Interaction between the membrane protein of a pathogen and insect microfilament complex determines insect-vector specificity


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Many insect-transmissible pathogens are transmitted by specific insect species and not by others, even if they are closely related. The molecular mechanisms underlying such strict pathogen–insect specificity are poorly understood. Candidatus Phytoplasma asteris, OY strain, line W (OY), is a phytopathogenic bacterium transmitted from plant to plant by sap-feeding insect vectors (leafhoppers). Our study focused on an abundant cell-surface membrane protein of the phytoplasma named antigenic membrane protein (Amp), which is not homologous with any reported functional protein. Immunofluorescence microscopy of the phytoplasma-infected insect showed that OY phytoplasma was localized to the microfilaments of the visceral smooth muscle surrounding the insect’s intestinal tract. The affinity column assay showed that Amp forms a complex with three insect proteins: actin, myosin heavy chain, and myosin light chain. Amp–microfilament complexes were detected in all OY-transmitting leafhopper species, but not in the non-OY-transmitting leafhoppers, suggesting that the formation of the Amp–microfilament complex is correlated with the phytoplasma-transmitting capability of leafhoppers. Although several studies have reported interactions between pathogens and mammalian microfilaments, this is an example of host-specific interactions between a bacterial surface protein and a host microfilament in insect cells. Our data also suggest that the utilization of a host microfilament may be a universal system for pathogenic bacteria infecting mammals or insects.

host determination | microbiology | pathogen–host interaction | phytoplasma

Insect-transmissible pathogens can cause devastating damage to humans, animals, and even plants, because these pathogens are transmitted rapidly over a wide area. In nature, most insect-transmissible pathogens are carried by specific insect vectors and not by other insects, even if they are closely related species (1, 2). Therefore, the scale of damage caused by a pathogen is determined largely by the number of insect-vector species that are capable of transmitting the pathogen (1). Elucidating the determination mechanisms of insect-vector specificity may enable the rapid discrimination of vector and non-vector insects and allow the monitoring or forecasting of the spread and infection route of the pathogen. Moreover, the development of chemicals to inhibit the interaction between a pathogen and an insect vector may be made possible. The mechanisms of pathogen–insect specificity, however, are poorly understood. The malaria parasite (Plasmodium falciparum) is the most studied insect-transmissible microorganism, and its specificity to its mammalian host has been examined extensively. An interaction between a ligand in mammalian tissues and a receptor protein on the parasite surface has been well characterized (3, 4). However, although several potential parasite receptors and insect ligands have been identified (5–7), the crucial interactions between a malaria parasite and a mosquito that determine its being either a vector or a nonvector in nature are still unclear.

The genus Phytoplasma comprises a group of phytopathogenic bacteria in the class Mollicutes. Phytoplasmas, transmitted from plant to plant by sap-feeding insect vectors (leafhoppers), infect >700 plant species and cause symptoms such as stunting, yellowing, witches’ broom (proliferating shoots), phyllody (leaf-like petals and sepals), virescence (greening of floral organs), and, sometimes, withered plants (1). Phytoplasmas propagate within the cytoplasm of both insects and plants. In plants, phytoplasmas inhabit nutrient-rich phloem tissues exclusively. Phytoplasmas are cell-wall-less and protein, and both their cell size (0.1–0.8-μm diameter) and genome size (0.5–1.3-Mbp) are the smallest among bacteria. The inability to culture phytoplasmas in vitro has hindered their characterization at the molecular level. We recently reported the complete 860-kb genome sequence of the Candidatus Phytoplasma asteris, OY strain, line M, and showed that the genome encodes an extremely limited number of genes, suggesting reductive evolution as a consequence of its being an intracellular parasite (8, 9). Phytoplasmas being the least complex of all bacteria facilitates the study of phytoplasma–host interactions. Elucidating the interactions between phytoplasmas and their hosts would contribute to the knowledge base regarding other parasitic or symbiotic microorganisms.

