BIOINFORMATION
Discovery at the interface of physical and biological sciences

open access www.bioinformation.net Hypothesis

Volume 8(21)

Computational analysis of transcriptome of Indian major carp, *Labeo rohita* (Hamilton-Buchanan, 1822) for functional annotation

Naresh Sahebrao Nagpure, Iliyas Rashid, Ajey Kumar Pathak*, Mahender Singh, Shri Prakash Singh & Uttam Kumar Sarkar

National Bureau of Fish Genetic Resources, Canal Ring Road, P.O.- Dilkusha, Lucknow-226002, India; Ajey Kumar Pathak – Email: pathakajey@rediffmail.com; Phone: +91-522-2442440, 2442441, Fax: +91-522-2442403; *Corresponding author

Received September 21, 2012; Accepted October 01, 2012; Published October 31, 2012

Abstract:
A total of 1671 ESTs of *Labeo rohita* were retrieved from dbEST database and analysed for functional annotation using various computational approaches. The result indicated 1387 non-redundant (184 contigs and 1203 singletons) putative transcripts with an average length of 542 bp. These 1387 transcript sequences were matched with Refseq_RNA, UniGene and Swiss-Prot on high threshold cut-off for functional annotation along with help of gene ontology and SSRs markers. We developed extensive Perl programming based modules for processing all alignment files, comparing and extracting common hits from all files on a threshold, evaluating statistics for alignment results and assigning gene ontology terms. In this study, 92 putative transcripts predicted as orthologous genes and among those, 44 putative transcripts were annotated with gene ontology terms. The annotated orthologous gene of our result associated with some very important proteins of *L. rohita* involved in biotic and abiotic stresses and glucose metabolism of spermatogenic cells etc. The unidentified transcripts, if found important in expression profiling can be vital resource after re-sequencing. The predicted genes can further be used for enhancing productivity and controlling disease of *L. rohita*.

Keywords: Expressed sequences tag, Functional annotation, Gene, *Labeo rohita*, Putative transcripts, Transcriptome

Background:

Indian major carp, *Labeo rohita* (Hamilton-Buchanan, 1822), commonly known as 'rohu' belongs to the family Cyprinidae is available in lakes, ponds, rivers and other water bodies of India and adjacent countries **[1].** It is a highly preferred carp and fetches high market price. India is by far the largest producer of rohu, followed by Bangladesh and Myanmar. Although a considerable work has been carried out on various aspects including aquaculture, biology, seed production etc., however the species has not been adequately studied with respect to its genome, proteome and transcriptome that may provide critical information for enhancing its aquaculture productivity, growth, disease resistance and other traits.

Identification and characterization of transcriptome of *L. rohita* can provide valuable information to undertake future research in functional genomics. Transcriptome explores the part of the genome that is functionally active in a particular tissue **[2].** An expressed sequence tag (EST) is a fraction of a transcribed cDNA sequence **[3]** and important for identification of transcripts of a gene **[4].** Large-scale EST data represents a snapshot of the transcriptome of an organism while cloning and sequencing of ESTs is also an effective approach for the recovery of full length cDNA, discovery of novel genes and molecular markers development **[5].** ESTs being one of the important genomic resources, their numbers in public databases are rapidly increasing. Globally, 32,300 fish species have been described [6] that offer a unique opportunity for studies of transcriptomics, genomics and evolutionary biology in fishes. Despite, the global importance of fishes, information on transcriptome is available only for few species [7, 8]. Therefore, the utilization of available biological data reported across these species should be a new starting point for expanding studies in functional genomics.

In the light of above, we performed transcriptome analysis and identified differentially expressed genes in different tissues of *L. rohita* by utilizing available EST information in other fish species. Homologous transcripts, similarity with mRNA and translated product of these ESTs were predicted by the sequential application of various methodologies. Initially, we downloaded ESTs of *L. rohita* from dbEST database with complete information. We further developed an EST database using MySQL database management system on Linux platform. The ESTs sequences were refined by removing vector contamination segments and assembled into contigs and singletons. A contig was referred to tentative consensus sequence [9], while both contig and singleton were collectively considered as putative transcripts (PTs) [10], which ultimately represented subsequence of individual gene. We also analyzed PTs for repeat motifs and stored them into database along with PTs sequences. This unique set of sequences were used as query sequence for analysis such as repetitive motif (microsatellites), RNA search, and homolog transcript search with zebrafish dataset and prediction of hypothetical protein and functional annotation. A total of 92 transcripts of *L. rohita* were putatively and functionally annotated using similarity searches, SSRs mapping and establishment of gene ontology [11] relationship.

