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Specific Amplification of Microsporidian DNA Fragments Using Multiprimer PCR

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Abstract

Using a mixture of several primers, the suitability of multiprimer PCR for the early and simultaneous detection of various kinds of infectious microsporidia of silkworms was evaluated. As a result, specific DNA sequences were amplified by PCR using several primers designed for this study only when genomic DNA of the target microsporidia was used as the DNA template. However, no PCR products were obtained when genomic DNA of the silkworms or other microorganisms was used as the DNA template. In addition, specific DNA sequences were amplified by multiprimer PCR even when silkworms were infected with various kinds of microsporidia. When genomic DNA extracted from silkworm eggs infected with *Nosema bombycis* was used as the DNA template, the specific DNA sequences were amplified by multiprimer PCR. In addition, similar results were obtained even when genomic DNA extracted from silkworms infected with *N. bombycis* was used as the DNA template. These findings suggest that multiprimer PCR using several primers designed for this study is suitable for pebrine inspection of silkworm eggs.

Discipline: Insect pest
Additional key words: *Nosema*, *Bombyx mori*, Silkworm pebrine, transovum transmission

Introduction

Microsporidia are obligate parasitic protozoa. Approximately 1,000 strains of microsporidia have been isolated to date[1], and most of them are parasitic on insects. Silkworm pebrine is caused by transovum transmission or peroral infection of microsporidian *Nosema bombycis*. *N.bombycis* is highly infectious and difficult to eradicate after the development of silkworm pebrine. Therefore, silkworm pebrine markedly decreases the production and quality of cocoons, thus affecting considerably the production of silk.

Previously, the development of silkworm pebrine had been prevented by pebrine inspection of mother moths, which was enacted by the Sericultural Industry Act especially in Japan. However, although the Sericultural Industry Act in Japan was abrogated because the number of silk yarn producing farms decreased, manufacturers other than expert dealers have recently been allowed to produce silkworm eggs. Unexamined silkworm eggs may spread silkworm pebrine, and pebrine inspection of silkworm eggs will be required to investigate the presence or absence of microsporidian infection in imported silkworm eggs.

Since silkworm pebrine is caused by transovum transmission or oral infection with *N. bombycis*, it is highly infectious. Therefore, once silkworm pebrine spreads, it is difficult to eradicate individual silkworms infected with *N. bombycis* alone. Until now, the development of silkworm pebrine had been prevented by pebrine inspection of mother moths. However, silkworm eggs cannot be examined using the same procedure because the number of microsporidia contained in silkworm eggs is limited, and sporulation may not be observed in most cases. Therefore, using silkworms and silkworm eggs infected with microsporidia, we determined whether the detection of silkworm pebrine by multiprimer PCR could be applied to pebrine inspection of silkworm eggs.

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Materials and methods

Microsporidia

Strains of *N. bombycis* NIS-001, *Vairimorpha* sp. NIS-M11, *Vairimorpha* sp. NIS-M12, and *Pleistophora* sp. Sd-NU stored at the Institute of Insect and Animal Sciences, National Institute of Agrobiological Sciences, Independent Administrative Institution were used in this study. *Vairimorpha necatrix* was kindly provided by the Laboratory of Applied Entomology, College of Bioresource Sciences, Nihon University.

Primers

PCR primers specific to microsporidia were designed by comparing DNA sequences registered to a database with newly analyzed DNA sequences of microsporidia (Table 1). NBEF 35F and NBEF957R were prepared based on DNA sequences of an elongation factor of *N. bombycis* (Accession No. AB009600). M11-96F and M11-822R were prepared based on DNA sequences of the small subunit rRNA gene of *Vairimorpha* sp. NIS-M11 (Acc. No. D85501). V70-176F and V70-1898R were prepared based on DNA sequences of the hsp70 gene of *Vairimorpha* sp. NIS-M12. PCR primers for NIS-M12 can also be used to detect DNA sequences derived from *V. necatrix* (Acc. No. AF008215). PSDF1 and PSDR450 were prepared based on DNA sequences of the small subunit rRNA gene of *Pleistophora* sp. Sd-NU (Acc. No. D85000).

