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Comparison of Amplified RNA Gene Sequences From Microsporidian Parasites (*Agmasoma* or *Thelohania*) in *Penaeus merguiensis* and *P. monodon*

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Abstract

The microsporidian parasites (*Agmasoma* or *Thelohania* sp) of *Penaeus merguiensis* and *P. monodon* have identical morphology with the light and electron microscopes, suggesting that a single parasite species infects both shrimp. However, cross-infection studies to test this hypothesis have not been possible because the intermediate host required for disease transmission is unknown. This study compared DNA extracts from microsporidian spores of infected *P. merguiensis* and *P. monodon* to help answer the question of conspecificity. In these tests, a part of the *Agmasoma* small-subunit ribosomal RNA (SSU rRNA) gene from each host was amplified by the PCR reaction using synthesized degenerate primers based on highly conserved sequences of the SSU rRNA gene from another microsporidian (*Vairimorpha necatrix*) and from *Escherichia coli*. After amplification, the fragments were inserted into the plasmid pGEM7Zf+ and amplified in *E. coli* JM109. The cloned fragments were verified as *Agmasoma* DNA by signal expression (Digoxigenin labelling) with *Agmasoma* DNA and by lack of expression with DNA from other organisms including the two shrimp hosts and two marine fish. The two *Agmasoma* recombinant plasmids were then sequenced using a tag dye primer cycle sequencing kit. The amplified regions of the SSU rRNA gene from the two sources were identical for 722 base pairs. The results support the morphological data and strongly suggest that a single microsporidian parasite infects both of these penaeid shrimp.

Introduction

The small-subunit ribosomal RNA (SSU rRNA) is an anciently and universally distributed molecule, functionally constant, and consists of moderately

conserved nucleotides. Therefore, the gene coding for this molecule has been used as a tool to study evolutionary relationships between various organisms (Brock and Madigan 1991). It can be particularly useful in determining evolutionary relationships among unicellular eukaryotic organisms which have few features that can be used for differentiation simply on morphological grounds.

The microsporidia are classified as protozoan eukaryotes (Levine et al. 1980), and indeed, do have nuclei enclosed in a nuclear membrane and undergo the characteristic eukaryotic cell division (Weiser 1990). However, they seem to lack some of the other general properties of eukaryotic cells and share some common characteristics with bacteria. For this reason they are sometimes referred to as ancient eukaryotes. For example, the SSU rRNA of the microsporidian, *Vairimorpha necatrix*, has been found to be 16S rather than 18S (Vossbrinck et al. 1987), as is characteristic of normal eukaryotic cells. It also lacks 5.8S rRNA (Vossbrinck and Woese 1986). At the same time, the SSU rRNA of *V. necatrix* consists of approximately 1,244 nucleotides which is shorter than the SSU rRNA of *Escherichia coli*.

In the case of the microsporidian parasites (*Agmasoma* sp.) of *Penaeus merguiensis* and *P. monodon*, morphological evidence from the light and electron microscopes suggests that a single parasite infects both host shrimp (Flegel et al. 1992). However, an unknown intermediate host is required for infection of the shrimp, so cross-infection studies are not possible using parasite spores derived from the two shrimp species. The purpose of this study was to compare the gene sequence of the SSU rRNA gene of spores derived from the two species of shrimp, *P. merguiensis* and *P. monodon*. If these spores were produced by a single parasite species, it was expected that the DNA base sequences for the genes from the two sources would be identical. This was done by amplifying the variable region of the SSU rRNA gene from the parasite in both shrimp species by the polymerase chain reaction (PCR) using degenerate primers. After the succeseful cloning of the PCR products, the DNA sequences of the microsporidia were compared.

Materials and Methods

Isolation of DNA

The chromosomal DNA of *Agmasoma* sp from *P. merguiensis* and *P. monodon* was extracted and purified from spores isolated from infected tissue of frozen shrimp and purified by the 20-40% sucrose gradient interphase method as described in Pasharawipas and Flegel (1994). Details concerning the source of specimens and extraction of DNA from other organisms, including algae, bacteria, crustaceans, fish, etc. are given in the same article.

