5.7 Generation of a multi-tissue transcriptomic dataset as a tool to identify potential molecular physiological markers for growth and reproductive studies in the Pacific halibut

Josep V. Planas and Claude Dykstra

Abstract

Monitoring physiological processes (e.g. growth, reproduction, performance, etc.) in Pacific halibut is important to understand the physiological status and condition of the fish in its environment. Furthermore, it is also important to be able to detect changes in the various physiological processes as a result of environmental, ecological or fisheries-induced influences. Therefore, the identification, validation and potential application of markers of physiological processes to monitor fish from a physiological point of view is important for our understanding of the biology of Pacific halibut and its response to external influences. The objective of this study was to identify molecular physiological markers through the use of state-of-the-art RNA sequencing of Pacific halibut tissues. This study has generated more than 300,000 transcripts in six tissues that, after annotation, has led to the identification of a large dataset of genes of known function in other species and that represent potential molecular marker genes. In summary, this study represents the first successful attempt at providing genomic resources for the Pacific halibut and, specifically, molecular tools that will allow us to better understand physiological changes related to growth and reproduction in Pacific halibut.

Introduction

One of the primary goals of the research program at International Pacific Halibut Commission (IPHC) is to improve our current knowledge of aspects of the biology of the Pacific halibut (*Hippoglossus stenolepis*) that have implications for stock assessment and the management of the fisheries. Current research efforts at IPHC are devoted to study applied aspects related to the growth, reproduction, migration and genetics in Pacific halibut as well as physiological conditions related to survival of bycatch and fish discarded from the directed fishery. Particularly important is our ability to monitor changes in biological processes over time and space given that these are potentially impacted by environmental and anthropological influences. Therefore, there is a great need to identify and develop novel indicators of biological changes in the Pacific halibut population to evaluate the immediate response of the population, as well as to predict biological changes in the future. In this regard, research approaches investigating the transcriptome (i.e. the collection of transcripts from expressed genes that in great part will be translated into proteins) are enormously useful to identify potential biological markers for a variety of reasons. First, given that the transcriptome is a reflection of the activation state of the genome (i.e. through the transcription or expression of the genome), transcriptomic assessment provides an opportunity to evaluate physiological responses of organisms to external influences at a molecular level. It is well known that gene expression changes represent some of the first and most important responses driving adaptive changes in organisms. Second, although not all genes present in a vertebrate genome (i.e. estimated to contain approximately 20,000 to 30,000 genes) are expressed at all times

nor in all cells, the number of genes that are expressed at any given time or tissue under specific physiological conditions is very high (e.g. in the order of thousands) and, therefore, transcriptomic approaches can identify large numbers of potential marker genes for specific biological processes or tissues. Finally, currently available RNA sequencing (RNA-seq) technologies have become increasingly powerful and economical to allow an unprecedented view of the transcriptome of any species (Ozsolak and Milos, 2011) and this is particularly relevant for species without a sequenced genome, such as the Pacific halibut. Similar technical approaches have been adopted to generate genomic and transcriptomic resources in other flatfish species (Benzeki et al., 2014; Ribas et al., 2013). Therefore, IPHC has embarked on an initial characterization of the transcriptome of the Pacific halibut that will not only represent the first genomic resources for the species but that will lead to the identification of potential useful markers to monitor growth, reproduction, performance and condition in this species.

Materials and Methods

Sample collection

Five adult Pacific halibut (4 females and 1 male) were captured and sampled between December of 2015 and January of 2016 by Levy Boitor on the *F/V Highliner* in the waters off Petersburg, AK. From each fish, tissue samples were excised with the use of sterile dissection equipment and placed in 1 ml of RNAlater in screw-cap microcentrifuge tubes. Tissues excised were gonads (testes in males and ovaries in females), liver, white skeletal muscle in all five fish, and red skeletal muscle and heart in only two fish. Samples were kept refrigerated until shipped to IPHC, where they were stored at -20 C until they were processed.