Preparation of silkworms infected with microsporidia

Silkworms were infected with microsporidia by the inoculation of approximately 10^4 of spores to newly exuviated silkworms at stages 3–4. That is, an artificial diet soaked in a solution containing spores of microsporidia was ingested by silkworms over 24 h. Subsequently, 1 silkworm was picked up and stored in a deep freezer every day from the second day after the inoculation of microsporidia.

Preparation of silkworm eggs infected with microsporidia

Silkworm eggs infected with microsporidia were prepared by the inoculation of 10^5 of spores to newly exuviated silkworm larvae at stage 3, 4 or 5. That is, an artificial diet soaked in a solution containing spores of microsporidia was ingested by silkworms over 24 h. Thereafter, silkworms were maintained normally to obtain silkworm moths and eggs.

Extraction of genomic DNA from microsporidian spores

To extract genomic DNA from spores of microsporidia, 10 mg of spores were initially placed in a 1.5-mL plastic tube, and then washed with a STE buffer solution containing 10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, pH 8.0, and 10 mmol/L NaCl. After washing, the spores were crushed by vortically mixing with 50 mg of glass beads (G-8772, SIGNA Co.) and 200 L of STE buffer solution for 30 s. Subsequently, the crushed spores were heated at 95ºC for 5 min, and then 150 L of the supernatant was recovered, from which DNA was extracted using the ethanol precipitation method[8].

Recovery of genomic DNA using a pen-type microhomogenizer

One silkworm stored in a deep freezer was placed in a 1.5-mL Eppendorf tube, and then homogenized using a pen-type microhomogenizer 233A2 (Iuchiseieido Co.). After homogenization, Distilled Water (D.W.) was added to adjust the total volume to 400 μL, followed by the addition of the same volume of Tris-Phenol. After gentle swirling, the sample solution was centrifuged to recover the aqueous phase. This procedure was repeated twice,

Table 1. List of PCR primers

Target strains	Primers	Sequence
Nb	NBEF 35F	5'-TGGCGCTGTTGATAAGAGATT-3
	NBEF957R	5'-AATTTAGCAACACAAGCCTTAT-3'
M11	M11-96F	5'-CTCGAATTAGAAAATTCTCTCAA-3'
	M11-822R	5'-TACTTTATTTAATGTACATTTGAAAA-3'
M12	V70-176F	5'-CAAATGACAGGGAAAGAAATAAGTTCCA-3'
	V70-1898R	5'-TTAAATATTTTGTGCTATAGCTTACTC-3'
PSD	PSDF1	5'-CACCAGGTTGATTCTGCCTGACG-3'
	PSDR450	5'-GCTCCGCCTCTCTTTCCGTCTCC-3'

Nb: *Nosema bombycis*, M11: *Vairimorpha* sp. NIS-M11, M12: *Vairimorpha* sp. NIS-M12, PSD: *Pleistophora* sp. Sd-NU.

followed by the addition of the same volume of chloroform. After gentle swirling, the sample solution was centrifuged again. Subsequently, 0.1 × volume of 3 mol/L sodium acetate buffer solution (pH 5.2) was added to the aqueous phase, followed by ethanol precipitation by the addition of 2.5 × volumes of 99.5% ethanol. The precipitate was recovered by centrifugation before dissolving in 50 μL of TE buffer solution.

Recovery of DNA from silkworm eggs using the KOH method

DNA was extracted from silkworm eggs using the KOH method[10]. That is, 1, 5, 10, and 20 silkworm eggs were independently placed in an Eppendorf tube, followed by the addition of 500 μL of 30% KOH. The sample solution was allowed to stand for about 20 min until the color of the silkworm eggs turned red. After confirming the color change, KOH was removed, and then the silkworm eggs were washed twice with 500 μL of D.W. and once with 1 mol/L HCl in order to return to the neutrality. The silkworm eggs were then washed twice with D.W., and gently swirled after the addition of 500 μL of 2% SDS solution. Subsequently, the same volume of Tris-Phenol was added, and the solution was centrifuged after gentle swirling to recover the aqueous phase. Phenol extraction was performed twice, and then ethanol precipitation was performed after washing with chloroform. In addition, the extracted DNA was rinsed twice with 70% ethanol before dissolution in 100 μL of TE solution.