The parasite factors involved in the interaction with insect cells are likely to be located on the outer surface of the parasite cell. Previous studies have suggested that an immunodominant membrane protein constitutes the major portion of the total cellular membrane proteins in most phytoplasmas (10–14). A gene encoding an immunodominant (antigenic) membrane protein (Amp) of Ca. P. asteris, onion yellow strain, line W (OY phytoplasma), was previously isolated and designated as amp (15). Three repeated coiled-coil sequences were barely detectable in the central hydrophilic domain of Amp. Because the coiled-coil structure is often responsible for protein–protein binding, it has been suggested that Amp functions by binding to another protein (15). In addition, Amp has been predicted to contain a ligand-binding sequence, and a ligand-binding function has been proposed for this protein (13). These potential sequences suggest that Amp is important in the host–phytoplasma interaction, although the biological function of Amp is still unknown.

In this study, we identified insect–host proteins that interact with the Amp of OY phytoplasma. Amp interacted with the

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Abbreviations: Amp, antigenic membrane protein; AM, Amp–microfilament; OY, Candidatus Phytoplasma asteris; strain, line W.

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microfilament complexes of leafhopper species that transmit OY phytoplasma, but not with those of leafhoppers that do not transmit OY phytoplasma. These data suggest that an interaction between Amp and the insect microfilament complexes is involved in phytoplasma transmissibility.

Results

Amp Is Colocalized with the Microfilaments. Intestinal tissues from five OY-infected leafhoppers and five healthy leafhoppers were treated with anti-Amp IgG and then treated with both FITC-conjugated anti-rabbit IgG and rhodamine-coupled phalloidin. Confocal laser scanning microscopic analysis was then performed to clarify the localization of Amp and microfilaments (actin filaments) in the insect intestine. In the OY-infected insect’s tissue, Amp was found to colocalize with the microfilaments of the visceral smooth muscle surrounding the insect’s intestinal tract (Fig. 1). The intestine consists of the epithelial cells surrounded by the connective tissue and the visceral smooth muscle. Strong Amp signals were observed, especially on the inner side of the intestinal muscle band. Interestingly, the Amp signals were remarkably concentrated around the terminal ridges of microfilaments (Fig. 1F). More than 30 microscopic fields were observed, and 84% of fields showed the colocalization of Amp and microfilaments in insect cells. In contrast, >30 microscopic fields of the control intestinal tissues of healthy insects did not show signals typical of Amp (Fig. 1H).

Separation of Insect Proteins Interacting with AMP in Vitro. The total protein extracted from 20 Macrosteles striifrons was loaded onto three affinity columns: Amp, SecA (a membrane protein of OY phytoplasma), or BSA columns. The proteins eluted from these columns were separated by using both SDS/PAGE (Fig. 2) and 2D electrophoresis (see Fig. 5, which is published as supporting information on the PNAS web site). Three proteins, P30 (~30 kDa), P42 (~42 kDa), and P200 (~200 kDa), were specifically detected in the Amp affinity column (Fig. 2) but were not detected in the BSA (Fig. 2) or SecA affinity columns (data not shown). Among these three proteins, P42 was separated into four major protein spots with similar charges (pH ~5.5) after 2D electrophoresis (Fig. 5).
MS Analysis and Peptide Sequences of Insect Proteins That Form a Complex with Amp. These protein spots were cut out from both the SDS/PAGE and 2D electrophoresis gels and digested by using trypsin. To identify these proteins, peptide mass fingerprints were performed using MALDI-TOF MS and analyzed by using the ms-fitt system. All P42 from the SDS/PAGE gel and four spots from the 2D electrophoresis gel showed identical mass spectra: 976.43, 1198.69, 1486.68, 1790.89, and 1968.10. All mass spectra were matched with spectra of the actins from brine shrimp (Artemia salina) and silkworm (Bombyx mori) in the mass database. In addition, the P42 protein band was excised from the SDS/PAGE gel, and peptide sequence analysis was performed. The amino acid sequences were also similar to the actins from brine shrimp, silkworm, and Arabidopsis thaliana (see Fig. 6, which is published as supporting information on the PNAS website). Subsequently, total proteins eluted from the Amp affinity column were subjected to Western blot analysis using an anti-chicken actin antibody. As shown in Fig. 3 A, a clear protein band of ∼42 kDa was detected, suggesting that all P42 protein spots are actin, because the amino acid sequences of actins are highly conserved from animals to plants.