RNA purification, RNA sequencing and bioinformatic analyses

Tissue samples in RNAlater were shipped to Omega Bioservices[1] and processed. Total RNA was purified from individual tissue samples and RNA quality was assessed by conventional means. Once individually purified, RNAs were pooled for each tissue and this resulted in RNA pools for ovary (four pooled individual RNA samples), testis (one individual RNA sample), liver (five pooled individual RNA samples), white skeletal muscle (five pooled individual RNA samples), red skeletal muscle (two pooled individual RNA samples) and heart (two pooled individual RNA samples). Pooled RNA samples, each corresponding to a separate tissue, were sequenced at Omega Bioservices at pair end mode 2 x 100 base pairs (bp) on an Illumina HighSeq2500 sequencing platform, with an expected sequencing coverage of 30 million reads per sample. Reads were assembled *de novo* by Omega Bioservices using the Trinity software into contigs. Contigs were then annotated using an iterative blast strategy by using, as a first step, blastx against the zebrafish (*Danio rerio*) protein database. As a second step, non-annotated transcripts were then subjected to blastx against the vertebrate RefSeq protein database. As a third step, contigs not annotated by any of the two previous steps were then subjected to tblastx against selected NCBI fish EST collections.

[1]Omega Bio-tek, Inc. dba Omega Bioservices, 400 Pinnacle Way, Suite 450, Norcross, GA 30071

Results

RNA sequencing statistics

The total number of filtered reads (i.e. useable sequences) that were obtained per each of the tissues sequenced (i.e. white muscle, liver, ovary, testis, red muscle and heart), combining the reads from either end (left or R1 and right or R2) of each product of the paired-end libraries, ranged between approximately 21,400,000 (liver) and 44,760,000 (red muscle) (Table 1). The sequence outcome in terms of the number of reads was consistent with the requested depth of 30,000,000 reads per sample. *De novo* assembly of the reads into contigs (i.e. contiguous DNA sequence assembled or reconstructed from shorter overlapping sequence reads; Fig. 1) yielded a large number of transcripts and a slightly lower number of non-redundant transcripts (named 'genes') (Table 2). The number of non-redundant transcripts or 'genes' ranged from 37,161 in white muscle to 74,363 in the testis. Contig N50 values, a metric that indicates the contig length of more than 50% of the contigs assembled for a particular tissue, ranged from 1,096 bp in the liver to 2,494 bp in the ovary. The median contig length also ranged from 385 bp in the white muscle to 582 bp in the ovary. Consistent with these previous values, the liver and the ovary had the lowest and highest average contig lengths, respectively, with values ranging from 692 to 1,240 bp.

Annotation of the contigs was performed by an iterative blast protocol, as described above and shown diagrammatically in Fig. 1. The number of contigs successfully annotated against the *Danio rerio* RefSeq protein sequence database was high and ranged from 13,233 from the liver to 25,341 contigs from the red muscle (Table 3). However, the percentage on annotated contigs against this database with respect to the total number of contigs was very similar among the different tissues, with values ranging from 26.98% (testis) to 35% (white muscle). All non-annotated contigs after the first round of annotation against the *Danio rerio* protein database that were subsequently annotated against the Uniprot database produced a modest number of annotated contigs, ranging from 2,547 contigs in the liver to 5,579 contigs in the red muscle. Similar to the initial mapping of contigs against Danio rerio, the second annotation step against Uniprot yielded a similar number of annotated transcripts among the different tissues, ranging from 6.24% (liver) to 7.09% (ovary) of the total number of contigs. The third annotation step consisted in mapping the remaining non-annotated transcripts (approximately 65% of the original number of contigs) against the expressed sequence tag (est) database at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). This last annotation strategy almost resulted in a complete mapping of the remaining contigs (Table 3), leaving only a very small number of contigs that did not map to any of the three databases, ranging from 0.09% to 0.22% of the total number of contigs.

Identification of potential markers for reproduction and growth in the Pacific halibut

An initial search was conducted by interrogating the testis, ovary and white muscle sequence datasets for an initial set of transcripts coding for proteins known for their important function in reproduction and growth. A selection of functionally important reproductive genes expressed in the Pacific halibut testis is shown in Table 4, that includes transcripts that code for proteins important in steroid and prostaglandin production (*star*, *ptgs1*), male sex differentiation (*dmrt1* and *sox9a*) receptors for hormones such as testosterone, progesterone, gonadotropic hormone releasing hormone and inhibin (*ar*, *inhbb*, *gnrhr1*, *pgr*), hormones such as follistatin (*fsta*) and spermatid, spermatogonial and germ cell markers (*rsbn1*, *nanos3*, *strbp*, *ddx4*). Table 5 shows a selection of genes expressed in the ovary, including genes involved in oogenesis (*acvr1ba*, *bmp1a*, *inhbb*),