Amplification and Cloning of PCR Products

A region of the small ribosomal RNA gene was amplified by using synthesized degenerate primers based on a highly conserved sequence of the SSU

rRNA gene of *V. necatrix* and *E. coli* (Vossbrinck et al. 1987). The couple primers were: TS1, the upstream primer, 5' GTCG**GAATTC**GCCAGCAGCCGCGGT 3', and TS2, the downstream primer, 5'CAGC**GGATCC**GTCAAATTAAGCCGC3'. The upstream primer contained an inserted EcoRI restriction sequence (bold letters) preceeded by inserted clamp nucleotides, and the downstream sequence contained an inserted BamHI restriction sequence (bold letters) preceeded by inserted clamp nucleotides. The amplification reaction was modified from previous methods described by Innis and Gelfand (1990) and Compton (1990). Specifically, the reaction was conducted in a 100 µl solution containing 30 ng of the *Agmasoma* DNA template with a buffer solution containing 20 mM Tris-HCL (pH 8.8, 25°C), 10 mM KCl, 4 mM MgSO4, 0.1% Triton X-100, 0.1 µg Bovine serum albumin, 30 pmol of each primer, and 50 µM of dNTP. Finally, 2.5 units of *Taq* polymerase enzyme was added and the mixture was heated to 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 55°C for 1.5 minutes and 72°C for 3 minutes. The reaction was concluded with a final extension at 72°C for 5 minutes. The amplified products were checked by running 1% agarose gel electrophoresis and by visualizing the completed gels under UV light.

In the amplified products of DNA from both shrimp sources, the EcoRI and BamHI sites intended for use in cloning with the plasmid vector pGEM7Zf+ could not be used directly as planned. The reason for this was an unexpected internal EcoRI site approximately 50 bps from the 3' end (BamHI end) of the amplified segment. This was proven by digestion of both amplified products with each enzyme separately. EcoRI digestion of the amplified fragments yielded a single fragment significantly smaller than the undigested fragments, while the BamHI digestion product was of the same length (data not shown). Therefore, digestion with EcoRI removed the BamHI sequence. At the same time, the EcoRI digestion product could not be cloned into dephosphorylated, EcoRI-digested pGEM7Zf+. This indicated that the EcoRI restriction site designed into the original primer was not functional after amplification. To circumvent these problems, the amplified DNA segments (after digestion by EcoRI) were successfully cloned into the EcoRI/SmaI sites of pGEM7Zf+, instead of the EcoRI/BamHI sites as originally planned. Finally, the ligated plasmids were transformed and amplified in *E. coli* JM 109 host cells.

Specificity of the PCR Products

The PCR products and the recombinant plasmids were checked as actually derived from each *Agmasoma* by using the Southern blot hybridization technique. The PCR products or the EcoRI/BamHI cleaved recombinant pGEM7Zf+ products were run on 1.2% agarose gel electrophoresis and transferred to nitrocellulose filters by the Southern blot technique. After baking at 80°C for 2 hours (Sambrook et al. 1989) they were hybridized with digoxigenin (Boeringer) labelled *Agmasoma* DNA from each source. Alternatively, dot blot hybridizations were carried out for convenience. Negative controls included PCR products derived from other organisms including both host penaeid shrimp and two marine fish (*Scatophagus argus* and *Priacanthus tayenus*). No sporozoan or protozoan DNA was included in this test.

Sequencing of the Recombinant Plasmids

The recombinant plasmids were prepared as single stranded DNAs using M13KO7 as a helper phage (Messing 1983). The single stranded DNAs were sequenced with an automated DNA sequencer, Applied Biosystems Model 373A, using a tag dye primer cycle sequencing kit (Biosystems) with an M13 forward primer (-21M13). Double stranded DNAs were sequenced with M13 reverse primers (M13RP1) after purification by the polyethelene glycol technique. Each sequencing operation was repeated using the chromosome walking technique to complete sequencing of both DNA strands with a ready reaction dye terminator cycle sequencing kit (Applied Biosystems). Design primers arose from the sequence results with the tag dye primer cycle sequencing method described above. Finally, results were analyzed with the assistance of the DNA-SIS program.

