Lat. Am. J. Aquat. Res., 46(2): 461-468, 2018
DOI: 10.3856/vol46-issue2-fulltext-21

Short Communication

Isolation and characterization of partial insulin-like androgenic gland hormone gene from distal terminal ampoules of three marine shrimp *Penaeus* species from the Eastern Pacific

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ABSTRACT. The expansion of the shrimp industry depends on scientific advances related to the understanding of reproductive endocrinology that allows the development of aquaculture programs based on biotechnology. An insulin like-androgenic gland hormone (IAG) is synthesized in the male-specific androgenic gland (AG) and it is responsible for sex differentiation in crustaceans. In this study, RNA was extracted from different tissues; amplified cDNA was obtained by reverse transcription-PCR. Nucleotide sequences were analyzed following a bioinformatic approach of the encoding insulin-like androgenic gland hormone gene. This contribution presents the isolation of the partial cDNA encoding IAG from *Penaeus vannamei* (Lv-IAG), *Penaeus occidentalis* (Lo-IAG) and *Penaeus stylirostris* (Ls-IAG) from the distal region of terminal ampoules. In addition, the molecular characterization of these sequences is provided and the resulting aminoacid sequences were used to establish the taxonomic positioning of the IAG gene among other Decapoda species. The isolation of these partial sequences provides evidence about the anatomical location of the AG in *Penaeus* and is the basis for further characterize the complete cDNA of the AG-specific insulin-like encoding transcript for all species of the genus *Penaeus* from the Pacific coast of America.

Keywords: *Penaeus*, Penaeids, IAG hormone, aquaculture, biotechnology, RT-PCR.

The androgenic gland (AG) is a unique organ of crustaceans, responsible for controlling the differentiation of primary and secondary sexual characteristics in males. First described by Cronin (1947), in the decapod *Callinectes sapidus*, and since then, many studies were undertaken to define its role. Charniaux-Cotton (1954) associated this gland with the regulation process of male differentiation and spermatogenesis in the amphipod *Orchestia gammarella*. This gland has been studied in many malacostracan crustaceans, including isopods and decapods, in order to improve the understanding of the reproductive mechanisms (Khalaila & Sagi, 1999). The AG produces a peptide hormone that plays a key role in the process of sexual differentiation. The peptide corresponds to the insulin-like family androgenic gland hormone (IAG) (Martin & Juchault, 1999). The isolation and molecular characterization of the insulin-like androgenic gland hormone gene have been conducted in different species of crustaceans. In amphipods, isopods and decapods, where the structure and location of the AG have been analyzed, it was suggested the existence of only one kind of gland, characterized by the presence of thin lines or lobes of compacted cells associated with the subterminal region of the spermatic duct in amphipods, or at the end of each testicular utricle in isopods. The location of the AG in penaeids has been studied in *Penaeus setiferus*, *Melicertus kerathurus* (Charniaux-Cotton & Payen, 1985), *Marsupenaeus japonicus* (Nakamura *et al*., 1992; Banzai *et al.*, 2011), *Fenneropenaeus indicus* (Sunilkumar, 1996), *Fenneropenaeus chinensis* (Li & Li, 1993; Li & Xiang, 1997; Li *et al*., 2012), *Penaeus vannamei* (Alfaro-Montoya, 1994; Vázquez-Islas *et al*., 2014; Campos-Ramos *et al*., 2016), *Penaeus stylirostris* (Alfaro-Montoya, 1994; Alfaro-Montoya & Hernández, 2012), *Penaeus occidentalis* and *Rimapenaeus byrdi* (Alfaro-Montoya & Hernández, 2012). Based on previous re-

Corresponding editor: Crisantema Hernández

ports, the AG of *Penaeus* is a lobular grape-like cellular structure mainly concentrated at the distal region of terminal ampoules, and it is associated with a cellular cord of blood vessel nature, extending from the ampoule, the descending and the ascending medial vas deferens (Vázquez-Islas *et al.*, 2014; Alfaro-Montoya & Hernández, 2012; Alfaro-Montoya *et al.*, 2016).