Similar MS analyses were performed on P200 and P30 proteins. The MS spectra of P200 (2101.14, 2938.29, 2986.77, and 3114.61) matched those of the myosin heavy-chain class V of Cryptococcus neoformans var. grubii. Because no specific candidate proteins were observed in the mass database for P30, we could not identify the P30 protein. Therefore, we performed a peptide-sequencing analysis for P30. The P30 protein spot was excised from the SDS/PAGE gel, digested by endoproteinase Lys-C, and fractionated by using a reversed-phase HPLC. Eight peptide fragments were collected, and 7–15 amino acid sequences from each fragment were determined. Six of these peptide sequences showed remarkably high homology with insect myosin light chain 2 (see Fig. 7, which is published as supporting information on the PNAS website).

Cloning of Genes Coding Insect Proteins That Form a Complex with Amp. To confirm the results of the mass spectrum and peptide sequence analyses, actin and myosin light-chain genes were cloned and sequenced from both the OY-transmitting leafhopper M. striifrons and the non-OY-transmitting leafhopper Ophiola flavopicta. The deduced amino acid sequences of the cloned actin or myosin light chain were aligned with the corresponding sequences from several organisms (Figs. 6 and 7). The actin sequences were very similar to each other (96% identity) and were highly homologous to the actin sequences from other organisms (96% identity with brine shrimp and 95% identity with A. thaliana). The myosin light-chain sequences were also very similar to each other (92% identity), and these showed high homology with the myosin light-chain sequences from other organisms (56% identity with Anopheles gambiae and 54% identity with Drosophila melanogaster). All peptide fragments analyzed with MS or peptide sequencing were completely consistent with the amino acid sequences encoded in the genes from the OY-transmitting leafhopper (Figs. 6 and 7), suggesting that Amp forms a complex with three insect proteins: actin, myosin heavy chain, and myosin light chain.

Amp Interacts with Insect Microfilament Complexes in Vivo. To confirm the interaction between phytoplasma Amp and insect microfilament complexes in vivo, total soluble extracts from 80 OY phytoplasma-infected leafhoppers or 80 healthy leafhoppers (M. striifrons) were loaded onto an anti-Amp antibody affinity column. Total proteins eluted from the column were subjected to SDS/PAGE, followed by Western blot analysis. Because phytoplasma proteins occur in very small amounts in insect tissue, no marked signal was detected in the SDS/PAGE analysis. Therefore, the Western blot analysis using an anti-actin antibody was conducted to detect the Amp–microfilament (AM) complex. A clear actin protein band was detected in the extract.
from OY-infected leafhoppers (Fig. 3B, lane 2) but not in that from healthy control leafhoppers (Fig. 3B, lane 1), suggesting that Amp and insect microfilament complex are associated within OY-infected leafhoppers.

**Correlation Between AM-Complex Formation and Insect Transmission.** Because it is known that the OY phytoplasma is transmitted by three leafhoppers (M. striifrons, Hishimonoides sellatus, and H. sellatus), but not by other leafhoppers (O. flavopicta or Nephrotettix cincticeps) (16), we investigated the correlation between the AM-complex formation and the phytoplasma transmission by insects. Insect bodies of 20 M. striifrons, 15 H. sellatus, 5 H. sellatiformis, 10 O. flavopicta, and 10 N. cincticeps were homogenized in 500 μl of Rx buffer. Total protein fractions were extracted from five insect species, and each was subjected to the Amp affinity column assay. The volume of the eluted fraction and 10 μl of the crude fraction from before loading onto the column were subjected to Western blot analysis with an anti-actin antibody. The AM complexes were detected in the samples from the OY-transmitting insect species (M. striifrons, H. sellatus, and H. sellatiformis) but not in those from the non-OY-transmitting species (O. flavopicta and N. cincticeps; Fig. 4), suggesting that the phytoplasma transmission by leafhopper species is correlated with AM-complex formation.