Multiprimer PCR

Table 1 shows the respective primers used for PCR. PCR was carried out as follows: after preheating of the reaction mixture ultimately containing 10 mmol/L Tris-HCl (pH 8.9), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTP, 0.5 μmol/L of the respective primers, and 10 ng of template DNA at 98ºC for 5 min and subsequent cooling on ice, 2.5 units of Z-Taq DNA polymerase (TAKARA Syuzo Co.) were added, followed by 35 cycles of denaturing at 94ºC for 2 s, annealing at 55ºC for 2 s, and extension at 72ºC for 10 s using GeneAmp PCR System 9700 (Applied Biosystems Co.). After PCR, a 5 μL aliquot of the PCR mixture was mixed with 5 μL TE buffer and 2 μL 40% sucrose containing 0.25% BPB and subjected to 2% agarose gel (Type-II medium EEO A-6877, Sigma Co.) electrophoresis with 200bp DNA size markers. The gels were stained with ethidium bromide.

Results

Evaluation by multiprimer PCR

PCR was carried out using primers designed for this study and genomic DNA conventionally purified and extracted from silkworms and several kinds of microsporidia as the DNA templates. As a result, PCR products specific to the respective strains of microsporidia were obtained when genomic DNA of the microsporidia was used as the DNA template. Subsequently, it was confirmed that target PCR products alone were obtained even when a mixture of several primers specific to the respective strains of microsporidia was used, resulting in amplification of specific DNA sequences alone without amplification of any other DNA sequences (Fig. 1).

Evaluation of the specificity of primers

It was confirmed that the PCR primers designed for this study did not react with genomic DNA extracted from silkworms, the host. Therefore, no PCR products were definitely obtained by multiprimer PCR when genomic DNA extracted from the silkworms was used as

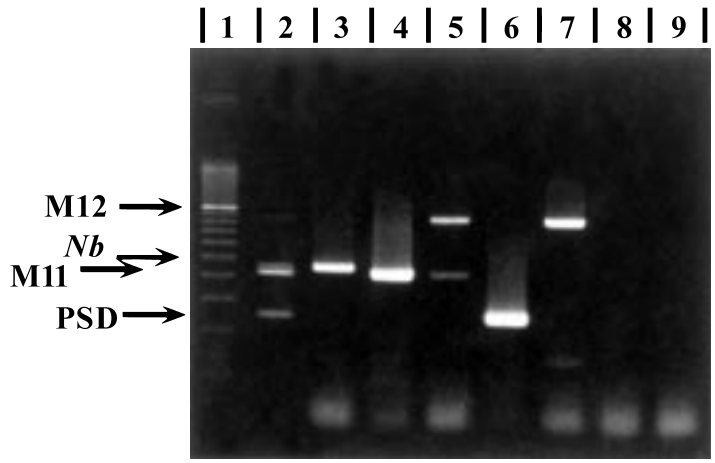

Fig. 1. Detection of microsporidia using multiprimer PCR

Lane 1: 200 bp. DNA marker,
2: product marker,
3: *N. bombycis* NIS-001,
4: *Vairimorpha* sp. NIS-M11,
5: *Vairimorpha* sp. NIS-M12,
6: *Pleistophora* sp. Sd-NU,
7: *V. necatrix*,
8: *Bombyx mori*,
9: primer only (control).

