multiplex PCR is a sensitive, specific, and rapid tool that can serve as a useful differential diagnostic tool for detecting *Vairimorpha* spp. and *Nosema* spp. in Lepidoptera insect.

Key words: Microsporidia, *Vairimorpha* spp., *Vairimorpha necatrix*, multiplex PCR, Lepidoptera

The term microsporidia is a nontaxonomic name used to describe protozoan parasites belonging to the phylum Microspora. Microsporidia were first recognized in 1857 when *Nosema bombycis* was described as a silkworm parasite [16] and have subsequently been reported as infecting major animal groups, from other protists to invertebrates and all classes of vertebrates; 143 genera and almost 1,200 species have been recognized. Currently, almost half of the 143 described genera of microsporidia have insects as the type of host [4, 12, 22, 23]. Several genera of microsporidia (*Nosema*, *Vairimorpha*, *Pleistophora*, and *Thelohania*) have been reported to be agents of disease in Lepidoptera and have been mainly detected as *Nosema* and *Vairimorpha* [1, 6].

The genus *Vairimorpha* was originally established by Pilley [20]. It comprises 2 types of spores, the *Nosema* type and the *Thelohania* type. The *Nosema*-type spores are formed through the spore formation as observed in the genus *Nosema*, whereas the *Thelohania*-type spores develop in a sporophorous vesicle into 8 spores. The ratio of *Nosema*-type to *Thelohania*-type spores varies, depending on the temperature to which microsporidia are exposed. Many microsporidia have, however, been identified as genus *Nosema* without checking spore development under different temperatures, so that some microsporidia of the genus *Vairimorpha* might have been mistakenly classified as *Nosema* spp.

Vairimorpha necatrix, a pathogen for fall armyworm (*Spodoptera frugiperda*), is considered a potential microbial insecticide for the control of several genera of Lepidoptera [3]. Their development takes place from direct contact with the host cell cytoplasm, and nuclei are paired throughout the entire life cycle. Most entomopathogenic microsporidia induce sublethal effects on hosts, resulting in reduced fertility, shortened longevity, and loss of vigor [2, 25].

The diagnosis of microsporidiosis has traditionally depended on direct visualization of the parasites through light or electron microscopy, but the sensitivity and specificity of these techniques are not known [27]. Several methods have been developed for detection of microsporidia in their hosts, including immunological [7, 11, 17, 18], Southern hybridization [9, 13], and PCR amplification with species-specific primers [5, 8, 10, 19]. Although each has its advantages, PCR is considered a superior method because it provides detection at very low levels of infection [28]. Here, the development of a multiplex PCR-based procedure for rapid, sensitive, and simultaneous detection of *Nosema* spp. and *Vairimorpha* spp. and

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differentiation of them, and the identification of the *Vairimorpha necatrix* is reported.

MATERIALS AND METHODS

Microsporidia

Four unidentified (*N.* sp C01, C02, C03, C21) microsporidia were obtained from *Pieris rapae* and *Papilio xuthus* larvae collected from an insectarium in Korea (Figs. 1, 2). All of the species were determined to be *Nosema* and *Vairimorpha* species by morphological features and DNA sequences of the V4 region of ribosomal RNA gene. Partial nucleotide sequences for the V4 region of ribosomal RNA gene of the microsporidia isolated from *P. rapae* and *Papilio xuthus* were deposited in the GenBank database under Accession Nos. AF485270, AY311589, AY311590, and AY311592. Infected larvae were macerated in phosphate-buffered saline and the resultant microsporidia spore suspension was filtered through cotton wool. Spores in the filtrate were purified by the method of Sato and Watanabe [21] using a gradient of neutralized Percoll (Sigma). After centrifugation at 73,000 ×*g* for 30 min, a band of purified spores was collected from the gradient. This band was rinsed twice and the final suspension of spores was stored in distilled water at 4°C until required. Strains of *Vairimorpha necatrix* and *Vairimorpha* sp. NIS-M12 were stored at the National Institute of Agricultural Science and Technology.

