Litopenaeus vannamei
Transcriptome Profile of Populations Evaluated for Growth Performance and Exposed to White Spot Syndrome Virus (WSSV)

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INTRODUCTION

The Pacific white shrimp Litopenaeus vannamei represents one of the main marine shrimp species commercialized worldwide (Gucic et al., 2013; FAO, 2014; Senapati et al., 2017). Despite its importance on aquaculture and fishery activities, the exposure of wild and captive stocks to pathogens, such as fungi, bacteria and mainly virus, has commonly leading to concerns on healthy and population viability, causing damages related to quality of the shrimp used for local and international trading and human feeding (Wang and Wang, 2013; Liu et al., 2014; Rao et al., 2015). The White Spot Syndrome Virus (WSSV), for instance, may spread very quickly in the open sea, with even more severe harms for the aquaculture stocks, sometimes leading to 100% mortality (FAO, 2017). In addition to the animal health, growth is also an important value for the shrimp aquaculture (Liu et al., 2005; Jung et al., 2013; Lv et al., 2014). This scenario causes large economic losses on the global shrimp farming (Chen et al., 2015) and also on the annual fishing catches (Xue et al., 2013).

Despite the remarkable importance of penaeid shrimp, few data about determinant genes related to growth and immunity are already described for crustacean (Jindra et al., 2013; Jung et al., 2013; Lv et al., 2014), added to the fact that molecular mechanisms involved in growth and immunity are not yet known in detail. In this manner, it becomes evident the need of expanding the knowledge about genes and transcripts involved in cellular responses and physiology of this group. The lack of a shrimp reference genome highlights the importance of studies addressing next generation sequencing (NGS) as a relevant source of information (Guo et al., 2013; Santos et al., 2014; Powell et al., 2015).

In this study, we used the RNA-seq approach for assembling a robust L. vannamei transcriptome obtained from muscle tissue of captive individuals evaluated for growth and high survival performances, and also from hepatopancreas and muscle of animals exposed to White Spot Virus. Sequencing was carried out using Illumina technology (San Diego, California, USA), and the transcripts blastX annotation against Swissprot database returned more than 20 thousand unigenes with known gene products, identifying a higher amount than those previously reported for other marine shrimp transcriptomes (Nguyen et al., 2016; Rao et al., 2016). These findings

Abbreviations: PLs, post-larvae; SPF, Specific Pathogen Free; WSSV, White Spot Syndrome Virus.
certainly constitute an important source of genetic information for further studies, considering gene differential expression analysis and single nucleotide polymorphism (SNPs) identification in crustacean species.

**VALUE OF THE DATA**

- *L. vannamei* has enormous commercial value among penaeid shrimp, which account for one-third of the annual global crustacean fisheries.
- The transcriptome reported herein was produced from hepatopancreas and muscle tissue samples of specific pathogen free (SPF) shrimp submitted to selective breeding programs for high rates of survival and fast growth; and to exposure to White Spot Virus.
- The percentage of unigenes presenting SwissProt hits described in this study is higher than those observed for other shrimp Illumina transcriptomes (e.g., Nguyen et al., 2016; Rao et al., 2016).
- These data certainly constitute an important source of genomic information for *L. vannamei* and other penaeid species in addition to provide resources for the aquaculture studies aiming higher growth gains and WSSV-resistant shrimp.