Results

Amplification Products

The amplification products of the ribosomal RNA genes of the two *Agmasoma* products were approximately 0.8 Kb as shown in Fig. 1. The degenerate primers were also able to amplify major single bands from other organisms including both penaeid shrimp hosts and marine fish, yielding amplified segments of approximately 0.6 Kb. As mentioned in the Materials and Methods section, the internal EcoRI site at the 3' end of the *Agmasoma* DNAs resulted in a final sequence of only 722 nucleotides (known by sequencing) after EcoRI digestion (Fig. 1). After the failure to clone the PCR products at the EcoRI/BamHI sites as first planned, the EcoRI-digested PCR products were cloned into pGEM7Zf+ at the EcoRI site and the SmaI site of pGEM7Zf+, as described in the Materials and Methods section.

Specificity of the Agmasoma *PCR Products*

The PCR products of *Agmasoma* DNAs were proven to be truly derived from the microsporidians by Southern blot hybridization using DIG labelled *Agmasoma* DNA as a probe. Positive hybridization signals were shown only to the PCR products which were amplified from *Agmasoma* DNA templates, and not to the amplified products of the host penaeid shrimp or marine fish (Figs. 2 and 3). Similar tests using recombinant plasmids containing the PCR amplified SSU rRNA region of *Agmasoma* gave the same results.

The Nucleotide Sequences

Amplified rRNA sequences of the microsporidia from both host shrimp are shown in Fig. 4. The sequences were completely homologous for 722 nucleotides of the *Agmasoma* DNA from *P. merguiensis* and *P. monodon*. Use of the

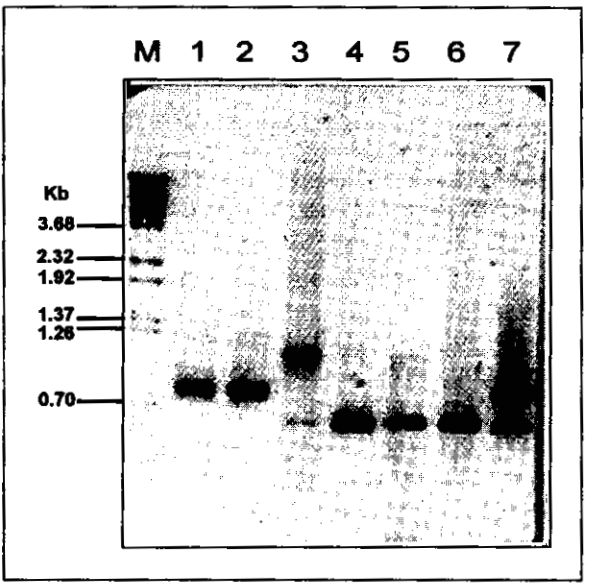
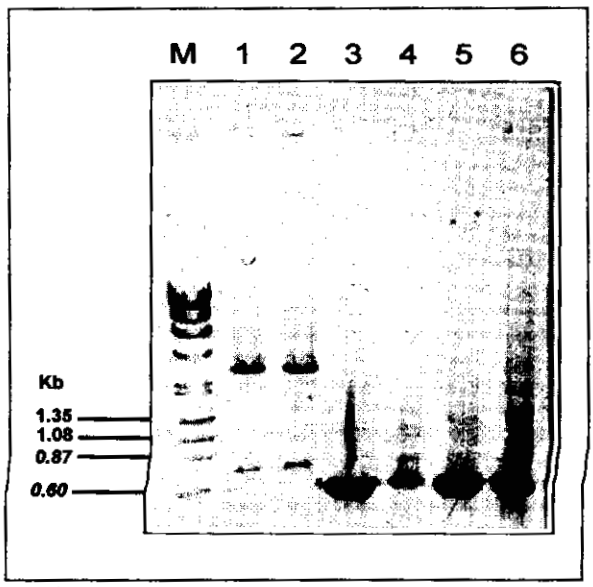