Primer Design for Specific Detection

The SSU rRNA gene was used for the simultaneous detection and differentiation among *Vairimorpha* spp. and *Nosema* spp. An MSSR primer was designed from the specific region of both *Vairimorpha* spp. and *Nosema* spp., and a VSSU primer was designed from the genus-specific region of only *Vairimorpha.* The oligonucleotide primers were as follows: MSSR-F/R, 5'-GTGCCAGCAGCCGCGGT-AAT-3'/5'-GATCCTGCTAATGGTTCTCC-3' (nucleotide position 395-414/1207-1226 after the GenBank No. AF485270); VSSU-F/R, 5'-GCCGACGATGTGATATG-ATAT-3'/5'-GTTCAAGTAGTTTTCA(T)CT A(T)AT-3' (positions 639-658/1156-1175 of the GenBank No. AY311592) (Fig. 3). VNAG primer was designed based on nucleotide sequences of the actin gene of *V. necatrix* accession No. AF031818. The oligonucleotide primers were as follows: VNAG-F/R, 5'-GAAGACTCAATCTAGCG-GG-3'/5'-CTCTCAGCAGGAGATACG-3'.

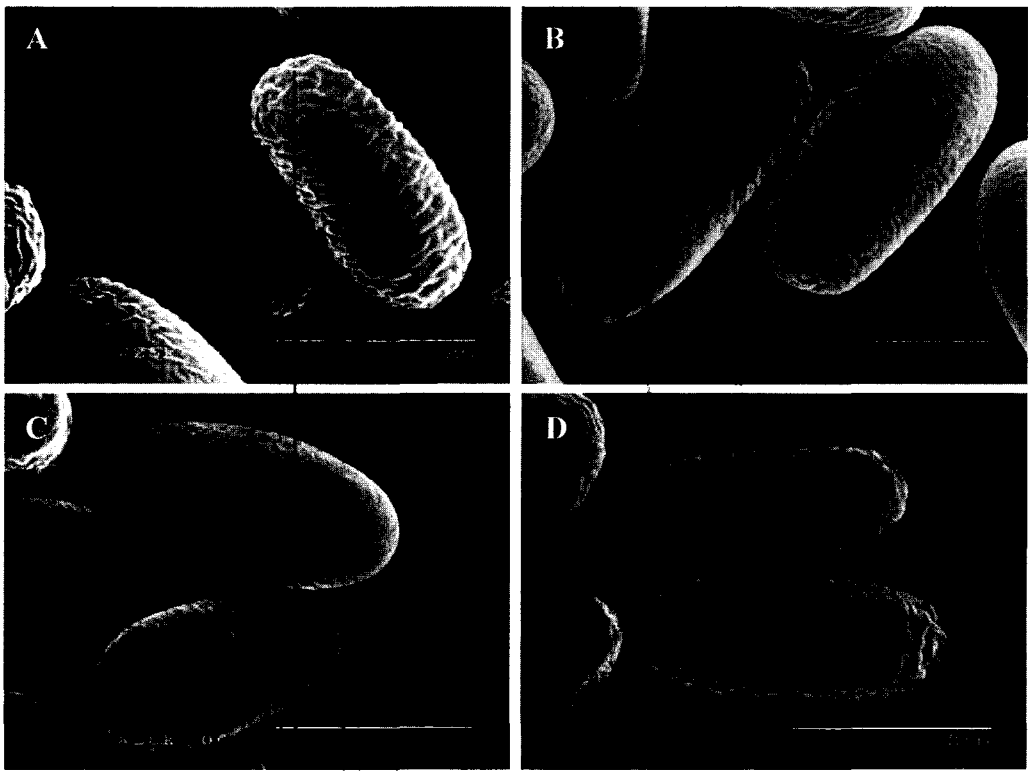

Fig. 1. Scanning electron micrographs of entomopathogenic microsporidia isolated from *Pieris rapae* and *Papilio xuthus* collected in Korea.

A, *Nosema* sp. C01 isolated from *Pieris rapae* in insectarium I; B, microsporidia isolated from *Pieris rapae* in insectarium II; C, microsporidia isolated from *Papilio xuthus* in insectarium III; D, microsporidia isolated from *Pieris rapae* in insectarium IV.