NIS-M12: *Vairimorpha* sp.NIS-M12,
NbNIS-001: *N. bombycis* NIS-001,
NIS-M11: *Vairimorpha* sp. NIS-M11,
PSD Sd-NU: *Pleistophora* sp. Sd-NU.

the DNA template. In addition, similar results were obtained even when a mixture of genomic DNA of microsporidia was used as the DNA template. Furthermore, we carried out multiprimer PCR using genomic DNA extracted from *Beauveria hassiana* and *Escherichia coli* as the DNA templates to confirm that these PCR primers did not react with other pathogenic microorganisms growing in a similar environment. As a result, no PCR products were obtained. Therefore, it was confirmed that the PCR primers designed for this study reacted specifically with microsporidia (Fig. 2).

Differentiation of complex infection with several strains of microsporidia

To confirm the suitability of multiprimer PCR for differentiating several strains of infectious microsporidia of silkworms, PCR was carried out using a mixture of genomic DNA extracted from *Vairimorpha* sp. NIS-M11, *Vairimorpha* sp. NIS-M12, and *Pleistophora* sp. Sd-NU as the DNA templates or using genomic DNA from these strains of microsporidia independently as the DNA template. As a result, similar results were obtained regardless of the presence or absence of complex infection with several strains of microsporidia. These findings indirectly suggest that complex infection with several strains of microsporidia does not influence the results of multiprimer PCR (Fig. 3).

Pebrine inspection of infected silkworms

When PCR was carried out using genomic DNA extracted from silkworms infected with microsporidia as the DNA template, PCR products were obtained 2 days after inoculation. As shown in Fig. 4, the amount of PCR products increased day by day, probably because infectious microsporidia proliferated every day in the host. These findings suggest that *N. bombycis* can be detected from the second day of infection. In addition, PCR products were definitely obtained when 10^4 of spores were inoculated to newly exuviated silkworms at stage 3 or 4 (data not shown).

Pebrine inspection of silkworm eggs using multiprimer PCR

When PCR was carried out using genomic DNA extracted from silkworm eggs infected with *N. bombycis* obtained from mother moths to which the microsporidia were inoculated at stage 5, PCR products were obtained even from 1 silkworm egg. The amount of PCR products increased with the number of silkworm eggs examined (Fig. 5), suggesting that *N. bombycis* infection can be detected regardless of the number of silkworm eggs examined. That is, *N. bombycis* infection can be detected using at least 1 silkworm egg. However, silkworm eggs could not be obtained from mother moths to

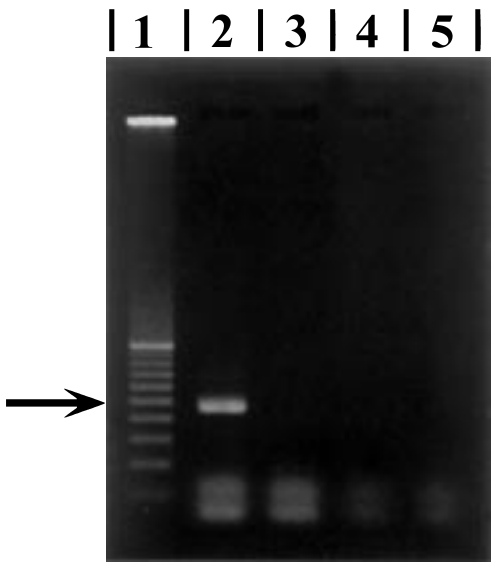

Fig. 2. Multiprimer PCR for genomic DNA from microorganisms other than microsporidia
Lane 1: 200 bp. DNA marker, lane 2: *N. bombycis* NIS-001, lane 3: *Bombyx mori* , lane 4: *B. bassiana,* lane 5: *E. coli.*

