

Characterization of growth-related genes in the south-western Atlantic pink shrimp *Farfantepenaeus paulensis* (Pérez-Farfante 1967) through a modified DDRT-PCR protocol

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Abstract

An exploratory methodology has been used to identify genes related to growth of the shrimp *Farfantepenaeus paulensis* (Pérez-Farfante, 1967). Six hundred postlarvae, with an average (\pm SD) initial weight of 0.022 g (\pm 0.008), were reared for 60 days in salinity 30 g L⁻¹. In the end, the 15 heaviest (> 1.2 g) and the 15 lightest (< 0.6 g) animals were frozen in liquid nitrogen. A modified differential display reverse transcription-polymerase chain reaction technique was used to generate expression profiles for all individuals. The resulting cDNA from reverse transcription were amplified by pairing 31 arbitrary primers with a reverse primer anchored to the cDNA tails. From the comparison between the expression profiles of the organisms in the two size classes, differences could be pinpointed. Bands of interest were collected, purified, cloned and sequenced. Despite the relatively small number of primers used, the methodology allowed the identification of three genes, not described previously for the investigated species and possibly related to growth. These partial sequences are likely to belong to genes that code for myosin heavy chain, cyclophilin and haemocyanin as revealed through an amino acid similarity search conducted using the BLASTP on-line tool.

Keywords: differential display, RNA, RT-PCR, prawn

Introduction

The pink shrimp *Farfantepenaeus paulensis* (Pérez-Farfante 1967) is a species native to the shallow south-western Atlantic waters. *Farfantepenaeus paulensis* uses the Lagoa dos Patos estuary, a large lagoon system in southern Brazil, as feeding grounds for its initial postlarvae stages. This Penaeid has demonstrated a strong potential for aquaculture in the region, being the subject species on a project aimed at the development of a technological package for the shrimp culture in estuaries using cages and pen enclosures (Soares, Peixoto & Benvenuti 2004). The work conducted so far has significantly advanced the knowledge on the reproductive biology of the pink shrimp, allowing the development of culture techniques with promising results for this species (Cavalli, Scardua & Wasielesky 1997; Peixoto, Cavalli & D'Incao 2003; Peixoto, Cavalli & Wasielesky 2004). The objective of the present study was to develop a methodology to investigate the expression of genes involved in the growth of the pink shrimp *F. paulensis*. There are several techniques available to generate gene

expression profiles that allow the identification of genes transcribed under specific conditions. Among them, the differential display reverse transcriptase-polymerase chain reaction method (DDRT-PCR) was described for the first time by Liang and Pardee (1992), with the aim of providing a tool that would allow the detection of individual species of RNA that were expressed differently in various types of eukaryotic cells, and clone the corresponding cDNA. This method can be used for prompt identification of differences in gene expression, allowing the determination of transcription profiles with qualitative or semi-quantitative accuracy (Kuhn 2001; Rahmanm, Vandingenen, Begum, Breuer, De Loof & Huybrencht 2003). Here, we present a new modification of this approach, which was designed to help identify genes related to growth in *F. paulensis*.

Material and methods

Sample production and storage

The shrimp used in this work were produced at the Marine Aquaculture Laboratory (EMA-FURG, RS, Brazil), from wild *F. paulensis* breeders captured in the southern coast of Brazil. Groups of 200 postlarvae, with an average (±SD) initial weight of 0.022 g (±0.008), were reared for 60 days in three 1 m³ tanks at salinity 30 g L⁻¹. Shrimp were fed *ad libitum* with a commercial diet supplied in trays twice a day. During the whole experiment, salinity and aeration were kept constant, under a natural photoperiod (14 h light:10 h darkness). Salinity, water temperature, pH and dissolved oxygen were monitored using, respectively, an optical refractometer from Atago (SP, Brazil) (±1), a thermometer (±0.1 °C) and a pH meter from Digimed (SP, Brazil) (±0.01). Total ammonia (TAN; NH₄⁺ + NH₃) concentrations were estimated according to UNESCO (1983). After 60 days of culture, shrimp were carefully counted, individually weighed and kept alive until selection for genetic analysis. Statistical analysis of variance (ANOVA) of shrimp data was conducted using STATISTICA® v. 5.0 (StatSoft 1998). The five heaviest (> 1.2 g) and the five lightest (< 0.6 g) animals from each of the three tanks were separated and all 30 animals were flash frozen in liquid nitrogen for posterior analyses.