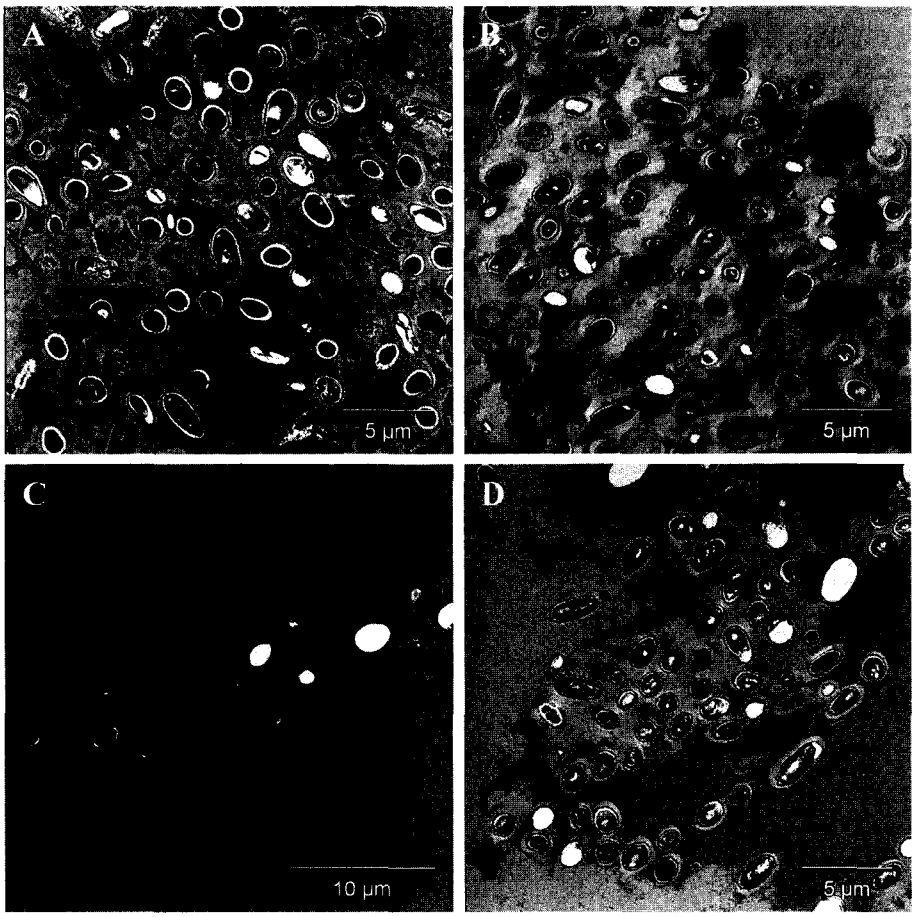

Fig. 2. Cross-section of the fat body of *Pieris rapae* larvae and *Papilio xuthus* larvae infected with entomopathogenic microsporidia. A, *Nosema* sp. C01 isolated from *Pieris rapae* in insectarium I; B, microsporidia isolated from *Pieris rapae* in insectarium II; C, microsporidia isolated from *Papilio xuthus* in insectarium III; D, microsporidia isolated from *Pieris rapae* in insectarium IV.

DNA Extraction and Multiplex PCR

DNA was prepared from spores using a QIAmp tissue kit (Qiagen, Germany). Spores were incubated in digestion buffer with 20 μl of proteinase K (Qiagen, Germany) and 0.4 U of chitinase (Sigma) at 56°C for 2 h. Glass beads (500 μg; diameter, 425 to 600 μm; Sigma) were added, and the mixture was vortexed every 15 min for 1 min [15]. DNA was isolated from the solution using QIAmp spin columns (Qiagen) in an eppendorf microcentrifuge following the manufacturer's instructions. PCR was performed on a Biometra T-gradient Thermoblock (Biometra; Germany) in a total volume of 20 μl consisting of a mixture of 1 μl (50 ng/1 μl) of extracted DNA solution, 250 M dNTP, 5 pmol MSSR, 20 pmol VSSR, 20 pmol VNAG, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase. The PCR cycling programs used with the different sets of primers are listed in Table 1. PCR conditions were optimized on the basis of theoretical calculations of melting temperatures of the primer pairs used and on the results of several amplification experiments.

Sensitivity of Detection

In order to investigate the sensitivity of PCR detection using the primers, serial ten-fold dilutions of the genomic DNA samples were prepared from the microsporidia spores. The reactions were prepared with genomic DNA. The PCR reactions were carried out as described above using MSSR, VSSU, and VNAG primer sets, and the products were electrophoretically separated on a 1.0% agarose gel and stained with ethidium bromide.