Following the discovery of the molecular structure of the androgenic hormone, it has been possible to identify genes involved in the development of the gonads and the organism by biotechnological techniques such as *in situ* hybridization, analysis of expression or gene silencing as well as developing monosex populations (Callaghan *et al*., 2010). Gender selection and subsequent development of a monosex population give industry the opportunity to reduce costs generated during the process and to obtain organisms with larger sizes, in populations with sexual dimorphisms, such as species of *Penaeus* (Alfaro-Montoya *et al.,* 2016). The characterization of the encoding insulin-like peptide-specific androgenic gland gene will reveal new aspects about the reproduction of penaeid shrimp, such as understanding the sex differentiation mechanisms and developing biotechnological breeding strategies for reversing females (WZ) into neomales (WZ) by androgenic hormone therapies (Vega-Alpízar *et al.*, 2016). The demonstration of sex reversal in males (ZZ) by the activation of the endogenous gene silencing mechanism for IAG (Ventura *et al.*, 2011) in penaeid shrimps has not been accomplished either. Here, we report for the first time the isolation and characterization of partial sequences encoding a putative AG specific insulin-like peptide in *Penaeus occidentalis* and *Penaeus stylirostris* using RT-PCR with conserved specific primers designed for *P. vannamei* reported sequences*;* these two species are commercially important for fisheries and aquaculture and promising models for RNAi based biotechnology for penaeid monosex culture in the Eastern Pacific.

Males of *P. stylirostris* (body weight, bw = 30 g) and *P. occidentalis* (bw = 30 g) were collected in the central Pacific coast of Costa Rica, in the Gulf of Nicoya, Puntarenas. Individuals of *P. vannamei* (bw = 25 g) were obtained from commercial production ponds as a positive IAG sequences specimen control. All individuals were kept under standard conditions of captivity at Estación de Biología Marina, UNA, Puntarenas (Alfaro-Montoya & Hernández, 2012). Hypertrophy of the AG was induced by bilateral eyestalk ablation (Khalaila *et al*., 2002). Two weeks post-eyestalk ablations, dissections of specific tissues were performed on anesthetized shrimps with cold water (10°C). Specific tissues were dissected and stored/stabilized with RNAlater® (Ambion®, Invitrogen) or immediately processed for total RNA extraction.

Total RNA was extracted from the distal section of the terminal ampules (Amp) (Alfaro-Montoya & Hernández, 2012; Alfaro-Montoya *et a*l., 2016), and from other tissues such as sperm duct section from medial descending and ascending vas deferens (Sd), abdominal muscle (Ms) and hepatopancreas (Hp). RNA extraction was performed from 100 mg of each tissue by using a commercial extraction kit Pure Link (Ambion®, Invitrogen) following the protocol according to the manufacturer. RNA extraction and analysis from selected tissues were performed in at least three males belonging to each species. Total RNA (~1ug) was used for the synthesis of complementary DNA (cDNA) using an oligo (dT) primer (5`-$T_{24}$VN-3`) by reverse transcription (RT-PCR). The procedure of the first strand synthesis was performed using M-MLV Reverse Transcriptase, RNase H Minus (Promega). The cDNA resulting from the reverse transcription reaction was amplified to double-stranded DNA by PCR under the following thermal cycling conditions: 3 min 94°C, 40 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 45 s and finally 72°C for 10 min. For RT-PCR assays, the essential and housekeeping ß-actin (AF300705) gene was used as positive control and integrity indicator of total RNA. Amplification of the partial IAG transcript of *P. vannamei*, *P. occidentalis*, and *P. stylirostris* was made using specific primers designed with Primer3 software (Rozen & Skaletsky, 2000) and sequences of the IAG reported in the GenBank, such as *F. chinensis* (accession number JQ388277) and *P. vannamei* (accession JQ670898 and KM066114) (Table 1).

RT-PCR obtained products were extracted and eluted from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and cloned and/or sequenced in both directions, on an automated ABI 3130 Genetic Analyzer (Applied Biosystems), located at Escuela de Ciencias Biológicas, Universidad Nacional, Heredia. The nucleotide sequences obtained were analyzed by a local alignment analysis (BLASTn) using the NCBI database (GenBank). Cleaning and editing of low-quality chromatograms were done by eye double checked and a multiple alignments of nucleotide and amino acid sequences were performed using Geneious R8 software (Biomatters Limited) with a Blossum 62 matrix including AG-specific insulin found sequences in *P. occidentalis*, *P. stylirostris*, and *P. vannamei* with other sequences previously described and reported in the NCBI database (GenBank) for penaeid species. The translation of the nucleotide sequence of amino acids was performed using online software EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Protein

Table 1. Primers used in RT-PCR reactions, designed based on the sequences described in GenBank IAG (JQ670898, KM066114) by Primer3 software. The primer ß-actin was designed from AF300705 sequence and used as a positive control reaction and checking the integrity of total RNA extracted.