Methodology:

Database design and development

A database 'lrest' was designed in MySQL on Linux to manage the downloaded EST data in GB and FASTA files. The data was then parsed through Perl parser according to schema and managed into database comprised of three tables: 'fishinfo' for physical information of fish, 'estinfo' holding the records related to ESTs information. The database also holds the results of the analysis in the third table 'analysis'. Relationships among the tables were created based on primary and foreign keys. The diagrammatic representation of data organization and other details have been explained in **(Figure 1).**

Primary analysis

ESTs collected from dbEST, NCBI in GB (for annotation) and FASTA (for sequences) format (February 2012) [12] were used for analysis. The downloaded EST sequences were cleaned by using SeqClean program [13] and UniVec database [14]. The resultant quality ESTs were assembled through CAP3 [15] assembly program which generated unique gene sequences. These unique sequences were used as query sequences for alignment analysis. Further, the singletons and contigs were analyzed to identify simple sequence repeats (SSR) motifs with the help of MIcroSAtellite identification tool (MISA) [16], a publicly available Perl script for SSR search within nucleotide sequences. This program produces types (simple perfect, compound perfect and imperfect), repeat unit length (mono- to hexa-nucleotide) and length and repeat sequence class of microsatellites. The result of Cap3 program related to contig and singleton and results of the MISA program were managed into the 'analysis' table of the database. We established relationship of the 'analysis' table with other tables through primary and foreign keys of database **(Figure 1).**

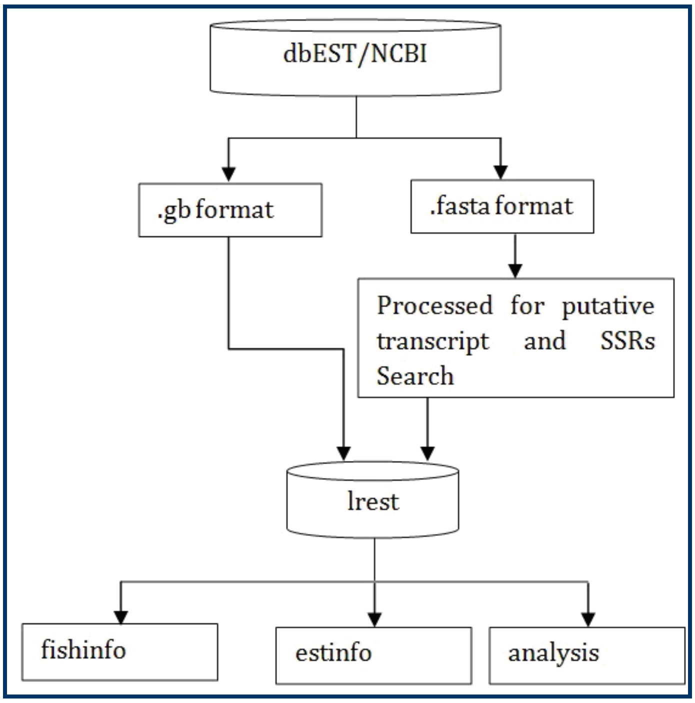


Figure 1: The dataflow diagram of 'lrest' database comprising with three tables 'fishinfo', 'estinfo' and 'analysis'.

Similarity search

The Refseq_RNA [17] and Swiss-Prot [18] databases provide more comprehensive, integrated, non-redundant and well-annotated set of sequences for similarity search. These two databases along with UniGene [17] dataset of zebrafish were downloaded from ftp server of NCBI (ftp.ncbi.nih.gov). The annotation of 1387 PTs was performed on the basis of significant hits using local BLAST [19] against Refseq_RNA and UniGene databases using BLASTn and Swiss-Prot protein databases using BLASTx. The Nucleotide search against Refseq_RNA and UniGene databases were obtained on high threshold score > 250 and identities > 85%, while putative transcripts were compared against the protein database using BLASTx with significant matches at a set threshold of score > 100 and identities > 70%.