Partial Ribosomal DNA Sequencing

PCR primers were selected to amplify partial nucleotide sequences spanning the V4 region of the small subunit ribosomal RNA (SSU rRNA) gene [14]. Primers had the following sequences: 5'-GTTGATTCTGCCTGAC-GTA-3' (*Vairimorpha necatrix* numbering as recorded in the GenBank database Accession No. M24612) and 5'-ACCCGACGTGCGCGTTATGT-3' [14]. The amplified DNA fragments were cloned in pGEM-T Easy Vector (Promega). DNA sequencing was performed using

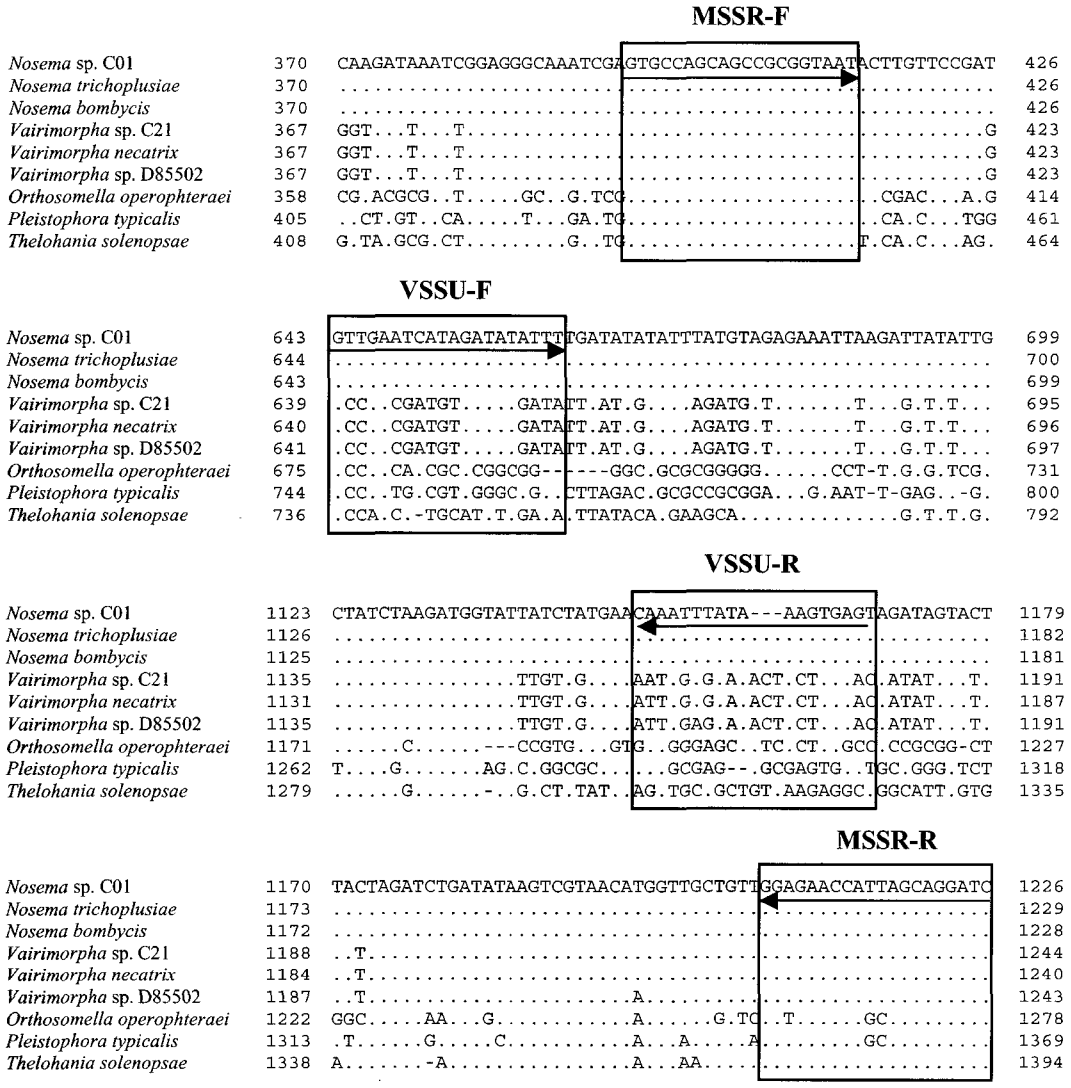

Fig. 3. Sequence of unique region and universal regions of the SSU rRNA genes of nine species of entomopathogenic microsporidia. Gaps indicated by a dash (-) are introduced into the sequence to optimize the alignment. Dots mark the same nucleotide sequences as *Nosema* sp. C01. The position and direction of primers for the multiplex PCR are indicated on the top of the nucleotide sequence with a box and an arrow. MSSR-F, entomopathogenic microsporidia (*Vairimorpha* spp. and *Nosema* spp.) specific forward primer; MSSR-R, entomopathogenic microsporidia (*Vairimorpha* spp. and *Nosema* spp.) reverse primer; VSSU-F, *Vairimorpha* spp. specific forward primer; VSSU-R, *Vairimorpha* spp. specific reverse primer.

an ABI 377 Genetic Analyzer (PE Applied Biosystems, U.S.A.). The sequence data were analyzed using the DS Gene software (Accelrys Inc., U.S.A.) and Blast searches (http://www.ncbi.nlm.nih.gov) against GenBank entries.

Electron Microscopy

For transmission electron microscopy (TEM), the tissue was fixed in 2.5% (v/v) glutaraldehyde phosphate buffer (0.1 M, pH 7.4) for 2 days and post-fixed in 1% osmium tetroxide (OsO_4) in the same buffer, dehydrated through

Table 1. PCR cycling programs applied with the three set of primers used in this study and sizes of the PCR products obtained.

Set of primers	Denaturation	Annealing	Polymerization	Size of PCR product (bp)
MSSR	94°C 30 sec	57°C 30 sec	72°C 30 sec	831
VSSU	94°C 30 sec	54°C 30 sec	72°C 30 sec	542
VNAG	94°C 30 sec	56°C 30 sec	72°C 30 sec	476
MSSR+VSSU+VNAG	94°C 30 sec	60°C 30 sec	72°C 30 sec	476, 542, 831

In all cases, the reaction mixture was first denatured at 94°C for 5 min followed by the series of amplification repeated for 35 cycles.