Primer name	Primer sequence 5'- 3'
Lv-IA_{GT}(f)	CCCTGCTCAACTGTTACTCGGAT
Lv-IA_{GT}(r)	CTCGCAATACTCCAGGATCTCC
Lv-IAG (f)	GAGTGTCAAGGTCAGCCGAT
Lv-IAG (r)	GGGTCCTCGCAATACTCCAG
Lv_B-actinF	CGAGGTATCCTCACCCTGAA
Lv_B-actinR	AGGGCATATCCCTCGTAGA

domains were obtained using Motif Scan, with PROSITE and PFAM models (http://myhits.isb-sib.ch/cgi-bin/motif_scan). A phylogenetic tree was performed to determine the taxonomic position of the aminoacid sequences obtained in this study (accession KX589057, KX589058, and KX589059) with the IAG sequences reported in the GenBank. Sequences were aligned using MAFFT 7.0 online program with an iterative refinement method (FFT-NS-i) and BLOSUM62 parameters for the amino acid scoring matrix (Silvestro & Michalak, 2012). The phylogenetic tree was obtained using maximum likelihood (ML) by randomized accelerated maximum likelihood graphical user interface (raxMLGUI v.7.4.2) software by PROT/BLOSUM62 substitution model for amino acids and 1000 rapid bootstrap inferences (Silvestro & Michalak, 2012).

The RT-PCR assay performed in all specific tissues, amplified a fragment of the housekeeping gene ß-Actin (~300 bp), demonstrating the effectiveness in isolating a messenger of RNA and optimizing the reaction of reverse transcription (Fig. 1). In order to assess the presence of the IAG in other tissues besides the distal terminal ampoule (site of transcription of IAG messenger), cDNA from abdominal muscle, hepato-pancreas and spermatic duct-derived from RNA extraction were used for RT-PCR. Positive amplification was obtained only from terminal ampoules in the three species, each fragment with a weight of ~400-500 bp (Fig. 1).

Molecular characterization of *Penaeus* IAG sequences: the length of sequences obtained from the partial transcripts for the IAG in *P. vannamei*, *P. occidentalis*, and *P. stylirostris* was 453 nucleotides (nt), 459 nt and 453 nt at the open ready frame region (ORF), respectively. Additionally, *in silico* nucleotide sequences translated into amino acids (151 aa polypeptide chain length in *P. vannamei* and *P. stylirostris*, and 153 aa in *P. occidentalis*) were obtained. Since complete sequences were not generated, it was not possible to observe the start codon (ATG) and the stop codon, so the hybridization of the primers in the target sequences of DNA occurs after the ATG codon and before the stop codon. In our sequences, it is possible to observe the translated ORF containing a partial signal peptide (20 aa), followed by an entire B chain (37 aa), a C chain (63 aa), and a partial section of A chain (31-33 aa), which are characteristic domains of the insulin-like peptide (Fig. 2). A multiple alignment of the amino acid sequences obtained in our study with sequences reported from decapod-IAG related sequences was performed to identify conserved regions including cysteine bonds between them. In penaeids, the B chain contains two cysteine residues in conserved locations, followed by the incomplete A chain with four conserved Cys residues. While in the decapods *M. lar, M. rosenbergii*, *M. nipponense* and *P. paucidens*, two additional cysteine bonds were found; one of them in B chain and one in the A chain. One N-glycosylation site is located in the 38 aa, in penaeids, while in the decapods *M. lar*, *M. rosenbergii*, *M. nipponense* and *P. paucidens*, it is located in the 173 aa. (Fig. 3a).

A phylogenetic tree was generated with the amino acid sequences of B, C and A chains to assess the phylogenetic relationship and the evolutionary distance between the *Penaeus* species studied with other species of the same family and other decapods. As expected, our species are taxonomically placed in the group of Penaeids, with a force of node of 99% (bootstrap value) and a taxonomic placement of 80% of bootstrap with species of *Penaeus* gender. Also, it shows that on an evolutionarily level, *P. stylirostris* is the most divergent out of the *Penaeus* species analyzed (Fig. 3b).

