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Source: Zoological Science, 33(4):366-371.

Published By: Zoological Society of Japan

DOI: <http://dx.doi.org/10.2108/zs150186>

URL: <http://www.bioone.org/doi/full/10.2108/zs150186>

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De Novo Assembly of the Transcriptome of *Turritopsis*, a Jellyfish That Repeatedly Rejuvenates

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In most animals, aging is an irreversible process; however the species *Turritopsis* sp. has been observed to undergo a rejuvenation process as many as 14 times. In the present study, we used multiplexed RNA libraries to obtain the transcriptome from four developmental stages (St) of *Turritopsis* sp., including (I) immature medusa, (II) dumpling, (III) dumpling with a short stolon, and (IV) polyp, which had recently rejuvenated. A total of 4.02 billion paired-end reads were assembled *de novo*, yielding 90,327 contigs. Our analyses revealed that significant blast hits were recovered for 74% of the assembled contigs, and 19% were successfully annotated with gene ontology (GO) terms. A BLAST search demonstrated that 32% of the contigs were most similar to *Hydra vulgaris* sequences. Raw reads from each sample were mapped against the contigs to find St-specific genes. This represents the first comprehensive set of *de novo* transcriptome data for this species, which may provide clues toward a better understanding of cyclical rejuvenation in multicellular animals.

Key words: jellyfish, RNA-Seq, rejuvenation, *de novo* assembly, *Turritopsis*

INTRODUCTION

Life cycle reversion, which is rejuvenation from a mature or young medusa to a polyp, is a unique feature of some cnidarians, including *Turritopsis* spp. (Bavestrello et al., 1992; Piraino et al., 1996; Kubota, 2005), *Laodicea undulata* (Kubota, 2006; Vito et al., 2006), *Linuche unguiculata* (Vito et al., 2006) and *Aurelia* sp.1 (He et al., 2015). Of these, *Turritopsis* sp., an undescribed species (Kubota, 2009), is the only one in which repeated cycles of rejuvenation has been observed, more than 10 times in a female colony from southern Japan (Kubota, 2011). Interestingly, the same colony has continued to repeat this reversal of its life cycle, suggesting a possibility that these animals are in some sense ‘immortal.’ This phenomenon, however, may in fact represent a form of regeneration, since dumpling-stage animals which have degenerated from a medusa develop into polyps without passing through the planula stage, which is typical of cnidarian juveniles. We have observed that two different states of dumplings punctured with a pair of stainless needles transformed into two different forms; one developed directly into a medusa and the other into a stolon (Kubota, 2016). We believe that the latter phenomenon is a form of rejuvenation, or “life-cycle reversal,” while the former

represents regeneration. Elucidating the mechanisms underlying rejuvenation in this species may help to clarify the processes that lead to transdifferentiation, dedifferentiation, and aging.

Recently, a number of transcriptome studies using next-generation sequencing (NGS) in cnidarians have been reported (Siebert et al., 2011; Kitchen et al., 2015; Sanders and Cartwright, 2015), in addition to whole-genome sequencing of *Nematostella vectensis* (Putnam et al., 2007), *Hydra magnipapillata* (Chapman et al., 2010), and *Acropora digitifera* (Shinzato et al., 2011). However, to our knowledge, no molecular biological studies in the Oceaniiidae have been performed. Next-generation sequencing is a useful strategy for the study of gene expression in *Turritopsis* sp., and it enables an important step in understanding rejuvenation in this species. In the present report, we present the now publicly available sequence of a repeatedly rejuvenating jellyfish, and highlight the genes that appear to be involved in rejuvenation.

MATERIALS AND METHODS

Sample collection and preparation

A total of 11 *Turritopsis* sp. medusae were collected on 14–15 August 2014 in Tanabe Bay, Japan (33.68°E, 135.36°N). All of the materials were collected along the wharf using a plankton net hand-towed from the bottom (7 m deep) to the surface. After collection, immature medusae were each punctured over 100 times with a pair of stainless steel needles, and transferred individually into 60 cc polystyrene containers filled with 5 µm of filtered natural seawater and placed in an incubator maintained at 25°C. After a few days, the polyp rejuvenated, transforming from an immature medusa to a polyp through the following four stages: (I) immature medusa, (II)

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Supplemental material for this article is available online.
doi:10.2108/zs150186

dumpling, (III) dumpling with a short stolon, in which neither the hydranth nor hydrocaulus had yet emerged from the stolon, and (IV) newly rejuvenated polyp, consisting of only a single zooid with tentacles on the extended stolon (Fig. 1).