Fig. 1. Agarose gel of PCR amplified SSU rRNA fragments stained with ethidium bromide and visualized with ultraviolet light. A black and white polaroid photograph of the gel was scanned into a computer. The resulting image was then reversed before printing of the figure shown here. Template DNA was derived from *Agmasoma* of *P. merguiensis* and *P. monodon* and from other sources. Lane M, molecular weight markers HindIII digested lambda DNA and HaeIII digested PhiX-174; lane 1, DNA of purified spores from *P. merguiensis*; lane 2, DNA of purified spores from *P. monodon*; lane 3, DNA from the gut of the fish *Priacanthus*; lane 4, DNA from the gut of the fish *Scatophagus*; lane 5, DNA from the muscle of *P. merguiensis*; lane 6, DNA from the muscle of *P. monodon*: lane 7, unpurified spores from *P. monodon*. The amplified SSU rRNA products from the *Agmasoma* DNA are about 0.79 Kb, while those of the fish and shrimp are about 0.6 Kb The prominant upper band in lane 3 and the fainter matching bands in lanes 4 to 6 are due to the presence of bacteria in the extracted tissues. Note that the DNA extract from an unpurified spore preparation (lane 7) shows two prominant bands, one for the *Agmasoma* DNA product (upper band) and one for the host DNA product.

Fig. 2. Agarose gel of PCR amplified SSU rRNA fragments, cloned or uncloned, stained with ethidium bromide and visualized with ultraviolet light. A black and white polaroid photograph of the gel was scanned into a computer. The resulting image was then reversed before printing of the figure shown here. Template DNA was derived from *Agmasoma* of *P. merguiensis* and *P. monodon* and from other sources. Lane M, molecular weight markers HindIII digested lambda DNA and HaeIII digested PhiX-174; lane 1, PCR fragment from *Agmasoma* of *P. merguiensis* cloned with pGEM7Zf+ and digested with EcoRI and SmaI; lane 2, PCR fragment from *Agmasoma* of *P. monodon* cloned with pGEM7Zf+ and digested with EcoRI and SmaI; lane 3, direct PCR fragment from gut DNA of the fish *Priacanthus*; lane 4, direct PCR fragment from gut DNA of the fish *Scatophagus*; lane 5, direct PCR fragment from muscle DNA of host *P. merguiensis*; lane 6, direct PCR fragment from muscle DNA of *P. monodon*. Note that the prominent upper bands in lanes 1 and 2 are from EcoRI/SmaI digested pGEM7Zf+, while the lower bands are PCR products.



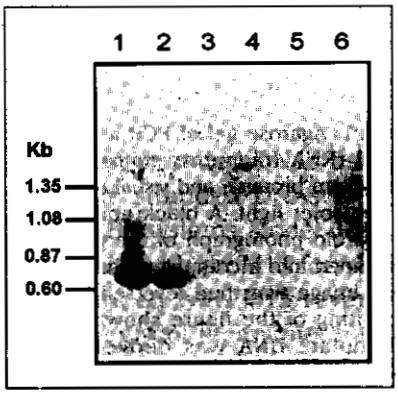


Fig. 3. Southern blot hybridization of DNA fragments from the gel shown in Fig. 2. The filter was probed with DIG labelled *Agmasoma* chromosomal DNA derived from the parasite in *P. monodon*. Only the cloned PCR product of *Agmasoma* DNA from the two host shrimp gave a strong hybridization signal with the probe.

DNA homology search (Genbank) showed that these amplified products were homologous only to other SSU rRNA sequences. Closest homology was to sequences from other microsporidians, that is (in order of highest to lowest homology), *Enterocytozoon bieneusi* (Zhu et al. 1993), *Encephalitozoon hellem* and *Encephalitozoon cuniculi* (Vossbrinck et al. 1993), and *V. necatrix* (Vossbrinck et al. 1987). The sequence of amplified *Agmasoma* SSU rRNA is aligned to the sequence of *V. necatrix* in Fig. 4.