**Discussion**

**Amp Forms a Complex with the Actin and Myosin Proteins of Its Insect Vector.** In this study, microscopic analysis showed that Amp of OY phytoplasmas was localized mainly in muscle cells surrounding the intestinal tract (Fig. 1), and, because Amp is a surface-membrane protein of OY phytoplasmas, its localization indicates the presence and localization of phytoplasma cells. Similar localization has been observed for spiroplasma, a plant pathogen and insect-transmissible bacterium phylogenetically related to phytoplasmas. EM has revealed that spiroplasma cells localize in the intestinal muscle cell of the spiroplasma-infected insects, suggesting that the intestinal muscle cells are the major infection sites of spiroplasma (17, 18). This finding supports the contention that the muscle cells of the insect are an appropriate niche for phytoplasmas. Moreover, the affinity column assay demonstrated that Amp forms a complex with the actin and myosin proteins of the insect vector (Fig. 2). Actin and myosin proteins are constitutive proteins of muscle organs, and this result is in good agreement with the microscopic analysis.

Intracellular parasitic microorganisms have been shown to often use host-cell proteins. In mammal-pathogenic bacteria, such as Shigella, Salmonella, Yersinia, and Listeria, several interactions between the surface membrane proteins of bacteria and the microfilaments of host cells have been reported (19–28). These interactions are essential to the invasion of host cells or to facilitate the intracellular motility of bacteria (29, 30). In addition, the motor proteins of host cells, such as myosin and dynein, are believed to be used in the infection process by some intracellular parasitic microorganisms. For example, chlamydiae interact with the host-cell’s dynein motor protein to move across the host cell (31, 32). These observations suggest that the interactions between a bacterial membrane protein and the microfilament of a host cell are important for successful bacterial infection. In this study, we demonstrated that Amp interacts with microfilament complexes both in vivo (Fig. 3) and in vitro (Fig. 2) and that this interaction is vector-specific (Fig. 4). These results suggest that an interaction between the phytoplasma Amp and the microfilament complexes of the insect host is important for infection to occur. Although several mammalian pathogens are known to interact with host microfilaments to infect a host, the phytoplasma is an example of an insect-transmissible bacterium that utilizes microfilaments in the insect cell.

**Affinity Between Phytoplasma Amp and Insect Proteins Is Involved in Vector/Nonvector Determination.** In nature, phytoplasmas are transmitted by specific leafhopper vectors and not by other leafhoppers (1). For example, M. striifrons transmits OY phytoplasma but cannot propagate rice yellow dwarf (OY) phytoplasma (Candidatus Phytoplasma oryzae, RYD strain). In contrast, N. cincticeps transmits RYD phytoplasma but cannot be infected with OY phytoplasma. Although this species-specific transmissibility has been verified, the molecular mechanism is thus far unknown. In this study, we demonstrated that Amp specifically interacts with the microfilament complexes of OY-transmitting leafhoppers but not with those of non-OY-transmitting leafhoppers (Fig. 4), suggesting that the formation of the AM complex is completely consistent with the transmissibility of OY phytoplasma by leafhoppers. Therefore, the interaction between Amp and insect microfilament complexes plays a major role in determining the transmissibility of phytoplasmas.

In general, each phytoplasma species is transmitted by a specific vector insect, whereas the plant-host ranges are broad. For example, phytoplasmas infect at least 700 species of plants in 98 families (1, 33), and aster yellows phytoplasma can infect >161 plant species in 120 genera and 39 families (33). The majority of phytoplasmas can infect the periwinkle plant (34). In contrast, the specificity for the insect host is usually much stricter (S.K., K.O., S.S., and S.N., unpublished data). Therefore, insect-vector determination is an important factor influencing the entire host range of phytoplasmas in nature. Combined with the results of the affinity column assay (Fig. 4), the formation of the AM complex seems to be important for the insect-vector specificity of phytoplasmas in nature, and elucidation of this mechanism could potentially reduce the severity and extent of crop damage.

In *Spiroplasma citri*, it has been reported that the defective mutant of immunodominant membrane protein (spiralin) was less effective in its transmissibility (35). However, there is no direct evidence of the relationship between spiralin and the insect-host specificity of spiroplasma, and molecular details of the function of spiralin are still unknown. The results of this study suggest that the phytoplasma Amp (immunodominant membrane protein) might be involved in transmission by insect vectors. Thus, although no homology was detected between Amp and spiralin (data not shown), we postulate that the immunodominant membrane proteins of phytoplasmas and spiroplasmas commonly play an important role in transmission by insect vectors.