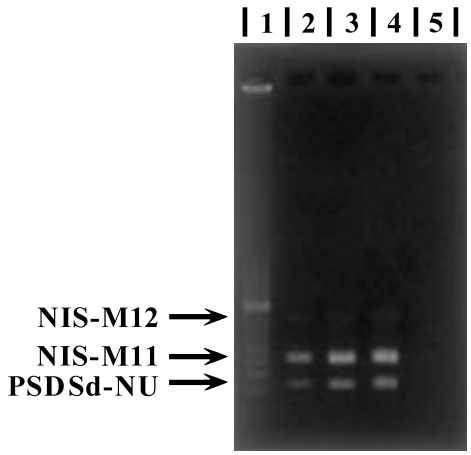

Fig. 3. Multiprimer PCR for complex infection with several strains of microsporidia
Lane 1: 200 bp. DNA marker, lane 2: mixture of 3 PCR products of genomic DNA from *Vairimorpha* sp. NIS-M11, *Vairimorpha* sp. NIS-M12, and *Pleistophora* sp. Sd-NU, lane 3: PCR products of a mixture of genomic DNA from *Vairimorpha* sp. NIS-M11, *Vairimorpha* sp. NIS-M12, and *Pleistophora* sp. Sd-NU, lane 4: PCR products of a mixture of genomic DNA from 3 different strains of microsporidia and genomic DNA from silkworms , lane 5: primer only (control).

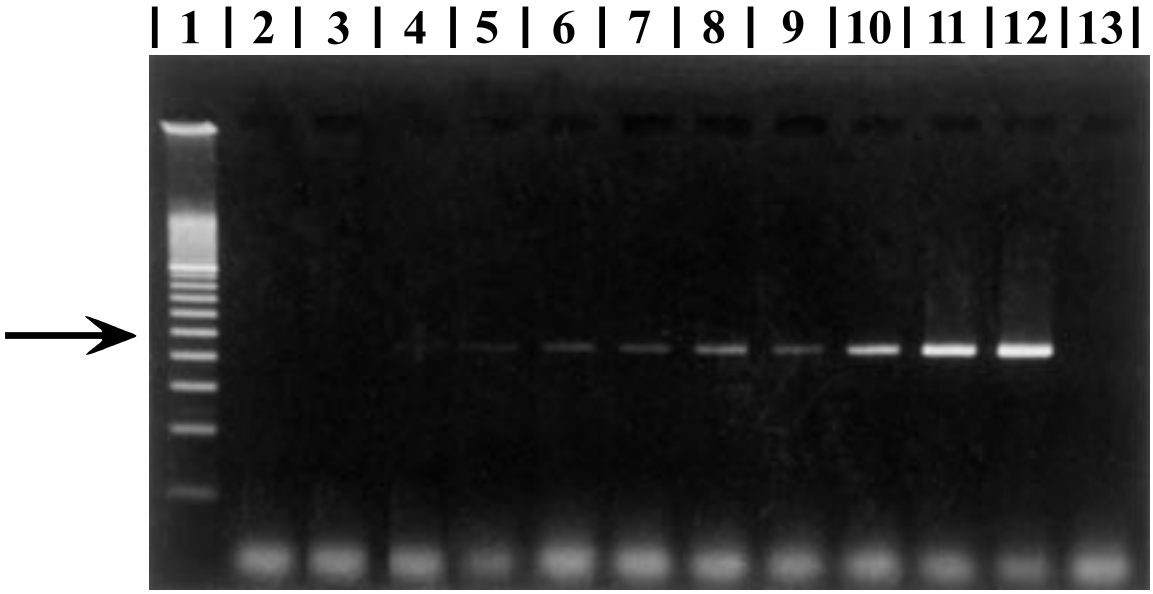

Fig. 4. Pebrine inspection of infected silkworms
Lane 1: 200 bp. DNA size marker, lanes 2–11: PCR products obtained on the respective days after inoculation, lane 12: *N. bombycis,* lane 13: primer only (control).

which *N. bombycis* was inoculated at stage 3 or 4. Although PCR products were not obtained by multiprimer PCR when genomic DNA extracted from 20 silkworm eggs infected with *Vairimorpha* sp. NIS-M11 was used as the DNA template, PCR products were obtained when 100 silkworm eggs infected with *Vairimorpha* sp. NIS-M11 were used (data not shown). Moreover, PCR products were not obtained by multiprimer PCR even when genomic DNA extracted from 100 silkworm eggs infected with *Vairimorpha* sp. NIS-M12 was used as the DNA template.