Messenger RNA isolation and cDNA synthesis

Total RNA was isolated from a 50 mg fragment of cephalothorax, including the muscle, carapace, digestive gland and whole eyestalk. RNA extraction

was conducted using TRIzol Reagent (Invitrogen, SP, Brazil), according to the manufacturer's protocol. RNA quantification was performed using ultraviolet (UV) spectrophotometry, and integrity was checked by electrophoresis on 1% agarose gels stained with ethidium bromide. Purification of messenger RNA was carried out using the MessageMaker Reagent Assembly Kit (Invitrogen). First-strand cDNA synthesis followed the recommendations from RT SuperScript III enzyme (Invitrogen), using 1 µL of 500 µg mL⁻¹ oligo(dT) primer AP [5'-GGCCAGGC GTCGACTAGTAC(dT)17-3'] in a 20 µL reaction. cDNA was purified using the GlassMAX cDNA Purification System (Invitrogen), diluted to a 1:10 ratio and stored at -20 °C for subsequent PCR.

Production of expression profiles

All 30 selected animals were examined by DDRT-PCR with each primer. Polymerase chain reaction reactions contained one of 31 arbitrary primers used (Table 1 – Operon Technologies, SP, Brazil) paired with the primer AUAP (5'-GGCCACGCGTCGACTAGTAC-3'). The 20 µL PCR reactions contained 1 × proprietary buffer (Invitrogen), 0.5 mg mL⁻¹ of purified bovine serum albumin (BSA), 1 µL of cDNA template, 1.5 mM MgCl₂, 50 µM each dNTP, 0.2 µM arbitrary primer, 0.2 µM of primer AUAP, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and RNase-free ultrapure water (Invitrogen). Cycling conditions consisted of an initial denaturing step of 60 s at 94 °C, followed by 40 cycles of 30 s at 94 °C, 60 s at 40 °C, 90 s at 72 °C, followed by a final extension step of 5 min at 72 °C. Polymerase chain reaction products were

Table 1 Sequences for the 31 arbitrary primers used

Primer	Sequence	Primer	Sequence
AB-01	5'-CCGTCGGTAG-3'	AB-17	5'-TCGCATCCAG-3'
AB-02	5'-GGAAACCCT-3'	AB-18	5'-CTGGCGTGTC-3'
AB-03	5'-TGGCGCACAC-3'	AB-19	5'-ACACCGATGG-3'
AB-04	5'-GGCAGCGTT-3'	AB-20	5'-CTTCTCGGAC-3'
AB-05	5'-CCC GAAGCGA-3'	AC-05	5'-GTTAGTGCGG-3'
AB-06	5'-GTGGCTTGGA-3'	AC-07	5'-GTGGCCGATG-3'
AB-07	5'-GTAAACCGCC-3'	AD-06	5'-AAGTGCACGG-3'
AB-08	5'-GTTACGGACC-3'	AD-07	5'-CCCTACTGGT-3'
AB-09	5'-GGGCGACTAC-3'	S-05	5'-TTTGGGCGCT-3'
AB-10	5'-TTCCTCCCA-3'	S-06	5'-GATACCTCGG-3'
AB-11	5'-GTGCGCAATG-3'	S-08	5'-TTCAGGGTGG-3'
AB-12	5'-CCTGTACCGA-3'	D-01	5'-ACCGCGAAGG-3'
AB-13	5'-CCTACCGTGG-3'	G-05	5'-CTGAGACGGA-3'
AB-14	5'-AAGTGCAGACC-3'	B-02	5'-TGATCCCTGG-3'
AB-15	5'-CCTCCTTCTC-3'	B-08	5'-GTCCACACGG-3'
AB-16	5'-CCC GGATGGT-3'		