The invasion of pathogens into the insect intestine is the first important step in determining the insect vector (insect-vector specificity) by insect-transmissible pathogens, including phytoplasma (36), the malaria parasite (37), and spiroplasma (17, 18). In the first step to infecting the insect host, phytoplasmas pass through a stylet containing phloem fluid, traverse an intestinal tract wall, multiply in hemolymph, and spread systemically. The phytoplasmas subsequently enter a salivary gland, multiply, exit the salivary gland cells into saliva, and are transmitted to a new host plant. A previous study has shown that for phytoplasma, the ability to pass through the insect intestine (including the epithelial cells, connective tissue, and visceral muscle) and salivary gland is an important factor in its vector determination (36, 38). Our results, together with these previous data, suggest that formation of the AM complex is necessary for passage through these host barriers.

Generally, it has been held that the host specificity of a pathogen is determined by the affinity between the pathogen surface and the host-cell surface (5, 39, 40). For example, mechanisms of host specificity are well characterized in some mammalian pathogenic viruses. These viruses recognize the carbohydrate chain on the surface of host tissue in the first
infection step, and differences in its structure are strongly correlated with virus-host specificity (39). Similar mechanisms have been suggested for the transmission of the malaria parasite by its insect vector (5). However, phytoplasmas are predicted to be able to attach to the surface of the gastrointestinal tract and salivary glands in host and nonhost insects (41). Moreover, our results suggest that the insect-vector specificity of phytoplasma is determined by the intracellular interaction between a host structural protein (microfilament) and a bacterial surface protein (Amp) and not by the affinity between the surface structures of the host and parasite.

In mammal-pathogenic bacteria, such as Listeria, Salmonella, and Shigella, the ability to form a complex with host-cell microfilaments seems to be deeply involved in host-cell determination. Bacterial motility within the cytoplasm of infected epithelial cells depends on the actin polymerization machinery through which the bacterium gains a propulsive force to spread within the cytoplasm and into adjacent epithelial cells (29). In Shigella, VirG (a surface-exposed outer-membrane protein of bacterium) is a critical virulence factor for this actin-based motility, and the interaction between VirG and its specific host ligand neural Wiskott–Aldrich syndrome protein (N-WASP), a protein that regulates the actin cytoskeleton, determines the host-cell type, allowing actin-based spreading (42). In agreement with this finding, we showed that the ability to form a complex between a bacterial surface protein and insect-vector microfilament is involved in vector determination. Interestingly, phytoplasmas, which infect insects and plants, are phylogenetically distant from Shigella, which infects mammals, and their host ranges and bacterial surface structures are very different, whereas their surface proteins share a specific interaction with host microfilaments, and these interactions determine the host species/cell. The utilization of a host microfilament for infectivity despite the phylogenetic distance seems to be a form of convergent evolution for the intracellular lifestyle of parasitic bacteria. It is possible that this host-determination system also occurs in other pathogens that interact with the microfilaments of their hosts. This study provides insights into the phytoplasma vector-determination mechanism, furthering our knowledge of not only phytoplasmas but also other pathogens. The mechanism of vector determination by the AM complex identified here is still unclear, and it is also not known whether additional factors involving the AM complex exist. Further investigation of this mechanism may help to elucidate the mechanism of vector–pathogen determination.

Materials and Methods

**Phytoplasma Lines, Plants, and Insects.** In 1982, OY phytoplasma was isolated from an onion, Allium cepa, in Saga Prefecture, Japan (43). The phytoplasma was maintained in garland chrysanthemum plants (Chrysanthemum coronarium) by serial transmission from infected to noninfected plants by using its leafhopper vector M. striifrons. All leafhoppers (M. striifrons, H. sellatus, H. sellatiformis, O. flavopicta, and N. cincticeps) and all plants were kept in a greenhouse.

**Immunofluorescence Analysis of an Insect’s Intestine.** Intestinal tissues from the OY phytoplasma-infected or noninfected M. striifrons were removed and washed in PBS. Tissues were fixed and permeabilized in PBS containing 0.2% Triton X-100 and 4% paraformaldehyde for 15 min at 37°C and washed four times in PBS. Subsequently, tissues were treated with anti-Amp-IgG in PBS containing 3% BSA (Sigma) for 1 h and washed four times in PBS containing 3% BSA. Then the tissues were treated with FITC-conjugated anti-rabbit IgG and, simultaneously, the microfilament was stained by using rhodamine-coupled phalloidin (Sigma) in PBS containing 3% BSA for 1 h. The tissues were washed and analyzed by using a confocal laser scanning microscope (Zeiss LSM5 Pascal).