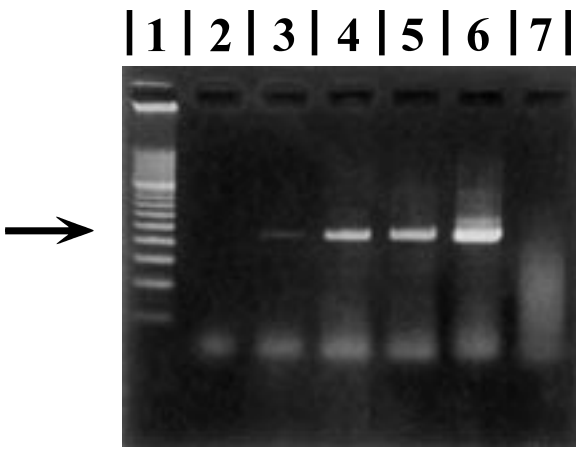

Fig. 5. Pebrine inspection of silkworm eggs using multiprimer PCR
Lane 1: 200 bp. DNA size marker, lane 2: PCR products of genomic DNA from 1 silkworm egg, lane 3: PCR products of genomic DNA from 5 silkworm eggs, lane 4: PCR products of genomic DNA from 10 silkworm eggs, lane 5: PCR products of genomic DNA from 20 silkworm eggs, lane 6: PCR products of genomic DNA from *N. bombycis* spores, lane 7: PCR products of genomic DNA from uninfected silkworm eggs.

Discussion

Previously, immunoperoxidase-staining methods[2], fluorescent antibody technique[9], and latex fluorescent antibody technique[4,7] had been used for pebrine inspection of mother moths. Since pebrine inspection of 1 silkworm was completed only by 1 procedure of multiprimer PCR, the sample volume was markedly limited compared to that of conventional procedures. Kawakami et al.[6] reported the specific detection of a toxic strain of *N. bombycis* using specific primers. They used DNA isolated from microsporidian spores. In this paper, we attempted to conduct pebrine inspection of silkworm eggs using the multiprimer PCR method. We developed this method considering the practical utilization.

As a result of pebrine inspection of silkworm eggs, it was confirmed that *N. bombycis* infection could be detected even in 1 silkworm egg. This finding supported the results of a study by Han and Watanabe[3] showing that silkworm pebrine was caused by transovum transmission of *N. bombycis*, and that *N. bombycis* could be detected regardless of the amount of silkworm eggs examined. In

addition, PCR products were not definitely obtained when genomic DNA extracted from 20 silkworm eggs obtained from mother moths infected with *Vairimorpha* sp. NIS-M11 were used as the DNA template. The rate of transovum transmission of *Vairimorpha* sp. NIS-M11 has been reported to be approximately 1.2%[3], suggesting that at least 100 silkworm eggs were necessary to detect DNA sequences derived from infectious *Vairimorpha* sp. NIS-M11. Furthermore, PCR products were not obtained even when genomic DNA, extracted from 100 silkworm eggs infected with *Vairimorpha* sp. NIS-M12, was used as the DNA template. Although Iwashita et al.[5] reported the results of a study on *Vairimorpha* sp. NIS- M12, no previous studies had confirmed the transovum transmission of *Vairimorpha* sp. NIS-M12. Therefore, it is likely that infection with *Vairimorpha* sp. NIS-M12 could not be detected because silkworms were not infected with *Vairimorpha* sp. NIS-M12 via transovum transmission, or because the amount of infectious *Vairimorpha* sp. NIS-M12 was markedly lower than the minimal detection range. Previously, the presence or absence of silkworm pebrine had been investigated by pebrine inspection of mother moths. However, the results of this study suggest that multiprimer PCR could be applied to the pebrine inspection of silkworm eggs.

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