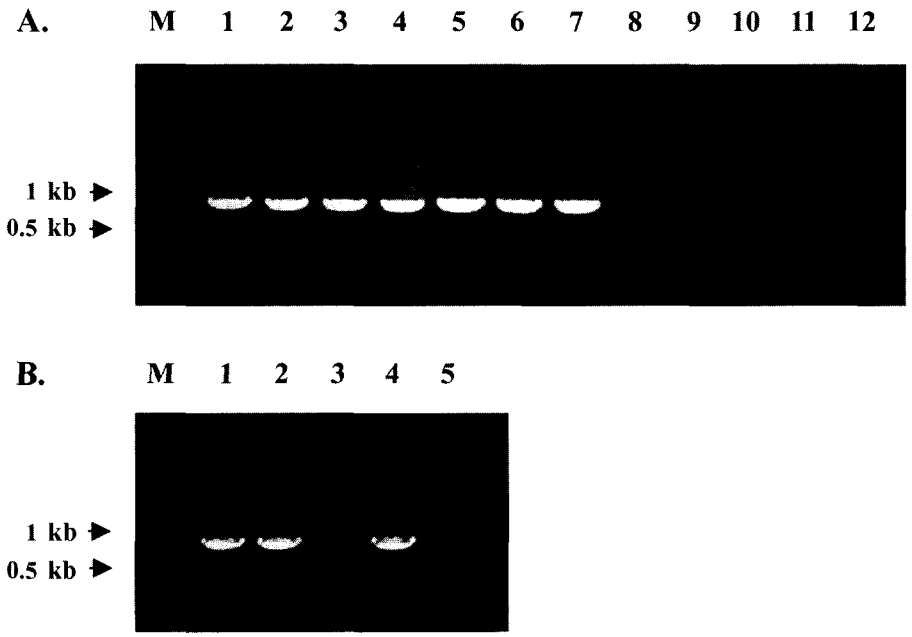

Fig. 4. Results of PCR analysis with the MSSR primer set for detection of microsporidia.
Lane M, DNA size marker (1 kb ladder); (A) Lane 1, *Vairimorpha necatrix*; Lane 2, *Nosema trichoplusiae*; Lane 3, *Nosema corneum*; Lane 4, *Encephalitizoon cuniculi*; Lane 5, *Vairimorpha* sp. NIS-M11; Lane 6, *Nosema ceranae*; Lane 7, *Nosema* sp. C01; Lane 8, *Metarhizium anisopliae*; Lane 9, *Paecilomyces teunipes*; Lane 10, *Pseudomonas* sp.; Lane 11, *Bacillus thuringiensis*; Lane 12, sterile water used as a negative control; (B) Lane 1, *Nosema* sp. C01; Lane 2, feces of infected larvae with *Nosema* sp. C01; Lane 3, uninfected larvae; Lane 4, infected larvae with *Nosema* sp. C01; Lane 5, sterile water used as a negative control.

ascending ethanol series and propylene oxide and embedded in Epon-Araldite. The ultrathin sections were cut with a diamond knife mounted on a ultramicrotome (Leica Ultracut UCT), stained in methanolic uranyl acetate and then lead citrate [24], and examined with an electron microscope (Zeiss LEO 912AB) operated at 100 kV.

RESULTS

Detection of Microsporidia

In order to detect both *Vairimorpha* spp. and *Nosema* spp., a PCR assay was developed using the primer MSSR. The MSSR primer sets were designed to unique areas of the

Table 2. Microsporidia strains and entomopathogenic fungi and bacteria used in this study, and evaluation of the specificity of multiplex PCR.

Strains	Strain No.	PCR results		
		VSSU	VNAG	MSSR
V. necatrix	isolate[a]	+	+	+
N. trichoplusiae	ATCC 30702[b]	-	-	+
N. corneum	ATCC 50505	-	-	+
E. cuniculi	ATCC 50503	-	-	+