In this study, cDNA encoding the insulin-like androgenic gland hormone precursors were isolated in *P. vannamei*, *P. stylirostris*, and *P. occidentalis*. The cDNA precursors used for RT-PCR were derived from RNA extracted from androgenic glands associated with the distal region of terminal ampoules (Alfaro-Montoya *et al*., 2016). The amplification of a fragment of the ß-actin gene demonstrates the effectiveness in isolating a messenger of RNA and the optimization of the reverse transcription. The amplicons obtained from the distal part of the terminal ampoule, are consistent with the results obtained by others authors (Alfaro-Montoya & Hernández, 2012; Vázquez-Islas *et al.*, 2014, 2015; Alfaro-Montoya *et al.*, 2016). In these previous studies, the histological analysis or gene expression indicated that the presumptive site for the production of the insulin-like androgenic gland hormone (IAG) was a glandular tissue associated with the terminal ampoule

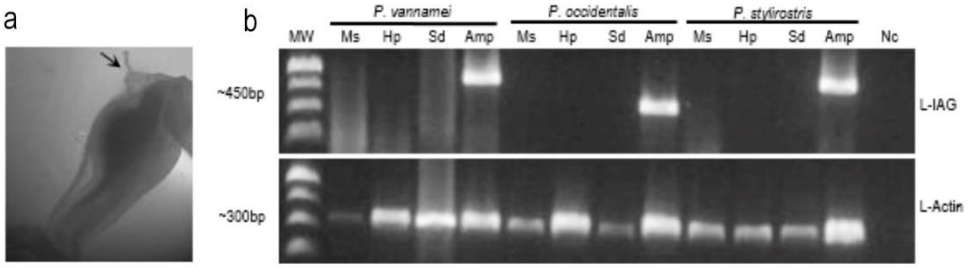

Figure 1. Presence/absence of expression of insulin-like androgenic gland hormone in the terminal ampoules of *P. vannamei. P. occidentalis* and *P. stylirostris* by RT-PCR. a) Location of the distal region of terminal ampoules (massive proliferation of androgenic cells, marked with an arrow) from *Penaeus* sp. induced by bilateral eyestalk ablation, b) IAG expression assay, top panel: Muscle (Ms), hepatopancreas (Hp), sperm duct (Sd), distal terminal ampoule (Amp), negative control (Nc). Bottom panel: Expression of the ß-actin transcript in the same tissue samples as integrity controls.

of the three species of *Penaeus* and *Rimapenaeus byrdi*. However, concerning the distal medial descending vas deferens, Vásquez-Islas *et al.* (2014, 2015) reported the expression of IAG at this region, but in the present study, IAG expression was not detected from tissue bulk of descending and ascending vas deferens in the three species of *Penaeus*.

The IAG precursor consists of a signal peptide, B chain, C peptide, and A chain, which is the common structure of all insulin-like peptides. The mature hormone has three disulfide bonds, two inter-chain bonds, and one intra-chain bond (within the A chain), also several insulin-like peptides require glycosylation for activity (Ventura *et al*., 2011). It is important to note that in proteins with an N-glycosylation site, the metabolic process has been highly conserved in all eukaryotes, playing an important role in protein viability. It functions by modifying appropriate asparagine residues of proteins with oligosaccharide structures, thus influencing their properties and bioactivities (Kukuruzinska & Lennon, 1998). This organization of the IAG precursor agrees with known amino acid sequences of the IAGs reported in *C. sapidus* (AEI72263), *P. paucidens* (BAJ84108), *P. clarkii* (ALX72789) *M. nipponense* (AHA33389), *M. rosenbergii* (ACJ38227), *M. lar* (BAJ78349), *P. monodon* (ADA67878), *F. chinensis* (AFU60548), *M. japonicus* (BAK20460), *S. verreauxi* (AHY99679), *J. edwardsii* (AIM55892), and others. Recently, cDNAs encoding insulin-like androgenic gland hormones have been identified and characterized in *Lysmata wurdemanni* (Zhang *et al.*, 2017), *Sagmariasus verreauxi* and *J. edwardsii* (Ventura *et al.*, 2014).