Analysis programs

For the detection of number of clustered sequences present in different contigs the CAP3 assembly files (.cap.ace) were analyzed using a Perl script (CAP3ResultParser.pl). We wrote another perl script (TissueStatsClust.pl) program for manipulation of total number of PTs and ESTs for individual tissues. A third program 'AlignmentAnalysis' was also written in Perl to generate the information about targeted databases and detail statistics about alignments by using 'lrest' database in the backend and alignment files. Initially the program took alignment files generated by BLASTn and BLASTx program for PTs query against UniGene set of zebrafish and Refseq_RNA database and Swiss-Prot database respectively. In its first step, the program separated the hits and non hits PTs sequences against corresponding target database and subsequently the program extracted only significant hits on the threshold Score >

250 and Identities > 85% for zebrafish and Refseq_RNA and alignment Score > 100 and Identities > 70% for Swiss-Prot. These significant results for all the databases were further stored in separate files. The program retrieved pre-computed SSRs from 'Irest' database and mapped same motif patterns into aligned sequences of zebrafish with corresponding PTs for providing strength to our putative annotation results. The program 'AlignmentAnalysis' used all the three alignments files and subsequently merged for PTs through corresponding ids, serving as primary key into table 'analysis' of 'Irest' database. Average length of PTs and length range of PTs were also processed by this program. We also downloaded two files 'gene2refseq' and 'gene2go' from Entrez Gene **[20]** for assigning the gene ontology terms like biological process, cellular component and molecular function to the putative transcript in functional annotation process. A program 'GOAssign.pl' written in Perl was used for assigning GeneID and GO terms to the annotation results with computational mapping of both files 'gene2refseq' and 'gene2go'**(Figure 2).**