**Affinity Chromatography.** The overexpression and purification of Amp or SecA and the production of an anti-Amp antibody have been described in refs. 15 and 44. The purified Amp, purified SecA, BSA, or an antibody against Amp was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biosciences) according to the manufacturer’s instructions, and affinity columns were set up by using this medium. Leafhoppers were homogenized with a mortar and pestle in Rx buffer (0.1% Triton X-100, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 1.25 mM EGTA, and 10 mM Hepes, pH 7.3) and centrifuged at 18,000 × g. All of the supernatant was loaded onto each affinity column, the columns were washed with Rx buffer, and specifically bound proteins were extracted with an elution buffer (0.1% Triton X-100 and 100 mM glycine-HCl, pH 2.5). All of the eluted proteins were subjected to SDS/PAGE, followed by gel staining or Western blot analysis.

**SDS/PAGE and Western Blotting.** SDS/PAGE, Coomassie brilliant blue staining, and Western blotting were performed by using methods described in ref. 15, except for the use of a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) and ECL-Plus detection reagent (Amersham Pharmacia Biosciences). A polyclonal antibody (Sigma) against the actin from chicken was also used.

**Two-Dimensional Gel Electrophoresis.** Two-dimensional gel electrophoresis using Immobiline strips (Amersham Pharmacia Biosciences) at a pH range from 4 to 7 was performed according to the manufacturer’s instructions. Gels were stained by using a silver staining kit (Daiichi Pure Chemicals, Tokyo) or SYPRO ruby (Molecular Probes) according to the manufacturer’s instructions.

**MS Analysis.** Protein spots of interest were excised from the 2D gel stained with the SYPRO ruby or the SDS/PAGE gel stained with Coomassie brilliant blue and subsequently digested by using trypsin. One microliter of the digested peptide solution was mixed with an equal volume of saturated α-cyano-4-hydroxy cinnamic acid solution, and the resulting samples were analyzed by using MALDI-TOF MS using a Voyager DE-STR mass spectrometer (Applied Biosystems). Peptide mass fingerprints were analyzed by using the program MS-FIT (http://prospector.ucsf.edu).

**Peptide Sequences.** Two major proteins, P30 and P42, were cut from the Coomassie brilliant blue-stained SDS/PAGE gel and digested by using Endoproteinase Lys-C (Roche). The resulting peptide fragments were fractionated by using reversed-phase HPLC (Amersham Pharmacia Biosciences). The amino acid sequence of the oligopeptide fragments digested from each of P30 (eight oligopeptide fragments) and P42 (two oligopeptide fragments) were determined by using the Edman degradation method with the Procise HT Protein Sequencing System (Applied Biosystems).

**cDNA Cloning of Actin and Myosin Light-Chain Genes from Leafhopper mRNA.** Total mRNA was extracted from leafhoppers by using the guanidinium thiocyanate method with a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biosciences). Then, a cDNA library was constructed from the mRNA preparation by using a ReverTra Plus RT-PCR kit (Toyobo, Osaka) according to the manufacturer’s instructions. To obtain the cDNA clone of the middle region of the actin and myosin light-chain mRNA, RT-PCR was performed using degenerate primer sets designed from the peptide sequences of actin and myosin.
myosin (actin, 5'-AAR MGN GGN ATH YTN ACN YTN AAR TA-3', 5'-TTY TAC TGN WSN GGN CGN RAN CGN TGN TA-3' and myosin light chain, 5'-TTY AAR GAR GGN TTY GCN TTY GCN TTY ATG-3', 5'-TTY YTC RTC DAT NAC CAT NGC YTC-3'). The amplified products were cloned into plasmids by using the pGEM-T Easy Vector cloning system (Promega), and the inserts were confirmed by DNA sequencing analyses (Applied Biosystems). To determine the remaining 5'- and 3'-terminal gene sequences, both 5'-RACE and 3'-RACE methods were performed using a 5'-Full RACE Core Set (Takara, Tokyo).

Full-length PCR products of both the actin and myosin light-chain genes were cloned into pUC119 and subsequently sequenced.

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