This study identified a partial sequence of the signal peptide (20 aa) in the three species, also the complete B chain (47 aa), the C chain (60 aa), and the partial A chain, which in *P. vannamei* and *P. stylirostris* had a size of 31 aa and in *P. occidentalis* 33 aa. The previously reported complete sequence of *P. vannamei* has an A chain of 35 aa. In the B chain it was found an N-glycosylation site, a casein kinase II phosphorylation site, and two cysteine residues, while the A chain was composed by a protein kinase C phosphorylation site and four cysteine residues. The multiple amino acid alignment (Fig. 3a) shows five cysteine residues in B (two) and A (three) chains in all the sequences analyzed; this cysteine skeleton is typical of the insulin family of hormones, and it is highly conserved. These cysteine residues fold the mature peptide through disulfide bridges in all decapod families (Ventura *et al*., 2014). On the other hand, Chung *et al.* (2011) suggested that the B chain on the insulin-like androgenic gland factor of *Callinectes sapidus* and others decapod crustaceans, is probably a species-specific region, due to the less conserved amino acid residues observed in this zone. This is consistent with our results for *Penaeus*-IAG, suggesting that nucleotide sequences of genomic DNA derived from the IAG-B chain could be used as a specific nuclear marker for phylogenetic analysis in decapod crustaceans. This characterization allows extending our knowledge of the molecular structure of insulin-like peptides related to sex differentiation in Malacostraca (Ventura *et al.*, 2009; Mareddy *et al.*, 2011; Vázquez-Islas *et al.*, 2014). Concerning the phylogenetic approach, the sequences reported here belong to the same family (Penaeidae), with other species such as *M. japonicus*, *P. monodon* and *F. chinensis* as expected, whereas species from different families such as *C. sapidus* (Portunidae), *Jasus edwardsii* (Palinuridae), *M. rosenbergii* (Palaemonidae) are relatively close to each other but far apart from IAGs from the family Penaeidae.

This study corroborates previous findings concerning the location of the AG in *Penaeus*. The isolation of mRNA of the IAG gene from the distal section of terminal ampoules belonging to three species