ovulation (*adamts2*, *mmp2*, *mmp9*), hormone signaling including receptors for estradiol and the pituitary gonadotropins follicle stimulating hormone and luteinizing hormone (*ar*, *esr2*, *fshr*, *gnrh4*, *lhcgr*), steroid and prostaglandin synthesis (*cy19a1a*, *hsd17b11*, *ptgs2b*), oocyte maturation (*egfr*, *pgr*) and hydration (*aqp10b*). A set of important potential markers for growth were initially identified and selected by searching the white skeletal muscle transcriptome dataset, as shown in Table 6. Selected transcripts represented growth processes involved in protein synthesis (*ar*, *eif4eb*, *mtor*) and atrophy (*fbxo32*, *trim63b*), force transmission (*camk2a*, *dmd*, *myoz1a*), energy metabolism (*ckma*, *gys1*, *ppargc1a*, *pygma*, *prkaa1*), muscle growth regulation (*igf2r*, *hdac1*, *mef2cb*, *myf6*, *myhz1*.3, *tgfb1a*) and lipid metabolism (*cpt1b*, *lpl*), among others.

Discussion

The present study has generated the first genomic resources for the Pacific halibut. By RNA-seq followed by *de novo* assembly, approximately 326,000 transcripts with an average length close to 1,000 bp have been obtained from six different tissues: ovary, testis, white and red skeletal muscle, liver and heart. Although this is a significant number of transcripts, the level of redundancy (i.e. different transcripts that correspond to the same gene) needs to be determined and will likely yield a much smaller number of unique or non-redundant transcripts. The acceptable size of the contigs assembled allowed for an annotation rate from 33% to 42% of the total number of transcripts. It is worth noting, however, that although mapping of the remaining transcripts against the 'est others' database was high (about 60%), the level of annotation was extremely poor since most of the matches corresponded to non-annotated expressed sequence tags from other teleost species.

An initial search for potential markers for reproduction and growth in the ovarian and testicular and white skeletal muscle datasets, respectively, identified a number of genes with known important functions in these processes. Therefore, the selection of identified genes may represent potentially relevant markers for reproduction and growth. Given that both males and females were captured during the spawning season, several reproductive markers identified could be used to monitor the reproductive status of the fish. For example, the spermatid markers *rsbn1* and *strbp* could potentially indicate the presence of spermatids, an advanced testicular germ cell type that matures into spermatozoa, and, therefore, testicular tissue at an advanced stage in spermatogenesis. Interestingly, the testicular dataset included male sex differentiation factors (e.g. *dmrt1*, *sox9a*) that could represent potentially useful genetic markers for sex. In fact, although the sex-determining gene in the Pacific halibut is not known, *dmrt1* could potentially be the sex determining gene in this species given its important role as the male sex determining genes in other teleost species (Pan et al., 2016). Similarly, in the ovary several potential markers of oocyte maturation and ovulation were found, most remarkably the receptors for progesterone (*pgr*) and for luteinizing hormone (*lhcgr*). These two hormones (i.e progesterone and luteinizing hormone) are known in other species to be required during the later stages of reproduction for the induction of oocyte maturation and ovulation (Nagahama and Yamashita, 2008). Furthermore, the presence of proteases known to participate in ovulation induced by progesterone and luteinizing hormone, including *adamts2*, *mmp2* and *mmp9*, that have a key role in the degradation of the extracellular matrix and the rupture of the follicle wall (Crespo et al., 2015; Ogiwara et al., 2005), was observed. Therefore, potential useful markers for maturity and ovulation in female Pacific halibut have been identified.

In white skeletal muscle, a tissue that is functionally linked to growth and swimming performance, a number of genes involved in these processes were identified and could represent potential molecular markers for evaluating muscle growth, muscle energy and performance. It is worth noting the identification of key genes involved in protein synthesis and, therefore, muscle accretion such as the key signaling molecules *mtor* and *eif4eb*, described in the literature as part of the main protein synthesis pathway in muscle (Laplante and Sabatini, 2012). An important set of molecules involved in growth regulation were identified, mostly related to growth factors and myogenic factors, such as *mef2cb*, known to be activated by the mTOR pathway and important in muscle fiber growth (Naya and Olson, 1999). Importantly, a number of genes involved in energy metabolism, either representing catabolic processes or anabolic, were identified including key molecules that sense the levels of energy in the cell, such as *prkaa1* and *ppargc1a*, and that could potentially be interesting markers to assess the energy condition of the fish (Kahn et al., 2005).