**MATERIALS AND METHODS**

**Experimental Design and Sampling**

The *L. vannamei* samples which were used in this work are SPF shrimp submitted to selective breeding programs for high rates of survival and fast growth in two shrimp commercial companies (Genearch and Aquatec), both located in Rio Grande do Norte (RN) state, Brazil. All samples were kept in a sanitary environment monitored for the presence of many pathogens, including WSSV and were collected in the first semester of 2015. The genetic improvement programs have been conducted based on performance of closed familiar lines, according to quantitative analyses and genetic gain parameters evaluated by the companies (data not available). For the sampling of individuals evaluated for growth and survival performance, 20 closed families in triplicates were assessed, and four families with the higher and four with the lower growth rates were selected. We sampled muscle tissue (pleopods) of one animal from each family, totaling eight animals evaluated for growth performance. The individuals were about 2 months aged and weighed around 1.5 g. For the WSSV exposed shrimp sampling, we used SPF individuals of a hybrid line from Aquatec, which was originated from the crossing of two different SPF lines, one from Aquatec and another from Genearch. These hybrid shrimps were exposed to WSSV in a farm pond with a high incidence of the disease. Before and after the exposure to the virus, shrimp hemolymph samples were submitted to quantitative real-time PCR (qPCR) analyzes in order to confirm the presence or absence of the WSSV, following a procedure proposed by Silva et al. (2011). The efficiency of qPCR amplification was determined by the method described by Pfaffl et al. (2002) and the specificity of the amplification was confirmed by the analysis of the dissociation curve (Ririe et al., 1997). After the contact with the WSSV and qPCR tests, we selected one positive animal showing WSSV symptoms, and a negative one with no symptoms, both weighting about 6 g. Later, hepatopancreas and muscle tissues from each individual were sampled, totaling four samples from these two shrimps evaluated to WSSV. Hepatopancreas and muscle tissues were chosen given their relevance to immune response in crustacean (Fan et al., 2016), and as a target for pathogen infections, including WSSV (Yu et al., 2017), respectively. The tissue samples were conditioned in RNA later (Thermo Fisher Scientific, Waltham, MA, USA) and kept in biofreezer (−80°C) for the isolation of total RNA.

**RNA Isolation and cDNA Library Preparation**

In total, 12 cDNA libraries were established. Eight libraries were constructed using muscle tissue of individuals evaluated for growth performance; the four remaining ones were constructed using muscle and also hepatopancreas from the two individuals exposed and evaluated to WSSV by qPCR. Total RNA isolation was performed using the Trizol®/chloroform protocol (Chomczynski and Mackey, 1995), modified for the centrifugation time in ethanol washing (12 min) and added a purification stage using lithium chloride (7.5 M) for elimination of phenol traces. The quality was assessed in a Qubit fluorometer (Thermo Fisher Scientific), quantity was evaluated in a NanoDrop spectrophotometer (Thermo Fisher Scientific) and samples with ratios between 1.8 and 2.2 were considered pure. The integrity of the samples was confirmed in a BioAnalyser equipment (Agilent Technologies Inc., Santa Clara, CA, USA) and those with RNA Integrity Number (RIN) ≥ 6 were stored at −80°C. A cDNA library was produced from each sample using a TruSeq RNA Library Preparation V2 kit (Illumina Inc., San Diego, California, USA).

**Sequencing, de Novo Assembly and BlastX Annotation**

Sample quantification was carried out in qPCR with the Universal Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA), prior to loading into the sequencer. The libraries were grouped and ran on the Illumina HiSeq 2500 platform with 2 × 100 bp paired-end, using a TruSeq SBS V3 kit (Illumina Inc., Thermo Fisher Scientific), at the Laboratório de Biotecnologia Animal, Escola Superior de Agricultura “Luiz de Queiroz”—Universidade de São Paulo (ESALQ-USP). The quality of the raw data generated after sequencing was visualized in the FastQC software (version 0.10.1) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All reads were filtered for Phred quality (QS) 23 (sequence inside) and 30 (sequence edges) and minimum length of 65 bp using the SeqyClean software (v.1.9.9) (https://github.com/ibest/seqyclean). The same package was also used to remove contaminant sequences (primers and vectors) using the Univec database (https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/). After filtering, the reference de novo assembly was performed in the Trinity software (Grabherr et al., 2011). The sequences were normalized using Trinity's
RESULTS AND DISCUSSION

The 488,681,310 transcripts from the 12 libraries were filtered on SeqyClean for contaminants, rRNA and low quality sequences. From these, about 30 million paired reads (29,376,967) remained after normalization in Trinity package, resulting in 28,654,475 (97.5%) fragments used in the reference transcriptome after filtering. We obtained 63,105 transcripts with 2,511 bp mean length and N50 of 3,464 bp. From this total, 20,865 (33%) showed blastX hits on SwissProt database (Supplementary Material 1; Table 1). The contigs filtered to <95% of similarity were considered unigenes after the reference assembly. The contig average size found here was greater than those previously reported in L. vannamei (Ghaffari et al., 2014), Penaeus monodon (Nguyen et al., 2016) and Macrobrachium rosenberguii (Rao et al., 2015, 2016) Illumina transcriptomes. The longest contig size observed in our study was equal to 20,061 bp, being superior to the value of 13,578 bp reported for P. monodon transcriptome (Nguyen et al., 2016). After elimination of the redundant contigs, low complexity sequences and isoforms, candidate-coding regions were identified, remaining 14,124 unigenes. The de novo assembly and the automatic annotation results are shown in Table 1.