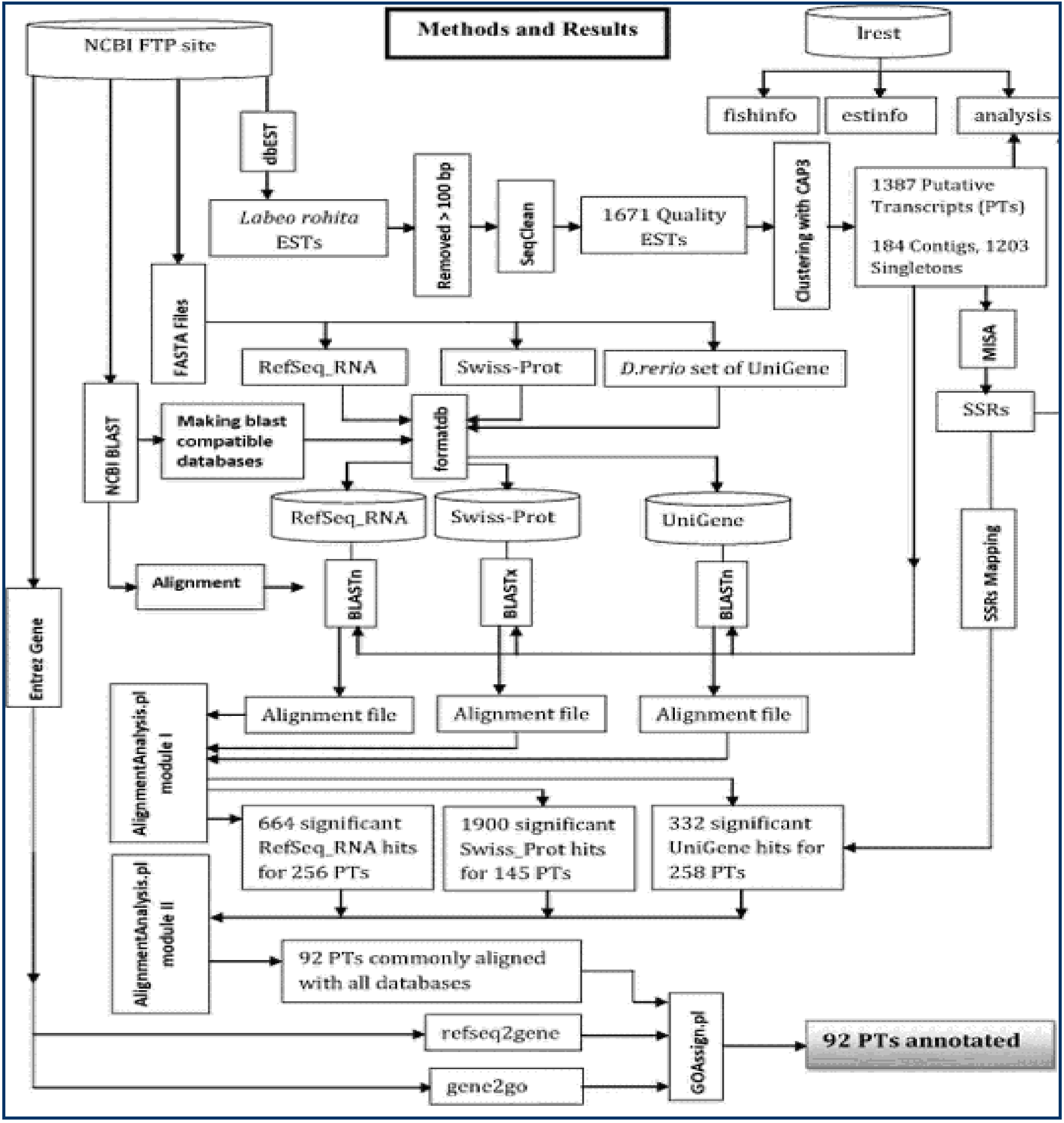


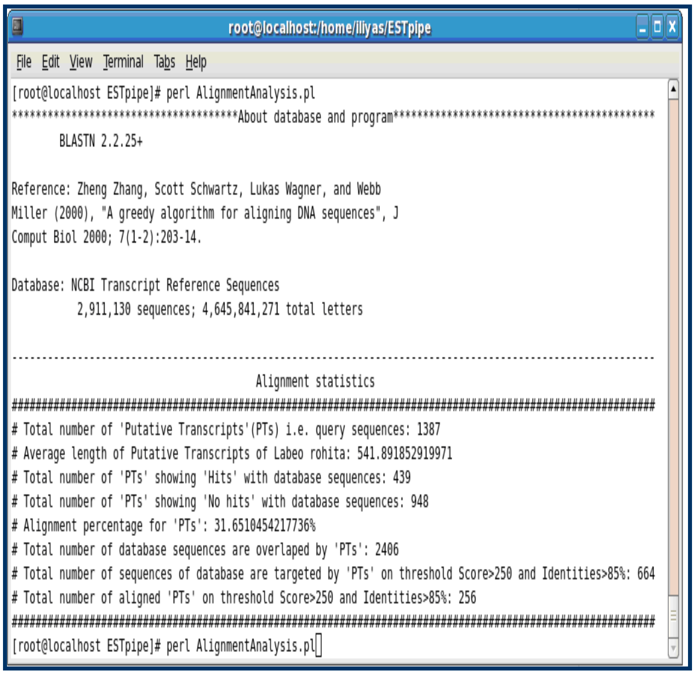
Figure 2: Flowchart for our method explaining functional annotation of transcriptome.

Results and Discussion:

Clustering and SSRs

The EST data were processed and managed into a database using MySQL on Linux platform. All cleaned EST sequences were assembled with CAP3 software that resulted in 184 contigs and 1203 singlets i.e. 1387 unique sequences putatively corresponding to as many genes with an average length of 542 bp. These 1387 PTs were used to identify SSRs motifs using MISA program and derived SSRs ranging in length from 2 to 6 bp were considered as repeat motifs. The minimum numbers of repeats were 6 for dinucleotides, 5 for trinucleotides, and 10 for tetranucleotides. In SSR analysis, observation was made on their types (di to tetra nucleotides), number of repeats, and percentage frequency of occurrences and their distribution in the sequence. The results of unique sequences (PTs) along with all SSR information was parsed and manipulated using Perl parser method according to database schema and data were managed into a separate table 'analysis' of 'lrest' database.