After the initial identification of relevant genes for the physiological processes under study (e.g. reproduction and growth), molecular assays have to be developed to validate the use of these potential molecular physiological markers for their application in subsequent field and captive studies with Pacific halibut. Future work will focus on the development of quantitative real-time PCR (qPCR) assays based on the sequences obtained to detect the expression of molecular markers to be tested and to subsequently quantify the expression levels of the selected markers under various physiological conditions. As an example, molecular growth markers will be tested and validated using skeletal muscle samples from juvenile Pacific halibut that grow under two different temperatures in order to generate fast versus slow growth patterns. In addition, molecular reproductive growth markers will be tested comparing ovarian or testicular samples from immature versus mature individuals. These studies will eventually lead to the development of assays for molecular markers that will allow us to better understand physiological changes related to growth and reproduction in Pacific halibut.

Importantly, one of the goals of a high-throughput gene discovery project as that outlined here is the deposition of the resulting sequences (hundreds of thousands of transcripts) in dedicated sequence databases that can be used to interrogate the deposited datasets and that is made public for use by the fisheries and scientific community.

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Table 1. Sequencing read filtering statistics.

Tissue	R1[1] Reads before filter	R2[2] Reads before filter	R1 Reads after filter	R2 Reads after filter	R1 Reads dropped	R2 Reads dropped
White muscle	15,147,002	15,147,002	15,141,056	15,141,056	5,946	5,946
Liver	10,508,353	10,508,353	10,501,969	10,501,969	6,384	6,384
Ovary	21,650,949	21,650,949	21,633,579	21,633,579	17,370	17,370
Testis	19,792,232	19,792,232	19,783,945	19,783,945	8,287	8,287
Red muscle	22,398,327	22,398,327	22,389,501	22,389,501	8,826	8,826
Heart	20,974,678	20,974,678	20,966,673	20,966,673	8,005	8,005

[1]First of two reads obtained by sequencing a paired end library.
[2]Second of two reads obtained by sequencing a paired end library.

Table 2. De novo assembly transcript statistics.

Tissue	Total 'genes'	Total transcripts	GC (%)	Contig N50 (bp[1])	Median contig length (bp)	Average contig (bp)	Total assembled bases
White muscle	37,161	39,638	47.76	1,198	385	721	28,598,382
Liver	38,143	40,814	46.02	1,096	398	692	28,237,340
Ovary	48,573	60,084	48.89	2,494	582	1240	74,513,854
Testis	74,363	87,644	47.10	2,004	489	1015	88,917,698
Red muscle	70,693	86,561	47.17	2,104	495	1052	91,050,930
Heart	57,697	70,338	47.58	2,322	534	1146	80,597,106

[1]bp: base pairs.

Table 3. Annotation statistics.

Tissue	Danio rerio	Uniprot	est[1] others	Total	Danio rerio (%)	Uniprot (%)	est others (%)	Not mapped (%)
White muscle	13,873	2,661	23,066	39,638	35.00	6.71	58.19	0.10
Liver	13,233	2,547	24,998	40,814	32.42	6.24	61.25	0.09
Ovary	18,426	4,259	37,267	60,084	30.67	7.09	62.02	0.22
Testis	23,644	5,539	58,303	87,644	26.98	6.32	66.52	0.18
Red muscle	25,341	5,579	55,466	86,561	29.28	6.45	64.08	0.20
Heart	20,824	4,715	44,681	70,338	29.61	6.70	63.52	0.17

[1]est: expressed sequence tags

Table 4. Selection of annotated important reproductive genes expressed in the Pacific halibut testis.

Annotation	Gene symbol	Length (nt)	Identity (%)	Function
Androgen receptor	*ar*	2574	71.15	Spermatogenesis
Cation channel sperm-Associated protein subunit gamma 2	*catsperg2*	248	68.75	Sperm activation
DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	*ddx4*	1411	78.57	PGC marker
Doublesex- and mab-3-related transcription factor 1	*dmrt1*	2573	65.77	Male sex differentiation factor
Follistatin a	*fsta*	2126	89.68	Hormone
Inhibin beta B	*inhbb*	624	80.56	Hormone receptor
Gonadotropin releasing hormone receptor 1	*gnrhr1*	310	79.61	Hormone receptor
Nanos homolog 3	*nanos3*	2899	82.09	Spermatogonial marker
Progesterone receptor	*pgr*	2763	83.42	Hormone receptor
Prostaglandin-endoperoxide synthase 1	*ptgs1*	345	74.56	Prostaglandin production
Round spermatid basic protein 1	*rsbn1*	234	97.83	Spermatid marker
Spermatid perinuclear RNA binding protein	*strbp*	3258	89.27	Spermatid marker
SRY (sex determining region Y)-box 9a	*sox9a*	635	92.67	Male sex differentiation factor
Steroidogenic acute regulatory protein	*star*	235	79.49	Testicular steroidogenesis

Table 5. Selection of annotated important reproductive genes expressed in the Pacific halibut ovary.