Total RNA extraction, paired-end mRNA library preparation, and sequencing

Each sample was initially stored in RNeasy RNA Stabilization Reagent (Qiagen, Venlo, Netherlands) at 4°C. The individuals processed on the same treatment day at the same developmental stage (St) were pooled for library construction. All libraries were constructed from 1–3 individuals (Table 1). Six libraries were prepared, consisting of one from St I, two from St II, two from St III, and one from St IV (Table 1). Within a week after RNeasy treatment, each specimen was transferred to 800 µL TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). The solution was homogenized using BioMasherII (Nippi, Tokyo, Japan) and incubated at room temperature for 5 min. After adding 200 µL chloroform and centrifugation, the aqueous phase was carefully transferred to a new tube after which 5 µg of glycogen (Life Technologies) was added to samples as a co-precipitant. RNA was precipitated by adding 600 µL of isopropyl alcohol. The RNA pellet was washed once with 75% ethanol and then dissolved in 15 µL RNase-free water and purified with the Agencourt RNAClean XP kit (Beckman Coulter). The quality and concentration of the RNA were verified with the Agilent 2100 Bioanalyzer and Qubit Fluorometer (Life Technologies), respectively. The final amount of each sample ranged from approximately 50 to 2,500 pg RNA. A 10-pg sample of purified total RNA was reverse transcribed and amplified to approximately 5 ng using SMARTer Ultra Low Input RNA Kit for Sequencing v3 (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's instructions. Amplified double stranded cDNA was sheared using Covaris, and then 1 ng of sheared cDNA was used for library preparation. Libraries were sequenced using the Illumina HiSeq 2000 (Illumina) and prepared with the KAPA Hyper Prep Kit (Kapa Biosystems) according to the manufacturer's instructions. An Illumina cBOT was used for cluster generation, and the cDNA libraries were sequenced on an Illumina HiSeq 2000 system performing 100-nt-long reads.

De novo transcriptome assembly and annotation

Prior to assembly, adapter sequences were removed from raw reads, and the trimmed reads were assessed for quality using FASTX Toolkit (v0.0.13) software. Base trimming was done from the 3' end of each read to remove bases with a quality below Q10 up to a minimum length of 50 bp. Reads shorter than 50 bp and the remaining orphan reads were removed before further analysis. The combined reads of all six libraries were assembled *de novo* using Trinity (Grabherr et al., 2011) (release 2014-07-17) with a minimum contig length of 200 bp and a k-mer size of 25. Gene ontology (GO) annotations of the contigs from the Trinity assembly were conducted through a BLASTx search against the NCBI NR protein database with a cut off E-value of 1.0E-6 using Blast2GO (v2.8).

The raw data and the *de novo* assembly have been deposited

in the DNA Data Bank of Japan (DDBJ), (accession nos. DRA004346, PRJDB4516, SAMD00045835-SAMD00045840, DRX048560-DRX048565, IAA01000001-IAA01090327).

RNA-Seq analysis

Each read of the stage-specific libraries was mapped to the contigs using Bowtie software (v2.0.6) with the default settings allowing for two mismatches (Langmead et al., 2009) and the resulting BAM data, which contained all transcript variants, was imported into a Strand NGS (v2.1, Agilent Technologies) for further analysis. After normalization of the trimmed mean of M-values (TMM) (Robinson and Oshlack, 2010) with the default settings, hierarchical clustering was performed on group stage-specific samples according to the gene expression patterns using the default settings. To identify the genes expressed only in specific St, TMM values for the contigs of samples from individual St (genes from St I and IV were obtained from a single library, whereas those from St II and III were identified from a total of four libraries, two from each St) were compared to the other three St samples, at which the fold-change cut-off was set at ≥ 2.0 . Subsequently, St-specific contigs were extracted, which were expressed only in one St and were reimported to Blast2GO to perform a statistical assessment of GO term enrichment compared to a reference group using a Fisher's Exact Test. The resulting data ($P < 0.01$) was then classified by GO terms, and are available at the following URL: <http://www.animalgenome.org/tools/catego/>.