The data of table 'analysis' were used for further application in transcriptome analysis study. After analyzing the ESTs, we found majority of them were from brain (1317) followed by liver (236), testis (77), spleen (1) and 40 ESTs from unknown tissue. The organ wise distribution of contigs, singletons and clustered ESTs were determined through Perl script 'TissueStasClust.pl' that uses computational mapping of accession id and GenBank annotation stored in 'lrest'. The contigs derived from more than one organ were not considered in this statistics obtained by the above program **Table 1 (see supplementary material).** Only 1625 ESTs were found distributed into 168 contigs derived from single organ and 1203 singletons.



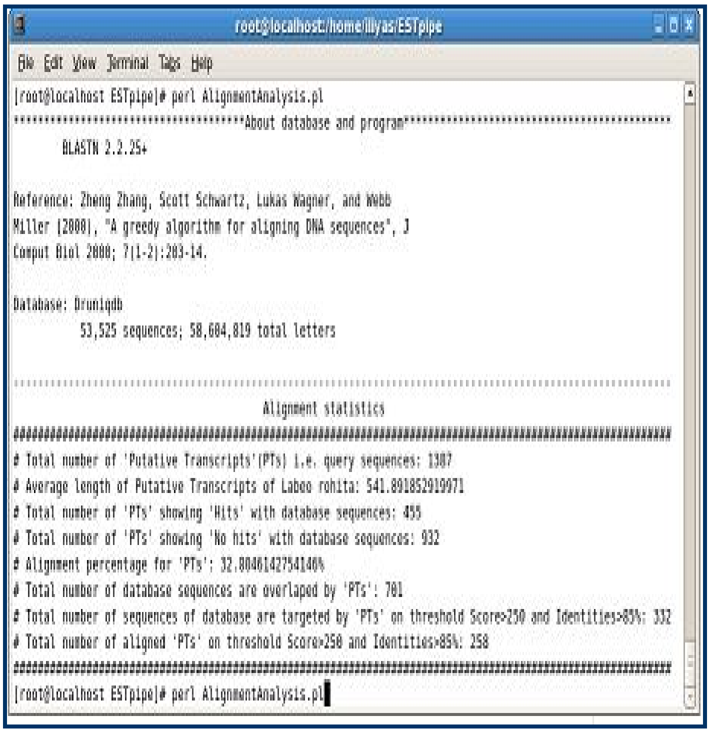
root@localhost:/home/iliyas/ESTpipe
File Edit View Terminal Tabs Help

```
[root@localhost ESTpipe]# perl AlignmentAnalysis.pl
*************************************About database and program*******************************************
       BLASTN 2.2.25+

Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb
Miller (2000), "A greedy algorithm for aligning DNA sequences", J
Comput Biol 2000; 7(1-2):203-14.

Database: NCBI Transcript Reference Sequences
          2,911,130 sequences; 4,645,841,271 total letters

--------------------------------------------------------------------------------------------------------------
                                      Alignment statistics
##############################################################################################################
# Total number of 'Putative Transcripts'(PTs) i.e. query sequences: 1387
# Average length of Putative Transcripts of Labeo rohita: 541.891852919971
# Total number of 'PTs' showing 'Hits' with database sequences: 439
# Total number of 'PTs' showing 'No hits' with database sequences: 948
# Alignment percentage for 'PTs': 31.6510454217736%
# Total number of database sequences are overlaped by 'PTs': 2406
# Total number of sequences of database are targeted by 'PTs' on threshold Score>250 and Identities>85%: 664
# Total number of aligned 'PTs' on threshold Score>250 and Identities>85%: 256
##############################################################################################################
[root@localhost ESTpipe]# perl AlignmentAnalysis.pl
```

Figure 3: The output of `AlignmentAnalysis.pl` perl script showing details on transcripts alignment statistics with database Refseq_RNA.