V. sp. NIS-M11	isolate	+	-	+
V. sp. C21	isolate	+	-	+
N. sp. C01	isolate	-	-	+
N. sp. C02	isolate	-	-	+
N. sp. C03	isolate	-	-	+
Metarhizium anisopliae	KACC 40969[c]	-	-	-
Paecilomyces teunipies	KACC 40503	-	-	-
Peudomonas sp.	isolate	-	-	-
Bacillus thuringiensis	isolate	-	-	-

[a]Isolated strains from National Institute of Agricultural Science and Technology.
[b]American Type Culture Collection, Rockville, MD, U.S.A.
[c]Korean Agricultural Culture Collection.

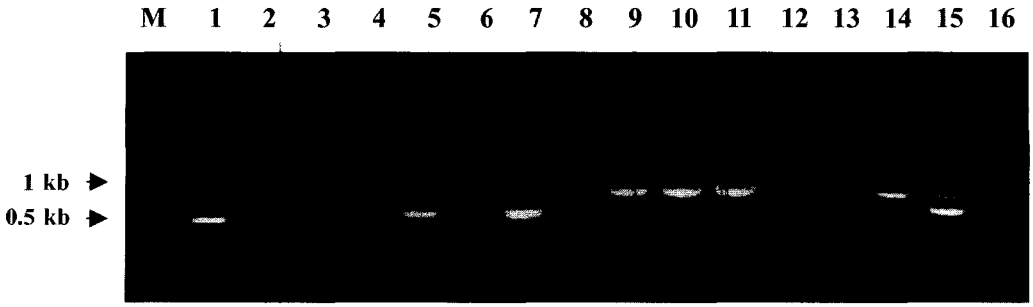

Fig. 5. Multiplex PCR assay with the three primer sets VNAG(A), VSSU(B), MSSR (C), and all primer sets (D).
M, DNA size marker (1 kb ladder); Lane 1, *Vairimorpha necatrix*; Lane 2, *Nosema trichoplusiae*; Lane 3, *Vairimorpha* sp. NIS-M11; Lane 4, sterile water used as a negative control.

small subunit ribosomal RNA gene from *Vairimorpha* spp. and *Nosema* spp. (Fig. 3). The primer MSSR amplified a 831 bp amplicon from the microsporidia, whereas the primer did not amplify any product from some of the typical entomopathogenic fungi nor from several other typical entomopathogenic bacterial strains (Fig. 4A). In spite of its similarity to ribosomal RNA genes [26], no product was amplified from the fungi strains. When PCR was carried out using genomic DNA extracted from infected larvae or feces of infected larvae, PCR products were obtained, while on the other hand, this primer did not amplify from uninfected larvae (Fig. 4B). These results suggest that MSSR is a good candidate for the specific detection of *Vairimorpha* spp. and *Nosema* spp. microsporidia in Lepidoptera insect.