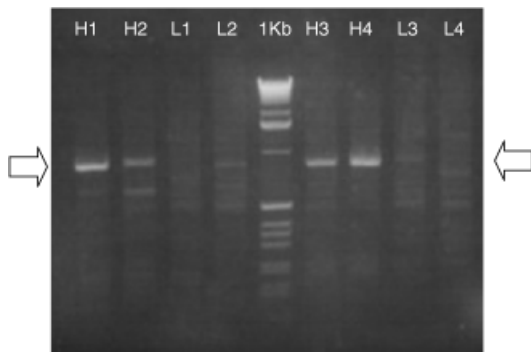


Figure 1 Example of typical differential display reverse transcription-polymerase chain reaction banding patterns obtained with an AB-4/AUAP primer combination. The arrows indicate the position of 900 bp bands showing different expression levels between heavier shrimp (H1, H2, H3 and H4) and lighter shrimp (L1, L2, L3 and L4).

electrophoresed on 2% agarose gels, containing ethidium bromide, and visualized under UV light. Differences in band brightness were considered to be caused by differential expression. Intense bands were identified visually (Fig. 1) and selected for cloning.

Retrieval, cloning and sequencing of differential display products

Differences in banding patterns between large and small shrimps were identified by visual examination. Bands that strongly displayed for larger shrimp and absent or with weak expression for smaller shrimp were extracted from the gel using the Concert Gel Extraction System (Invitrogen), according to the manufacturer's protocol. Successfully extracted products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen), following the manufacturer's instructions. Bi-directional sequencing was performed using primers M13F and M13R (Invitrogen) and read on the automated sequencer MegaBACE 1000 (Amersham Biosciences, Sunnyvale, California, USA), at the Genomics and Molecular Biology Center (CBGM, PUC, RS, Brazil). The nucleotide sequences obtained were translated into amino acid sequences using the standard genetic code and were screened for homology to known sequences using the BLASTP on-line tool (Altschul, Madden, Schäffer, Zhang, Zhang, Miller & Lipman 1997).

Results

During the growth experiment, the average values (\pm SD) for temperature, dissolved oxygen, oxygen sa-

turation, TAN and pH were, respectively, 25.88 °C (\pm 2.66), 5.0 mg L⁻¹ (\pm 1.02), 59%, 0.045 mg L⁻¹ (\pm 0.019) and 8.03 (\pm 0.09). At the end of the experiment, shrimp were counted and weighed, showing an average survival of 97%. The differences in weight observed between the replicas were not significant on an ANOVA test ($P > 0.05$). The average final weight (\pm SD) of all animals was 0.62 g (\pm 0.15), while the average weights of the 45 lightest and 45 heaviest shrimp were 0.41 g (\pm 0.00) and 1.36 g (\pm 0.01) respectively.

Among displays produced for 31 RAPD primers, for 30 individual samples, a few presented no band pattern, while most of them showed about two or three clearly expressed amplicons. Eight bands could be identified as having a clearly stronger expression for heavier shrimp. Bands with strong expression only in the lighter shrimp have not been studied further this time. An example of the observed pattern of differential expression between size classes is shown in Fig. 1. The differential gene expression analysis has shown that probably several genes had higher expression levels in the heavier shrimp group.

Among the purified amplicons, three could be re-amplified and sequenced successfully, from reactions using the primers G-05, S-08 and AB-04 (Table 1), and corresponding to amplicons of approximately 300, 600 and 900 bp respectively. BLASTP homology searches for the sequenced amplicons indicate that they correspond to known proteins. G-05 amplicon (GenBank accession number DQ115397) is likely to be a part of the myosin heavy-chain gene, as shown by the high similarity between the presented sequence and those from other crustaceans, such as the red lobster *Homarus americanus* (77%) and the snap shrimp *Alpheus lottini* (76%). The S-08 amplicon sequence (GenBank accession number AY775150) seems to correspond to cyclophilin, despite the fact that the only arthropod present in the resulting homology list was the limulid *Tachypelus tridentatus* (74%). Finally, the AB-4 amplicon sequence (GenBank accession number AY775151) showed significant similarity to known decapod haemocyanin, especially to those belonging to close genera, such as *Penaeus monodon* (86%) and *Litopenaeus vannamei* (84%).