| Transcripts used in the construction of the reference after filtering on SeqyClean |
|---------------------------------------------|
| Tissue |
| Muscle (8 libraries) |
| Growth | Muscle (2 libraries) | WSSV-exposure |
| Condition | Higher growth | Lower growth | WSSV-negative | WSSV-positive | Hepatopancreas (2 libraries) |
| Paired-end fragments | 142,094,916 | 150,593,354 | 44,029,454 | 44,972,016 | 59,850,408 | 47,141,162 |
| Total | 292,688,270 | 89,001,470 | 106,991,570 | 195,993,040 |
| 488,681,310 |

| Transcripts after normalization in Trinity |
|---------------------------------------------|
| Reads number after normalization | 29,376,967 |
| Remaining reads number after low quality and adaptors filtering/Reads number used in reference de novo assembly | 28,654,475 (97.5%)* |

| Reference transcriptome analysis |
|---------------------------------------------|
| Total number of contigs produced | 63,105 |
| SwissProt blastX hits unigenes | 20,865 (33%)** |
| Average size | 2,511 bp |
| N50 | 3,464 bp |
| Longest transcript (bp) | 20,601 |
| Transcripts number > 1,000 bp | 27,887 |
| Number of non-redundant unigenes with ORFs | 14,124 |

(*) from total reads number after normalization and (**) from total number of contigs produced.
Following the sequences alignment against SwissProt database, the focus was given to genes that showed hits for arthropod species and fitness-related functions, such as growth and disease resistance. Among the most frequent proteins exclusively identified in the animals evaluated for growth performance are those related to myosin (MYSa) (41%), tropomyosin and myosin heavy chain (9%), chitinase (6%) and molt-inhibiting hormone-like (2%) genes (Figure 1). Myosin acts on the formation of the muscular myofibrils and, along with the paramyosin, is responsible for the stabilization of the sarcomere (Liu et al., 2005). There is evidence that increased myosin production and reorganization of myofibrils is associated with growth during and after shrimp molt (Cesar and Yang, 2007; Li et al., 2014). Chitin constituent proteins are among those responsible for the digestion of arthropod chitin exoskeleton, enabling molt and animal growth (Li et al., 2015). Among the most frequent genes in the WSSV-exposed shrimp samples returning blastX hits were mainly those related to crustacyanin 1 (CRA1) and 2 (CRA2) (13%), lectins (8%) and hemocyanin B (HCYB), and C (HCYC) heavy chain (6%) (Figure 1). Hemocyanin and crustacyanin proteins have a wide range of action against viruses, especially WSSV, acting on animal stress and survival responses (Fan et al., 2016), agglutination of the pathogen on hemolymph and cell lysis (Shockey et al., 2009; Zheng et al., 2016). On the other hand, lectins act on the pathogen specific recognition through molecular structures to a given molecule (Song et al., 2010).

The de novo transcriptome assembly from muscle and hepatopancreas of non-infected and infected shrimp, enabled the identification of important genes with potential fitness-related functions involved in growth and immunity. Such assembly strategy, gathering two different tissues with diverse functions, was performed in the effort to reach a wider range and representativeness of transcripts, producing the most robust transcriptome dataset for L. vannamei reported to date. It becomes of particular interest for the species lacking a reference genome, such as the majority of crustacean, including L. vannamei. Considering the high accuracy and wide coverage of RNA-seq produced here, this dataset can be useful for a gamma of further approaches, such as differential gene expression studies, analyses of isoforms originated from alternative splicing (Rao et al., 2015; Sun et al., 2015), SNPs calling (Cui et al., 2014; Santos et al., 2014; Yu et al., 2014), with potential to high-density chip and linkage map (Baranski et al., 2014), apart from genome wide association (GWAS) studies (Yu et al., 2017).

**AUTHOR CONTRIBUTIONS**

CS has contributed to the transcriptome experiments, data analysis, and article writing and discussion; SA has conducted the bioinformatics analyses and participated in the article writing and discussion; KK has performed the qPCR analyses and the experiments related to the WSSV exposure; JR, FF, AT, and AG have conducted the selective breeding programs in the Genearch and Aquatec companies; PG has collaborated in the article writing and discussion; PF has lead the work, participating in the data analyses, article writing and discussion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00120/full#supplementary-material

Supplementary Material 1 | All the blastX hits found for 20,865 transcripts in the SwissProt database obtained for Litopenaeus vannamei.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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