Putative annotation

The annotation of the unique 1387 PTs was done on the basis of the best match data with Refseq_RNA database using BLASTn. Only 439 (31.66%) sequences of PTs showed alignment with 2406 sequence of Refseq_RNA database and remaining PTs showed no hits. Out of 2406 alignments with Refseq_RNA, 664 alignments were obtained for 256 PTs on threshold with identities > 85% and score > 250 that could be considered as highly significant homologs. The score > 250 was used so that the alignment coverage reached 50% of query length to the target, while identities > 85% provided highly conserved and homologous sequences. **Figure 3** describes the result of above alignment generated by `AlignmentAnalysis.pl` which used blast alignment file for parsing and calculating the statistics of alignment between query (putative transcript) and target sequences (selected database). Similarly, 1387 PTs sequences of *L. rohita* were aligned with *D. rerio* dataset of UniGene collections using a BLASTn with score > 250 and sequence identities > 85%. A total of 455 (32.81%) PTs overlapped, while remaining PTs could not align with any of the transcripts of *D. rerio*. Out of 701 alignments with *D. rerio* sequences, 332 alignments were obtained for 258 PTs sequences on above threshold values. The aligned sequences on above threshold parameters were considered as highly significant homologs. **Figure 4** describes the result of above alignment generated by our own script `AlignmentAnalysis.pl`. The pre-computed SSRs motifs stored in the database were used to map these motifs as a marker of *D. rerio* homologous sequences corresponding to the PTs. In this map we found a strong support to annotate the gene of *L. rohita*. Identification of repetitive elements and nucleotide polymorphisms in query as well as target sequences provided strong support for annotation.



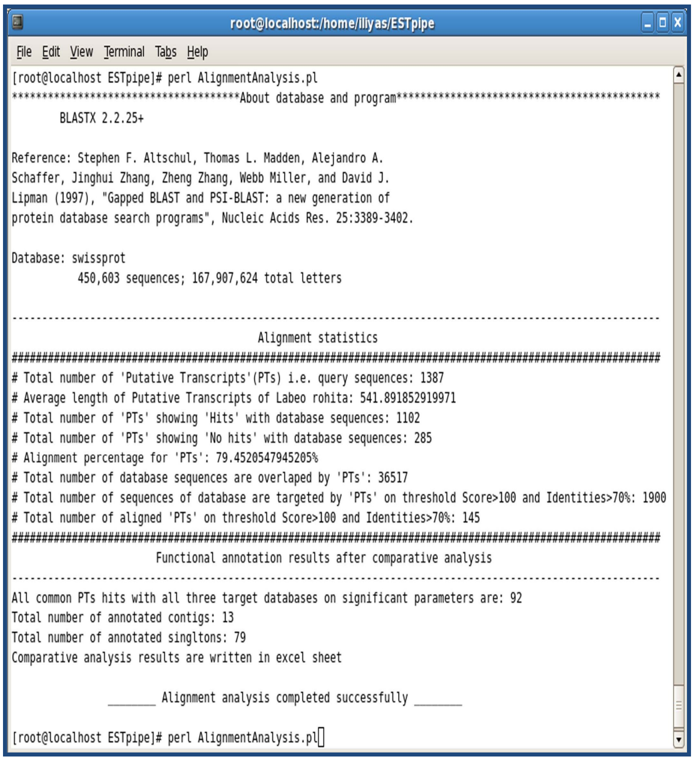
root@localhost:/home/iliyas/ESTpipe
File Edit View Terminal Tabs Help

```
[root@localhost ESTpipe]# perl AlignmentAnalysis.pl
*************************************About database and program*******************************************
       BLASTN 2.2.25+

Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb
Miller (2000), "A greedy algorithm for aligning DNA sequences", J
Comput Biol 2000; 7(1-2):203-14.

Database: Druniqdb
          53,525 sequences; 58,604,819 total letters

--------------------------------------------------------------------------------------------------------------
                                      Alignment statistics
##############################################################################################################
# Total number of 'Putative Transcripts'(PTs) i.e. query sequences: 1387
# Average length of Putative Transcripts of Labeo rohita: 541.891852919971
# Total number of 'PTs' showing 'Hits' with database sequences: 455
# Total number of 'PTs' showing 'No hits' with database sequences: 932
# Alignment percentage for 'PTs': 32.8046142754146%
# Total number of database sequences are overlaped by 'PTs': 701
# Total number of sequences of database are targeted by 'PTs' on threshold Score>250 and Identities>85%: 332
# Total number of aligned 'PTs' on threshold Score>250 and Identities>85%: 258
##############################################################################################################
[root@localhost ESTpipe]# perl AlignmentAnalysis.pl
```

Figure 4: The output of 'AlignmentAnalysis.pl' perl script showing details of transcripts alignment statistics with database Druniqdb (*D. rerio* dataset of UniGene).