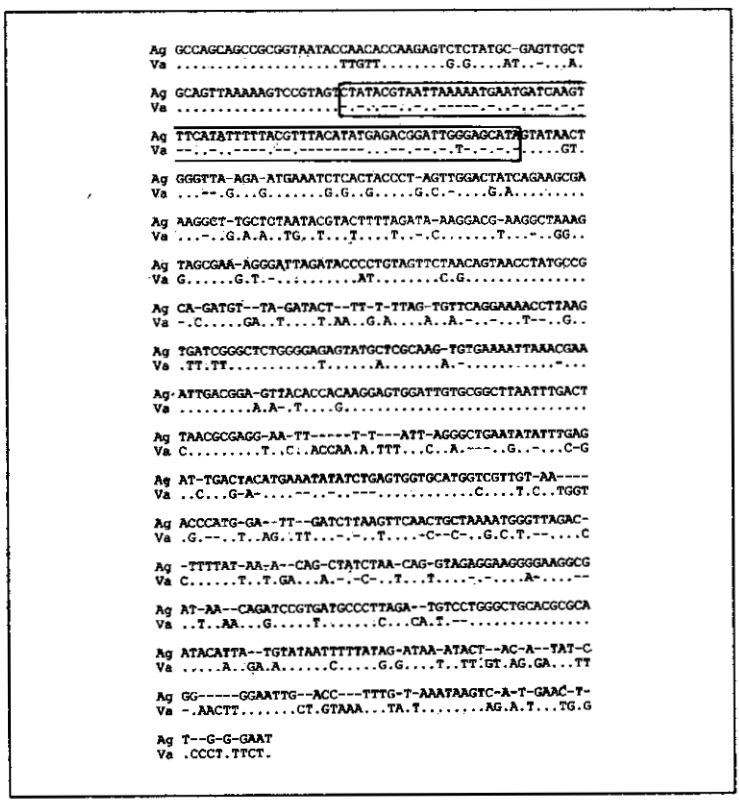


Fig. 4. The amplified SSU rRNA gene fragment of *Agmasoma* from *P. merguiensis* (*P. monodon* identical) aligned for comparison with the corresponding region of the SSU rRNA gene of *Vairimorpha necatrix*. The box in the figure identifies the regions of the two fragments that correspond to the region 590-650 in the *E. coli* SSU rRNA gene. Those positions of both sequences that have the same composition are shown as dots; dashes signify that no base occurs at those positions (Ag = *Agmasoma*, Va = *Vairimorpha*)

Discussion

The amplified products of the *Agmasoma* DNA isolated from spores purified by the 20-40% sucrose gradient technique gave a single major band upon agarose gel electrophoresis. The lack of minor bands might have been due to highly specific binding of the designed primers to the template DNA and to optimal conditions for the amplification procedure. Alternatively, the result might have been due to the efficiency of elimination of any contamination during the spore purification procedure, especially contamination of shrimp cells. The latter seems to be the most likely explanation, since amplification using unpurified *Agmasoma* spores resulted in two amplified products, one of which corresponded to the *Agmasoma* DNA and the other to shrimp DNA (Fig. 1).

The degenerate primers used to amplify *Agmasoma* DNA in this study were based on sequences of the ribosomal RNA gene from *V. necatrix* coding for rRNA helix regions 19 and 33 of the *V. necatrix* SSU rRNA. It is apparent that the corresponding SSU rRNA sequence of *Agmasoma* is longer than the comparable sequence of *V. necatrix* (i.e., approximately 790 bps for *Agmasoma* versus 738 bps for *V. necatrix*). However, this does not mean that the whole SSU rRNA for *Agmasoma* will be longer than that of *V. necatrix*. It would be necessary to sequence the whole SSU rRNA gene of *Agmasoma* before any conclusion could be made in this regard.

It was proven, by the Southern blot hybridization technique, that the amplified SSU rRNA gene fragments produced in this study were derived from the chromosome of *Agmasoma* sp. That is, the DIG-labelled *Agmasoma* DNA gave a positive hybridization signal only with the amplified product from *Agmasoma*, and not with the amplified products from penaeid shrimp and marine fish. The PCR amplification process with the penaeid shrimp and fish used in this study sometimes gave a product which corresponded to an amplified SSU rRNA gene sequence from *E. coli* (Fig. 1). This indicated that the template DNA extract contained DNA from *E. coli* or other bacteria. For the fish DNA extracts prepared from gut tissue, this is understandable, but for the DNA extracts prepared from shrimp muscle, the result is unexpected. It is possible that the shrimp specimen used had a nonsymptomatic bacterial infection or that the extract became contaminated with bacteria during handling.