Specificity of the PCR for Detecting *Vairimorpha* spp. and *Vairimorpha necatrix*

The VSSU primer set was chosen from the SSU rRNA gene and was used to specifically detect *Vairimorpha* spp. (Fig. 3). Genus *Vairimorpha* gave a 542 bp amplicon with the VSSU primer sets (Table 2). No amplified product was found for the *Nosema* strains. The VNAG primer was based on the sequences of the actin gene from *V. necatrix*. This primer set was evaluated for specificity in detecting *V. necatrix* (Table 2). The primer amplified a 476 bp fragment from the *V. necatrix* strain, whereas the primer did not amplify any product from the other *Vairimorpha* species nor from several other microsporidia strains. These results suggest that the actin gene is a good candidate for the specific detection of *V. necatrix*.

Specificity of the Multiplex PCR

To assess the specificity of the multiplex PCR, the primer sets VSSU and VNAG were used in the same reaction mixture, together with MSSR primer specific for all *Vairimorpha* spp. and *Nosema* spp. microsporidia species, including *V. necatrix*. Four distinct results were obtained from the multiplex PCR: three amplified products of 476 bp, 542 bp, and 831 bp for *V. necatrix*; two products of 542 bp and 831 bp for genus *Vairimorpha*; one amplified product of 831 bp for genus *Nosema* microsporidia species; and no amplified product for the negative control (Fig. 5).

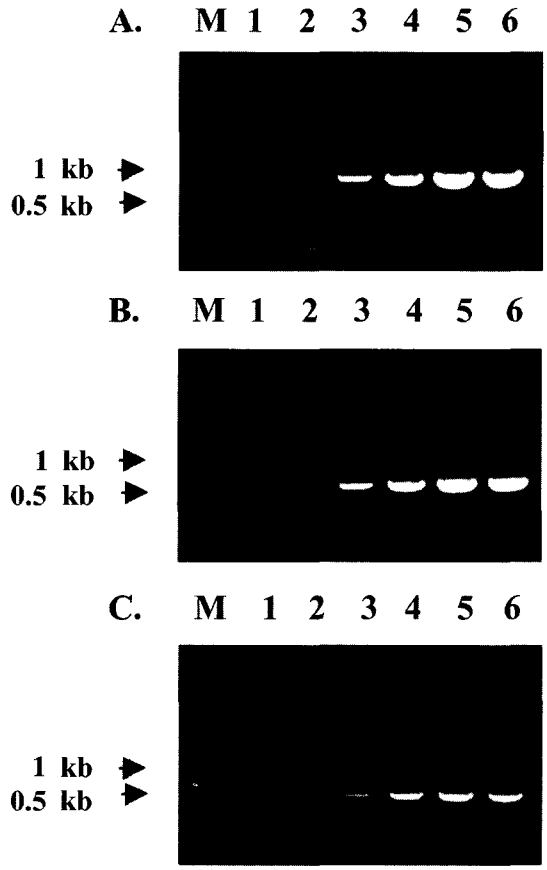

Fig. 6. Sensitivity of DNA detection of *Nosema* spp.. (A), *Vairimorpha* spp. (B), and *Vairimorpha necatrix* (C) spores in saline, determined by PCR and by ethidium bromide staining.
Templated DNAs were prepared from 1 ml of saline containing spores as follows: M, DNA size marker (1 kb ladder); (A) Lane 1, 1 spore/ml; Lane 2, 10 spore/ml; Lane 3, 10^2 spore/ml; Lane 4, 10^3 spore/ml; Lane 5, 10^4 spore/ml; Lane 6, 10^5 spore/ml; (B) Lane 1, 1 spore/ml; Lane 2, 10 spore/ml; Lane 3, 10^2 spore/ml; Lane 4, 10^3 spore/ml; lane 5, 10^4 spore/ml; Lane 6, 10^5 spore/ml; (C) Lane 1, 1 spore/ml; Lane 2, 10 spore/ml; Lane 3, 10^2 spore/ml; Lane 4, 10^3 spore/ml; Lane 5, 10^4 spore/ml; Lane 6, 10^5 spore/ml.