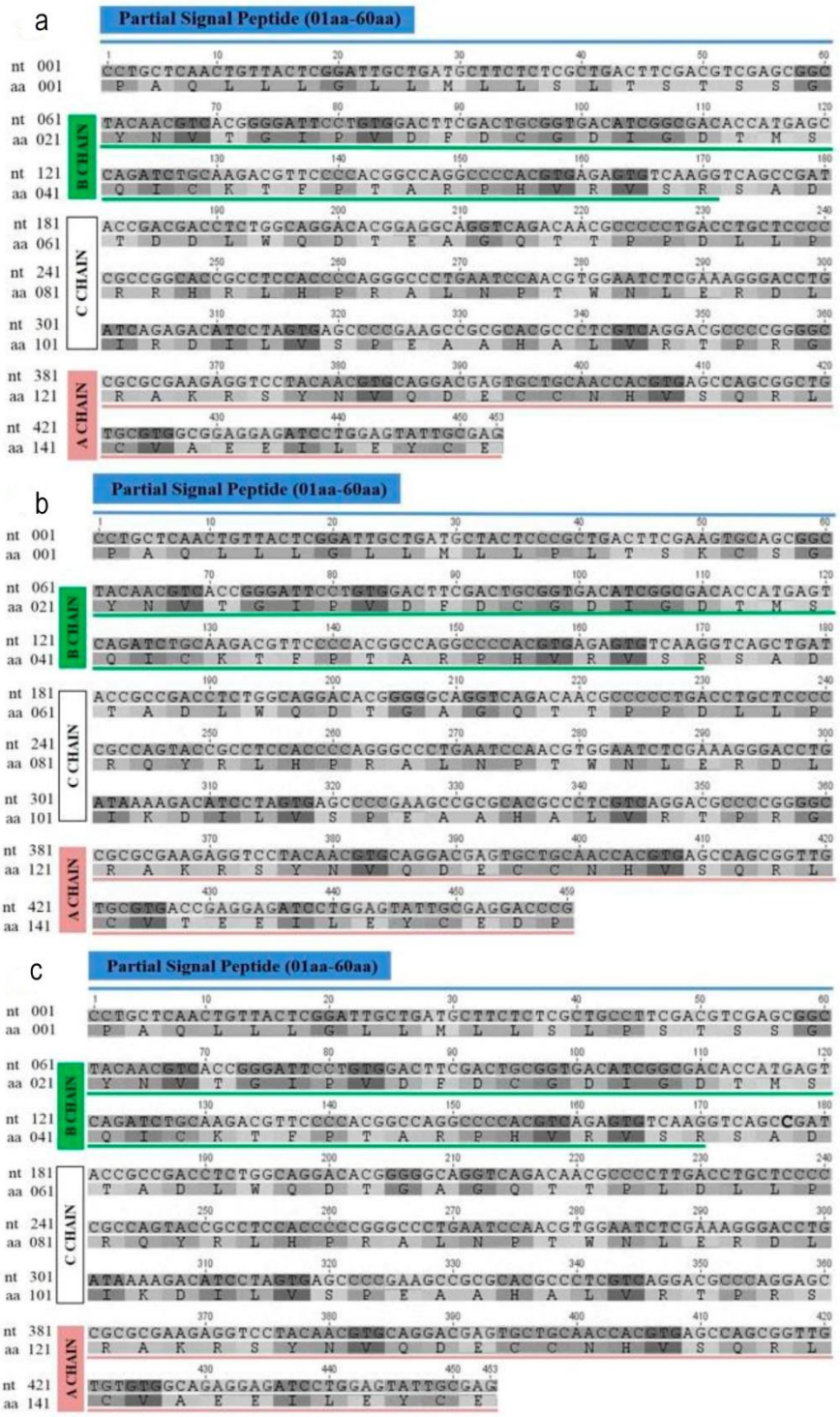

Figure. 2. Nucleotide and deduced amino acid sequence of a partial insulin-like androgenic gland hormone in a) *Penaeus vannamei* (Lv-IAG), b) *Penaeus occidentalis* (Lo-IAG), and c) *Penaeus stylirostris* (Ls-IAG) by RT-PCR. The signal peptide is represented in blue, the green line represents the putative B chain, the putative C peptide is unflagged, and the red line represents the putative A chain.

provides evidence about the distal terminal ampoule as the major site for androgenic hormone release. This knowledge is a fundamental advance on the anatomical description of the male reproductive system of *Penaeus* (Alfaro-Montoya, 2010). Finally, this study has allowed characterizing and comparing the partial sequences of