The initial failures in cloning the PCR amplified products into the EcoRI/BamHI sites of plasmid pGEM7Zf+ was believed to be due to the unexpected occurrence of an internal EcoRI restriction site in the amplified fragments. The existence of this site, approximately 50 bps from the 3' end (BamHI end) of the amplified segment, was eventually proven by sequencing of the fragments. The reason why the EcoRI-digested fragments could not be cloned into EcoRI digested and dephosphorylated pGEM7Zf+ is not clear. However, since cloning was eventually successful using the internal EcoRI site and a blunt end, into the EcoRI/SmaI sites of pGEM7Zf+, it appeared that the synthesized EcoRI site designed into the original primer sequence did not function.

From molecular genetic results, there are three reasons to support the hypothesis that the *Agmasomas* infecting both of these penaeid shrimp are conspecific. Firstly, sequencing results in this study showed that the amplified SSU

rRNA gene fragments derived from *Agmasomas* of *P. merguiensis* and *P. monodon* were identical over an interval of 722 nucleotides. The degenerate primers used to produce these fragments bracket the corresponding helix regions 19-33 of the *V. necatrix* SSU rRNA (Neefs et al. 1991) and this region corresponds to a variable region of the SSU rRNA gene. The fact that the fragments from the two sources are identical over this variable region supports the contention that they are derived from a single species. Secondly, it was found that the PCR products (SSU rRNA) of both *Agmasomas* cross-hybridized with the same amount of signal for the same amount of DNA. Finally, in a related study (Pasharawipas and Flegel 1994), the Southern filter of Sau3AI partially digested DNA from both *Agmasomas* gave identical hybridization patterns with each of two different DIG-labelled probes, one derived from *Agmasoma* of *P. merguiensis*, the other derived from *Agmasoma* of *P. monodon*. This information strongly suggests that the DNA of the parasite from both shrimp species is identical. If so, then it should come from a single parasite species.

This conclusion supports the hypothesis cited earlier for conspecificity based on morphology by the light and electron microscopes (Flegel et al. 1992). It is not uncommon for microsporidia to parasitize several different hosts. For example, *Encephalitozoon cuniculi* parasitizes a wide range of host mammals (Canning and Hollister 1987). The *Agmasomas* in the two shrimp studied here are also morphologically similar to that reported for other penaeid shrimp, as *Agmasoma penaei* (Lightner 1988). It is possible that a single microsporidian infects all of these shrimp. The amplification and cloning system described here could easily be used to help answer the question.

Using the DNA homology search data base of GenBank, it was found that sequences from the amplified products of the two *Agmasoma* sources showed homology only to SSU rRNA gene sequences of other organisms (at least 200 known SSU rRNA sequences). This confirmed the assumption that the amplified products were actually derived from SSU rRNA genes. Although the closest homology was to other microsporidian species, the degree of homology was not high (e.g., roughly 60% homology for the closest sequence from *Enterocytozoon bieneusi*). However, the length of the sequences compared was rather small and there is too little information to make any conclusions regarding evolutionary connections.

Curiously, the *Agmasoma* fragment studied here contained a sequence (72 bp box in Fig. 3) that corresponded to the 590-650 bp (*E. coli* numbering) region of the SSU rRNA gene of *E. coli*. Vossbrinck et al. (1987) found this sequence absent in *V. necatrix*. They stated that the 590-650 bp sequence of *E. coli* was relatively conserved in prokaryotes and that comparable sequences from eukaryotic SSU rRNA showed little or no homology to it. The comparable eukaryotic sequences are also much longer (Neefs et al. 1991). Like other eukaryotes, and unlike *V. necatrix*, *Agmasoma* has corresponding bases to the *E. coli* sequence, but like other eukaryotes, it shares little homology with the *E. coli* sequence (50% AT in *E. coli*, 71% AT in *Agmasoma*). On the other hand, the length (72 bp) of the *Agmasoma* sequence is relatively close to that of the corresponding *E. coli* sequence (58 bp) and much shorter than the corresponding sequence from eukaryotes (e.g., 224 bp in *Saccharomyces cerevisiae*, Neefs

et al. 1991). This sequence seems to account for the larger size of the *Agmasoma* fragment when compared to the corresponding fragment of *V. necatrix*. Again, it is difficult to speculate on the differences between these two microsporidians, but the existence of a sequence that is neither characteristic for eukaryotic length nor homologous for prokaryotes, further supports the contention that microsporidians are intermediate forms between eukaryotes and prokaryotes.

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