RESULTS AND DISCUSSION

Illumina sequencing and *de novo* transcriptome assembly

To generate a broad survey of the transcripts associated with rejuvenation, a cDNA library was constructed from a very small amount of RNA extracted from small individuals at various stages (Fig. 1). Illumina sequencing of the six libraries from four stages (composed of 11 individuals) yielded 566 million paired-end 100-bp reads (Table 1). After filtering was performed to remove low-quality and orphan reads, a total of 402,312,548 clean reads (71.1%) were obtained from six libraries. The *de novo* assembly yielded 90,327 contigs with an average length of 913 bp and a N50 of 1,464 bp (Fig. 2, Table 2). Analysis revealed that 74.2% of the contigs had significant blast hits against the NCBI nr protein database (Table 3). Importantly, although a proportion of the short contigs in no blast hit increased, i.e., in the 201–300 bp range (11,268 of 24,076 contigs), the substantial long-read contigs remained (average 419 bp, range 201–4440 bp). Of the contigs with hits, the species that was most often a match was *Hydra vulgaris* (21,407 hits), followed by *Nematostella vectensis* (4,007 hits), which belong to the same phylum as *Turritopsis* sp. in Cnidaria (Fig. 3). The hits for these two species represented 38.0% of the

Table 1. Sample information and sequencing yield.

Sample	Rejuvenation Stage	Sample ID	No. of individuals used	Raw Reads
Immature medusa	I	1	1	85,275,248
Dumpling	II	2	1	100,534,214
Dumpling	II	3	3	83,660,368
Dumpling with a short stolon	III	4	1	96,403,688
Dumpling with a short stolon	III	5	3	106,513,140
Polyp	IV	6	2	93,627,712

Table 2. Assembly statistics of Illumina paired-end data.

Total length (bp)	82,460,655
Number of contigs	90,327
Average length (bp)	913
Median length (bp)	533
Max length (bp)	85,699
Min length (bp)	201
N50 (bp)	1,464
GC content	38.67%

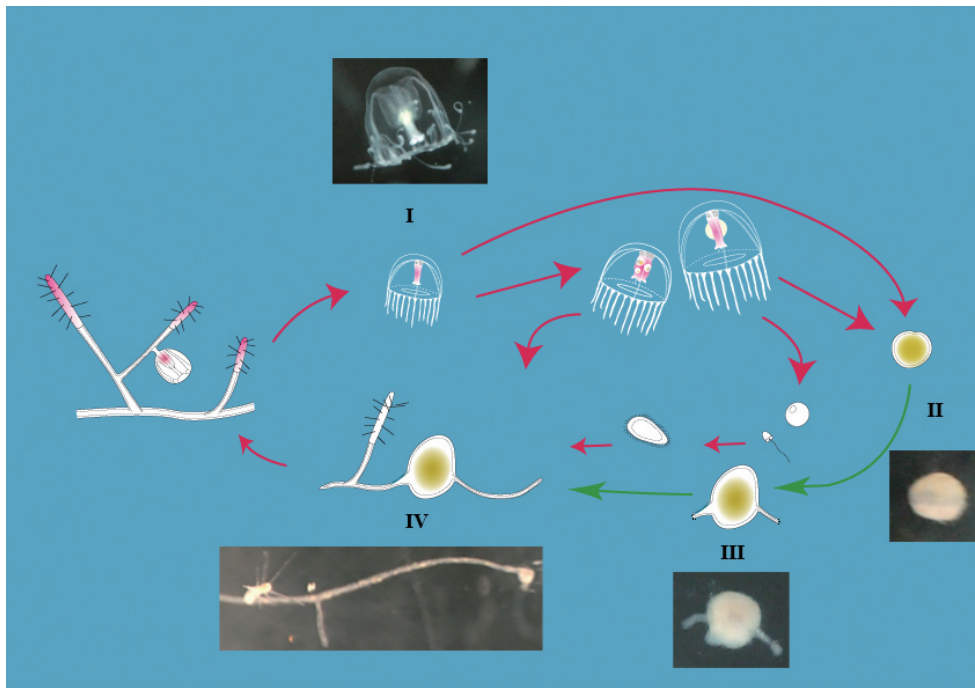


Fig. 1. Life cycle reversion showing rejuvenation from mature or young medusa to a polyp in *Turritopsis* sp. Developmental stages analyzed include the (I) immature medusa; (II) dumpling; (III) dumpling with a short stolon; and (IV) polyp (newly rejuvenated).