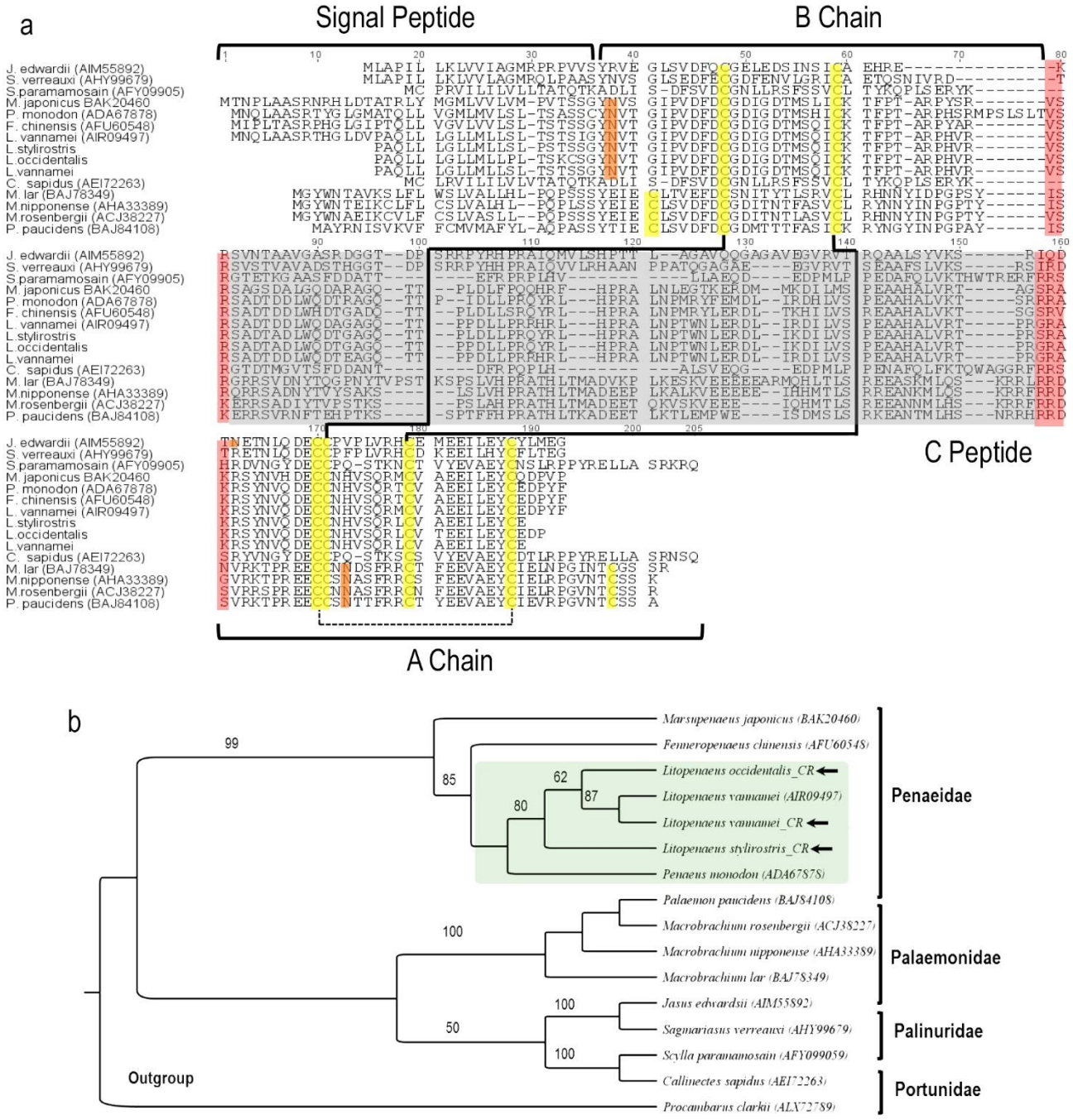

Figure 3. Comparison of insulin-like factors (IAGs) of *P. vannamei*, *P. occidentalis* and *P. stylirostris* (our study) with other reported Decapod AG-insulin-like sequences by using a multiple sequences alignment. a) All IAGs were aligned by BLAST using blosum62 substitution matrix. The predicted arginine (R) endoproteinase cleavage sites (RxxxR, KxxR or xR) are shown in red boxes. The C peptide is located between the cleavage sites, and it is represented in grey. The conserved cysteine residues are highlighted in yellow, which gives rise to predicted disulfide bridges (black lines), the dotted line represented one intra-chain disulfide bonds. Predicted N-glycosylation sites are highlighted in orange. b) Maximum-likelihood phylogenetic tree based on partial and complete sequences of the insulin-like androgenic gland factor obtained from GenBank (13 accessions) and amino acid sequences from this study (*Penaeus*_CR pointed by arrows). Numbers at branching points represent bootstrap values over 1000 repetitions (the bootstrap value lower than 50% not shown). *Procambarus clarkii* insulin-like androgenic gland factor was set as outgroup. GenBank accession numbers are given in parenthesis.

the IAG in the three species of *Penaeus* from the Eastern Pacific: *P. vannamei,* as a species with a known sequence, *P. stylirostris*, and *P. occidentalis.* Two of them have great importance for aquaculture at regional and world level, and one (*P. occidentalis)* is important for regional fisheries. The almost complete ORFs of these three *Penaeus* species are similar among them with over 93% of identity with respect to *P. vannamei* (AIR09497). The fundamental understanding of the gene and its role in sex differentiation and the development of sex reversal techniques can potentially lead to the application of technologies for female culture in penaeids. The synthesis of dsRNA against the IAG gene to produce neo-females (ZZ) will demonstrate the plasticity of sex reversal in shrimps. So far, sex reversal has not been demonstrated in any penaeid species; therefore, the identification and characterization of IAG in species of commercial importance and the general knowledge of the superfamily of genes involved in insulin-like reproductive processes are major advances for the commercial industry of penaeid aquaculture.

ACKNOWLEDGMENTS

This research is part of the project: Reversión sexual del camarón marino de cultivo, *Penaeus vannamei*, and the post-graduate studies of Carolina Sancho Blanco. It was financially supported by Consejo Nacional de Rectores (CONARE), Costa Rica, and was scientifically supported by the research group of Prof. Amir Sagi from Ben-Gurion University of the Negev, Israel. The authors wish to thank Luis Hernández Noguera for his help in the obtaining of samples. All authors declare no conflict of interest.

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Received: 28 April 2017; Accepted: 20 September 2017

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