Annotation	Gene symbol	Length (nt)	Identity (%)	Function
Activin A receptor, type IBa	*acvr1ba*	3754	89.31	Oogenesis
ADAM metallopeptidase with thrombospondin type 1 motif, 2	*adamts2*	585	74.74	Ovulation
Androgen receptor	*ar*	3062	82.07	Hormone signaling
Aquaporin 10b	*aqp10b*	1654	76.81	Oocyte hydration
Bone morphogenetic protein 1a	*bmp1a*	1680	94.97	Oogenesis
Cytochrome P450, family 19, subfamily A, polypeptide 1a	*cyp19a1a*	976	70.78	Aromatase (estrogen production)
DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	*ddx4*	2580	76.09	Oogonia marker
Epidermal growth factor receptor	*egfr*	232	71.62	Maturational signaling
17-beta-dehydrogenase 11	*hsd17b11*	209	66.67	Steroidogenesis
Estrogen receptor beta	*esr2*	4620	80.88	Hormone signaling
Follicle stimulating hormone receptor	*fshr*	287	79.79	Hormone signaling
Forkhead box L2	*foxl2*	2006	80.39	Female sex differentiation
Gonadotropin releasing hormone receptor 4	*gnrhr4*	306	70.83	Hormone signaling
Inhibin, beta B	*inhbb*	1391	71.32	Oogenesis
Lutropin-choriogonadotropic hormone receptor	*lhcgr*	1466	62.65	Hormone signaling
Progesterone receptor	*pgr*	771	82.1	Maturational signal
Prostaglandin-endoperoxide synthase 2b	*ptgs2b*	2592	80.26	Prostaglandin synthesis
Matrix metallopeptidase 2	*mmp2*	3164	77.49	Ovulation
Matrix metalloproteinase-9	*mmp9*	292	76.92	Ovulation

Table 6. Selection of annotated important growth genes expressed in the Pacific halibut white skeletal muscle.

Annotation	Gene symbol	Length (nt)	Identity (%)	Function
Androgen receptor	*ar*	4426	81.48	Protein synthesis
Calcium/calmodulin-dependent protein kinase II alpha	*camk2a*	2342	87.27	Force transmission
Creatine kinase, muscle a	*ckma*	2256	89.76	Energy metabolism
Carnitine palmitoyltransferase 1B	*cpt1b*	762	81.82	Lipid metabolism
Dystrophin	*dmd*	1282	75.23	Force transmission
Eukaryotic translation initiation factor 4eb	*eif4eb*	1168	85.19	Protein synthesis
F-box protein 32	*fbxo32*	695	86.25	Protein atrophy
Glycogen synthase 1	*gys1*	3328	89.47	Energy metabolism
Histone deacetylase 1	*hdac1*	2490	96.35	Muscle repressor
Insulin-like growth factor 2 receptor	*igf2r*	511	70.62	Growth regulator
Insulin-like growth factor binding protein 5b	*igfbp5b*	1372	81.5	Growth regulator
Lipoprotein lipase	*lpl*	1789	60.48	Lipid metabolism
Myocyte enhancer factor 2cb	*mef2cb*	5841	79.8	Muscle growth
Myostatin b	*mstnb*	789	95.74	Growth regulator
Mechanistic target of rapamycin	*mtor*	1153	97.92	Protein synthesis
Myogenic factor 6	*myf6*	819	76.19	Muscle growth
Myosin, heavy polypeptide 1.3, skeletal muscle	*myhz1.3*	246	86.42	Muscle growth
Myoblast determination protein 1 homolog	*myod*	2497	72.67	Muscle development
Myozenin 1a	*myoz1a*	795	74.6	Force transmission
Nuclear factor of activated T-cells, cytoplasmic 3	*nfatc3*	1587	62.96	Muscle activity
Paired box 3a	*pax3a*	269	75	Muscle development
Paired box 7b	*pax7b*	297	85.71	Muscle development
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	*ppargc1a*	519	88.7	Energy metabolism
Protein phosphatase 3, catalytic subunit, alpha isozyme	*ppp3ca*	3407	83.69	Muscle activity
Protein kinase, AMP-activated, alpha 1 catalytic subunit	*prkaa1*	1925	70.96	Energy metabolism
Phosphorylase, glycogen, muscle	*pygma*	5514	90.91	Energy metabolism
Serum response factor	*srf*	4393	63.81	Muscle development
Transforming growth factor, beta 1a	*tgfb1a*	561	77.04	Growth regulator
Tripartite motif containing 63b	*trim63b*	2117	81.16	Protein atrophy