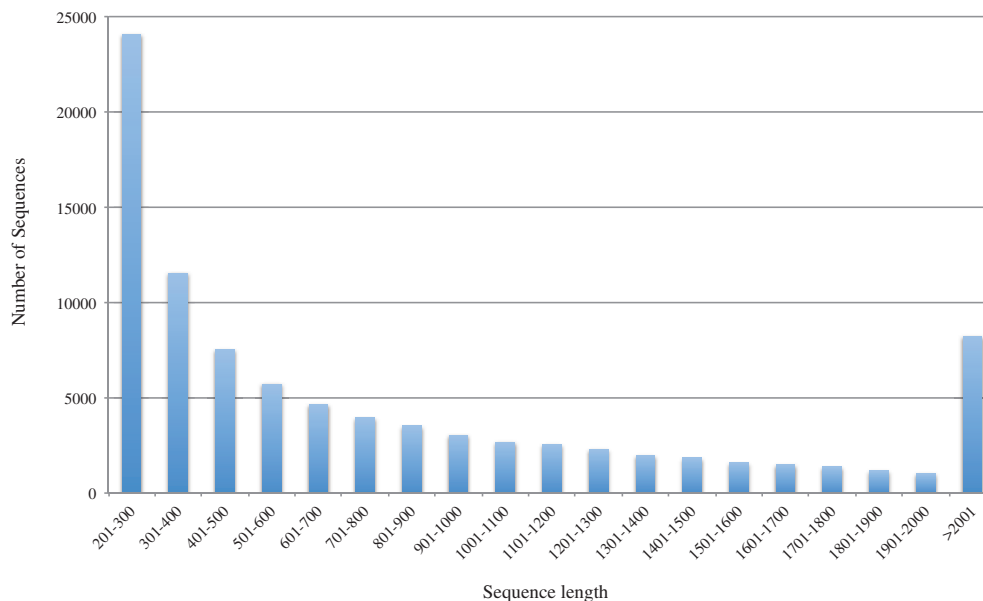


Fig. 2. Distribution of the size of assembled transcripts from *Turritopsis* sp. transcriptome libraries.

total hits, suggesting the high quality of the assembled genes and, notably, their conservation among Cnidaria.

Expression level analysis

In order to visualize the expression patterns of transcripts at the different stages, we conducted a hierarchical clustering analysis (Fig. 4). The expression profile across all transcripts demonstrated that it was largely divided between the medusa (St I) and all other stages (St II–St IV), and secondly revealed that the polyp (St IV) differed from the dump-

lings (St II) and dumpings with short stolons (St III).

One of the most important findings of this transcriptome sequencing project was the identification of St-specific genes from the four life stages (Supplementary Tables S1–S4 online). We found that immature jellyfish (St I) expressed the highest number of St-specific genes, followed by dumpling (St II), polyp (St IV), and dumpling with short stolon (St III) (Fig. 5). A detailed analysis of St-specific expression states revealed that the majority of the genes in St I was expressed over $10 \times$ more than in those in St II, III and IV, which were characterized by a low level of expression (Fig. 5). In particular, Wnt signaling is regulated by a conserved pathway in metazoans and has been reported to play an important role in development (Sanders and Cartwright, 2015), the regeneration process (Chera et al., 2009; Petersen et al., 2015), and axial patterning (Petersen and Reddien, 2009) in cnidarians. There is an example of St-specific expression linked to the Wnt pathway, which is expressed mainly found in St I. Specifically, Wnt3, which is secreted by apoptotic cells at the head regeneration site and induces regeneration after injury in *Hydra* (Chera et al., 2009), is expressed during St I and IV, but not St II or III. The expression values after TMM normalization of for Wnt3 from contigs c74698_g1_i1 and c77967_g1_i1 from St I were 153.8 and 165.9, respectively, and that of c63687_g1_i1 from St IV was 98.6. These results suggest that although the Wnt signaling pathway is not involved at the dumpling stage, it could play an essential role after the medusa is injured (for example, after being wounded with a needle). It is also possible that this pathway is involved in the wound healing response to mechanical disruption; therefore, we are currently planning to obtain additional St samples for further analyses of transcription level changes in the life stages of *Turritopsis* sp.

Table 3. Summary of *de novo* reference transcriptome annotation statistics using the blastx algorithm with Blast2GO software.

Total number of transcripts	90,327
Successful sequences after BLAST step	66,984
Successful sequences after Mapping step	44,022
Successful sequences after Annotation step	17,488

Mapping step is the process of retrieving GO terms associated to the hits obtained after a BLAST search.
Annotation step is the process of selecting GO terms from the GO pool obtained by the Mapping step and assigning them to the query sequences (a cut off E-value of 1.0E-6).