Discussion

The analysis of gene expression for organisms with little genomic information available still prevents the use of modern techniques when approaching specific

questions. Cheaper alternative methodologies for gene identification are still a matter of research today, and well-known approaches in molecular biology are still subject to development and improvement. An example of a relatively simple technique, which is still in use to approach the less-studied marine invertebrates, are those based on the development of molecular markers using RAPD primers (e.g. Garcia & Benzie 1995). In the present work, we have improved the targeting capability of the RAPD-based differential display technique using a reverse primer anchored to the cDNA tail. Thus, the strategy used here has reduced the number of bands displayed in each PCR reaction considerably. This strategy has allowed obtaining profiles from 2% agarose gels, containing ethidium bromide, instead of denaturing polyacrylamide gels, which are usually associated with labour-intensive autoradiography or silver staining. Another positive aspect of this approach is that the translation direction is intrinsically known. Despite the fairly reduced number of primers used in this work, the methodology presented has proven to be efficient and cost effective, allowing the detection of three genes not described previously for *F. paulensis*, which are very likely related to growth performance under aquaculture conditions.

Role of the identified genes in *F. paulensis* growth

Myosin forms a complex molecule in the actin-based molecular motors. It is ubiquitously expressed as multiple isoforms in all eukaryotic cells and it is the most abundant contractile protein present in the skeletal muscle (Denardi, Ausoni & Moretti 1993; Jung, Lieber & Ryan 1998). If the differences in weight and size are related to the production of muscle tissue, it is expected that myosin will be among the expressed genes. It was observed here that the group of heavier shrimp actually displayed a higher myosin heavy-chain expression. However, an insight as to why this occurs should emerge from the study of hormone-mediated processes, possibly from ion balance processes. Cyclophilin is an abundant and ubiquitous cytosolic protein that has been conserved throughout evolution from bacteria to vertebrates. It has an affinity to immunosuppressive drugs (Caroni, Rothenfluh, Mcglynn, & Schneider 1991; Martinez-Gonzalez & Hegardt 1995) and it is involved in plant pathogen response (Fidantsef, Stout, Thaler, Duffey & Bostock 1999; Zhang, Zhu & He 2004) and vertebrate

immunological system activation (Hamilton & Steiner 1998; Gothel & Marahiel 1999). Cyclophilins are also modulators of response to oxidative stress (Bugli, Khattab, Vigneti, Butler, Cioli & Klinkert 1998; Guedes, Vitorino, Domingues, Tomer, Correia, Amado & Domingues 2005). The high expression of a cyclophilin-like gene observed for *F. paulensis* might suggest better immunological conditions for the group of heavier shrimp. Haemocyanin is the heaviest polypeptide known and one of the largest molecules in nature (Rainer & Brouwer 1993). The role that haemocyanin plays in oxygen transport, in arthropods and clams associates it immediately with metabolic rates and growth. This gene has been observed to express strongly in the heavier shrimp, while it was considerably reduced or absent in the lighter animals. Haemocyanin is one of the best-studied genes of invertebrates, because of its central role in metabolism.

The results reported here show that the methodology presented can be used for the identification of genes of interest for Brazilian native shrimp aquaculture. Three genes, coding for myosin, cyclophilin and haemocyanin, have been strongly expressed in heavier shrimp while, for lighter shrimp, they were weakly expressed or absent, suggesting that these genes might be involved in growth performance. These results represent an initial approach towards a better understanding of the genetic regulation of growth in cultured *F. paulensis*.

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