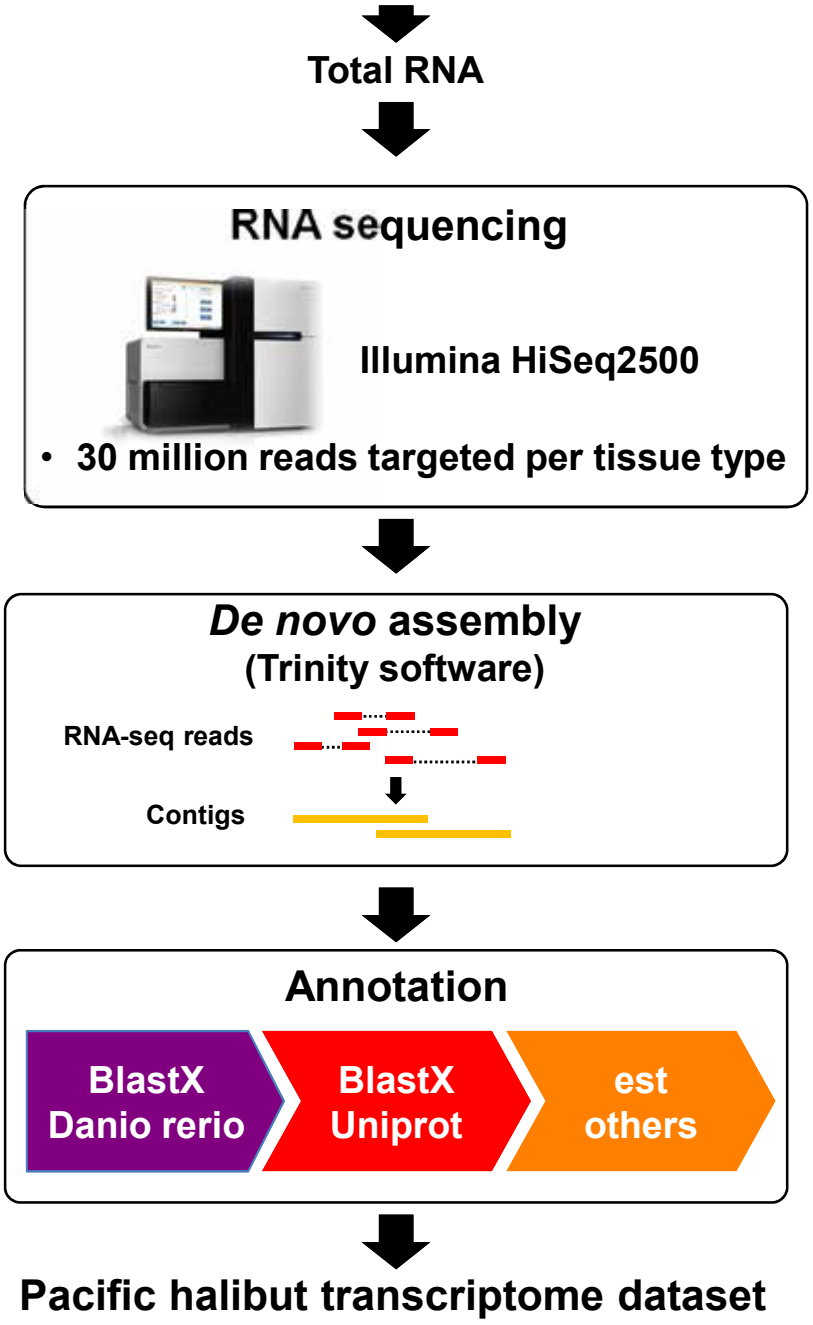

Figure 1. Schematic diagram of the RNA sequencing process. Pooled samples (n=1-5) for each tissue were pooled and processed for total RNA purification and subsequent RNA sequencing on an Illumina´s HighSeq2500 sequencer. Reads per tissue were independently *de novo* assembled and annotated according to an iterative blast strategy.