GO term enrichment revealed the St-specific functional-related gene groups (Fig. 6). St IV harbored the smallest number of enriched pathways in all three of the domains identified, most notably those in the cellular component group. Two GO terms (“cell communication” and “signal transduction”) in the biological processes domain were relatively similar in abundance in all stages other than St II. However, four GO terms, “catabolism” and “secondary metabolism” in the biological process domain, along with “catalytic activity” and “DNA binding” in the molecular function domain, were found in relatively similar levels of abun-

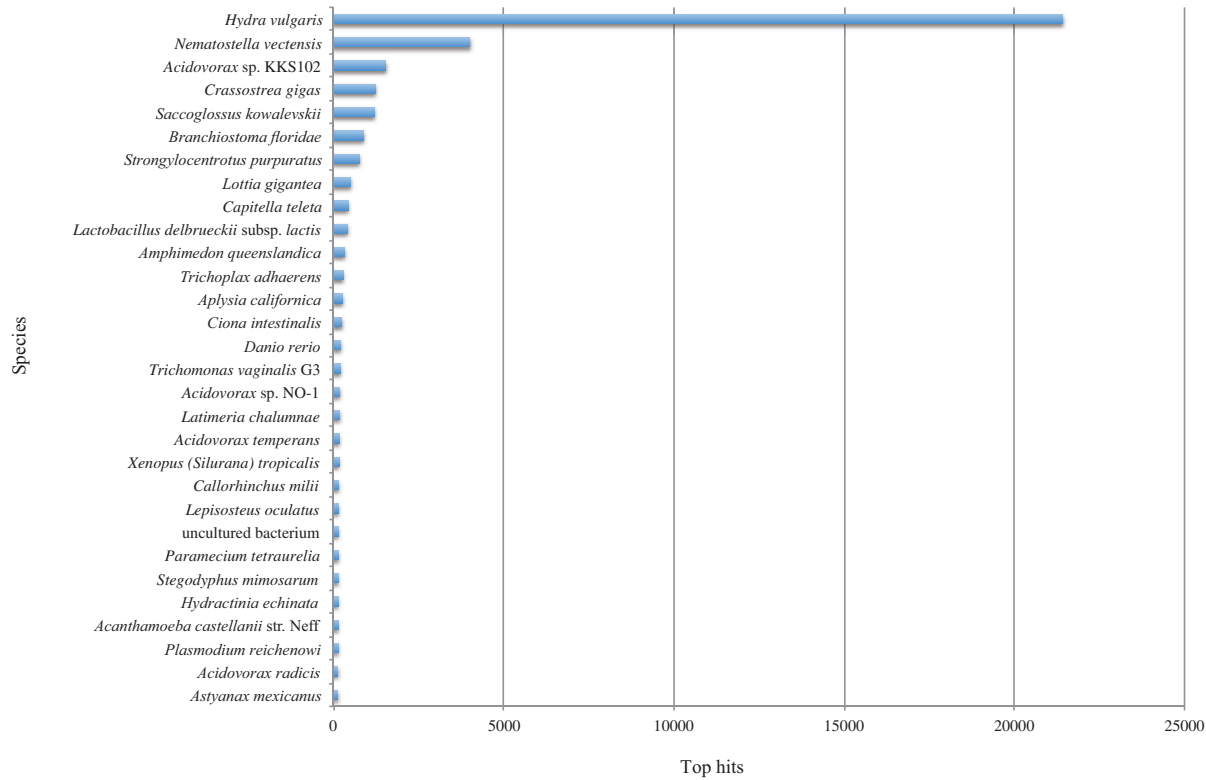


Fig. 3. Species distribution of the top hits identified by Blast2GO annotation using *Turritopsis* sp. sequences.