Thus, multiplex PCR yielded an amplified product which allows the differentiation of genus *Nosema* and genus *Vairimorpha* and the identification of *V. necatrix*.

Sensitivity of PCR Assay for Detection of Entomopathogenic Microsporidia

To examine the detection sensitivity, serial 10-fold dilutions of *Nosema* spp., *Vairimorpha* spp., and *Vairimorpha necatrix* spores were made in distilled water. PCR samples were prepared from each of the diluted samples as described in Materials and Methods. The PCR assay described here demonstrated a detection limit of approximately 10 spores per milliliter in saline for each of the strains (Fig. 6). It was also found that the intensity of the bands was in direct proportion to the amount of the template DNA used in the PCR reactions.

DISCUSSION

Microsporidiosis is an infection caused by one of many species of the genus *Nosema*, *Vairimorpha*, *Thelohania*, *Pleistophora*, and *Orthosomella* in Lepidoptera, and the most common pathogenic genus is *Nosema*. However, the number of infections with the recently identified genus *Vairimorpha* has increased, particularity in *Pieris rapae* (Lepidoptera) [14]. The various signs and symptoms were associated with microsporidiosis in insects from obvious tissue manifestations to abnormal developmental and behavioral change [2]. The two most infected insect tissues from which microsporidia have been reported are the fat body and midgut epithelium.

In this study, it was demonstrated that PCR is a sensitive and specific method for diagnosing microsporidia infections in insects. Among the molecular techniques such as PCR, restriction mapping, and hybridization probes, PCR has been the most widely employed for microsporidia diagnosis and epidemiologic studies [28].

The SSU rRNA gene sequences of many microsporidia have been elucidated and found to diverge greatly from other eukaryotes; the sequence is shorter and shares little homology with other eukaryotes [28]. Analysis of SSU rRNA has been possible to identify microsporidia at the species level without the use of ultrastructural examination [14]. Thus, the SSU rRNA genes of the microsporidia possess characteristics amenable to molecular detection. The genus *Vairimorpha* and genus *Nosema* microsporidia SSU rRNA gene-specific primers that were developed successfully amplified an 831 bp fragment from DNA preparations from purified microsporidia spores. Conversely, this primer pair did not amplify DNA preparations from uninfected larvae, several other fungi, and bacteria (Fig. 4A). Therefore, the MSSR primer sets exhibit specificity toward genus *Vairimorpha* and genus *Nosema* microsporidia.

The genus *Vairimorpha* SSU rRNA gene-specific primers that were developed successfully amplified a 542 bp fragment from genus *Vairimorpha* DNA (Fig. 5). Conversely, this primer pair did not amplify DNA preparations from other genus microsporidia (Table 2). Therefore, the VSSU primer set exhibits specificity toward genus *Vairimorpha*. The VNAG primer set, specific for the actin gene of *V. necatrix*, served as a positive control in multiplex PCR with the *V. necatrix*-specific primer set. These results suggest that the SSU rRNA gene and actin gene are good candidates for the specific detection of *Vairimorpha* spp. and *V. necatrix*, respectively.

A multiplex PCR system offers some advantages for the detection of *Vairimorpha* spp. and *V. necatrix*, such as detecting coinfections simultaneously, simplifying diagnostic procedures, and saving labor time and costs. In this study, three sets of primers were used; one was specific for *V. necatrix*, the other specific for *Vairimorpha* spp., and a third specific for *Vairimorpha* spp. and *Nosema* spp. microsporidia. The products amplified from *Vairimorpha* spp. and *V. necatrix* with these primers were sufficiently specific to allow for the differentiation of genus *Nosema* and genus *Vairimorpha* and the identification of the *V. necatrix*.

The multiplex-PCR assay developed in this study is expected to reduce the time for routine diagnosis. In conclusion, a multiplex PCR assay has been developed to detect all *Nosema* spp. and *Vairimorpha* spp. microsporidia and to discriminate between genus *Nosema* and genus *Vairimorpha*. With the primer set used in this study, *Nosema* spp. and *Vairimorpha* spp. could be detected at a concentration of 10 spores per milliliter. Although more trials are needed concerning the detection of other *Vairimorpha* species in Lepidoptera, it is expected that the multiplex PCR assay with the primers that were used will be a useful and valuable tool for the detection of the genus *Nosema* and *Vairimorpha* microsporidia.

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