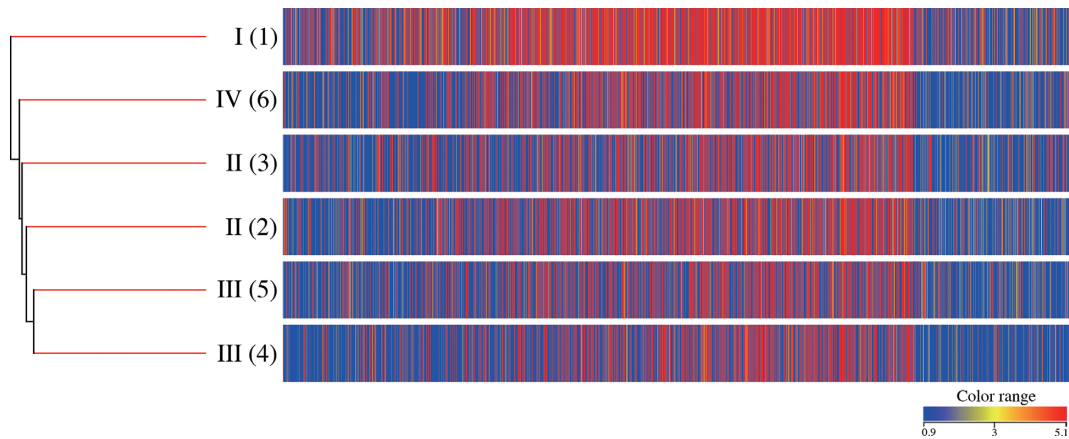


Fig. 4. Heat map comparing expression levels among all transcript libraries of *Turritopsis* sp. in the four developmental stages analyzed. I, II, III, and IV indicate the developmental stages whereas the number in parenthesis is the sample ID shown in Table 1. Similarity between developmental stages with hierarchical clustering is shown above the heat map. The analysis was performed using Strand NGS, with the Hierarchical Clustering algorithm, Euclidean distance metric, and Ward’s linkage rule.

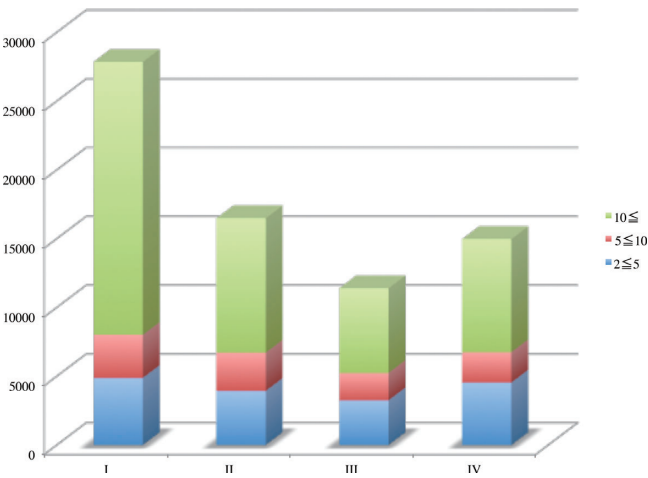


Fig. 5. Relative expression of developmental stage-specific expression for various *Turritopsis* sp. contigs. Relative levels of expression in comparison to the three other stages are identified as follows: the blue bar indicates 2–5 times, red indicates 5–10 times, and green indicates a minimum of 10 times greater than that in the other three stages.

dance only in St II. These results may aid in the identification of genes that play essential roles in rejuvenation.

Conclusion

In this study, we identified genes and pathways expressed in specific developmental stages of *Turritopsis* sp., and were thus able to provide an inventory of the genes that may be involved in the rejuvenation process. One-third of the contigs from *Turritopsis* sp. harbored blast hits to *Hydra vulgaris*. *Hydra* is considered another example of immortality due to its constant mortality and fertility over age (Schaible et al., 2015). *Turritopsis* sp. and *Hydra* could possibly share multiple pathways, although significant differences among them may allow for a unique function in *Turritopsis* sp. In order to elucidate the mechanism of the initial reaction to the rejuvenation phenomenon, it is effective to investigate the combination of St II-specific expression and/or silent genes using the same method as that previously implemented to identify iPS factors (Takahashi and Yamanaka, 2006). It is also important to determine whether rejuvenation occurs only in specialized cell types, such as stem cells. While here we identified candidate genes and pathways related to rejuvenation, we suggest that it is pos-

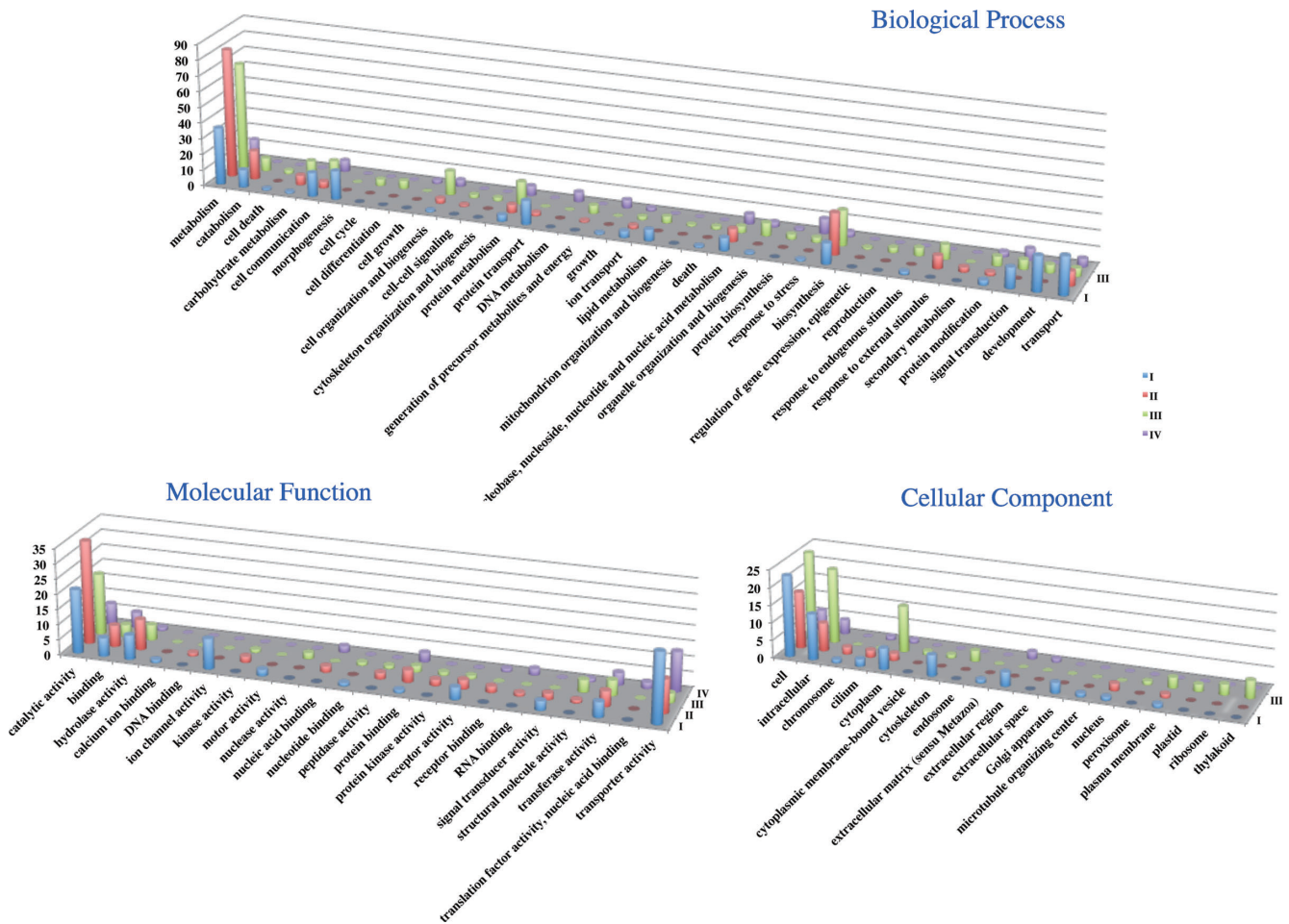


Fig. 6. Comparison of the level 2 gene ontology (GO) term enrichment analysis among the developmental stage-specific gene expression in *Turritopsis* sp. ($P < 0.01$, Fisher's Exact Test).

sible that as many as one-fourth of the contigs with no blast hit also play major roles in this process. In fact, analysis of 90,327 Trinity contigs using ESTscan (Iseli et al., C, 1999) produced 64,191 ORFs, and one contig was assigned an ORF for the first time. Moreover, of the 23,343 contigs with no blast hit, 98 harbored extended lengths of over 100 bp (data not shown). We are currently working towards obtaining a whole-genome sequence for this *Turritopsis* sp., the immortal jellyfish. The transcriptomes presented here will enhance attempts at fully assembling the genome, and allow for the elucidation of the molecular mechanisms underlying immortality and repeated rejuvenation in *Turritopsis* sp.

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(Received November 17, 2015 / Accepted April 6, 2016)