Hypothetical protein analysis

Swiss-Prot databases were used for hypothetical protein prediction for 1387 transcripts query with the help of BLASTx and program aligned 1102 PTs hits with 36517 protein sequences with alignment percentage 79.46%. Further, the set threshold values i.e. score > 100 and identities > 70% for Swiss-Prot resulted in alignment of 145 PTs with 1900 protein sequences **(Figure 5).**


root@localhost:/home/iliyas/ESTpipe
File Edit View Terminal Tabs Help

```
[root@localhost ESTpipe]# perl AlignmentAnalysis.pl
**************************************About database and program********************************************
       BLASTX 2.2.25+

Reference: Stephen F. Altschul, Thomas L. Madden, Alejandro A.
Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J.
Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of
protein database search programs", Nucleic Acids Res. 25:3389-3402.

Database: swissprot
          450,603 sequences; 167,907,624 total letters

---------------------------------------------------------------------------------------------------------
                                       Alignment statistics
#########################################################################################################
# Total number of 'Putative Transcripts'(PTs) i.e. query sequences: 1387
# Average length of Putative Transcripts of Labeo rohita: 541.891852919971
# Total number of 'PTs' showing 'Hits' with database sequences: 1102
# Total number of 'PTs' showing 'No hits' with database sequences: 285
# Alignment percentage for 'PTs': 79.4520547945205%
# Total number of database sequences are overlaped by 'PTs': 36517
# Total number of sequences of database are targeted by 'PTs' on threshold Score>100 and Identities>70%: 1900
# Total number of aligned 'PTs' on threshold Score>100 and Identities>70%: 145
#########################################################################################################
                        Functional annotation results after comparative analysis
---------------------------------------------------------------------------------------------------------
All common PTs hits with all three target databases on significant parameters are: 92
Total number of annotated contigs: 13
Total number of annotated singltons: 79
Comparative analysis results are written in excel sheet

                ________ Alignment analysis completed successfully ________

[root@localhost ESTpipe]# perl AlignmentAnalysis.pl
```

Figure 5: The output of 'AlignmentAnalysis.pl' showing details on transcripts alignment statistics with Swiss-Prot database along with results of functional annotation.

Comparative analysis and functional annotation

Alignment of ESTs with Refseq_RNA, *D. rerio* transcripts and Swiss-Prot resulted in 256 PTs, 258 PTs and 145 PTs respectively as significant hits in the similarity search analyses. The pre computed and stored SSRs of related PTs sequences were mapped with the aligned transcripts of zebrafish and the presence of SSRs motifs in corresponding target transcripts provided strength to this annotation. All significant alignments were merged with their PT ids and found that only 92 PTs were common hit for all the three databases and referred to as annotated on the basis of gene and protein prediction and mentioned into the supplementary 'S1.xls'available with us. Functional categorization of predicted protein sequences for 92 PTs was done through gene ontology terms like biological process, cellular component and molecular function. The Program 'GOAssign.pl' efficiently mapped gene ontology terms for 44 PTs sequences and result about gene ontology has been mentioned into the supplementary file 'S2.xls' available with us.

Our final result based on putative and functional annotation of transcriptome of *L. rohita* for 92 PTs was executed and the analysis was supported by several approaches for transcriptome and proteome based similarity searches as well as computational mapping of SSRs and gene ontology. The annotated results were explained in the supplementary file S1. We identified several genes and proteins along with function, process and location based on high identities (85-94%) and matches (70-95%) in the similarity search, e.g. Contig93 (HO758335; HO758369; HO758347) as protein L40-1, Contig120 (GR977092; HO758682) as L29, Singlet1101 (HO762402) as L5a, Singlet1176 (JK492853) as L28-like and L32, Singlet290 (GR958016) as L17, Singlet478(GR977096) as L36a, Singlet595 (HO758410) as L38, Singlet965 (HO762207) as L35, Singlet826 (HO758770) as S6 ribosomal protein or transcript variant 1, Singlet79 (GR426887) as ribosomal protein S16, Singlet814 (HO758752) as ribosomal protein S33. Additionally few important orthologous genes viz. Singlet288 (GR958013), Singlet1001 (HO762255), Singlet686 (HO758554) on high identities > 92% were also annotated as heat shock protein (HSPs) associated with cell stress and cell growth **[21]**. Similarly, we identified protein specific for biotic stress viz. Singlet311 (GR958037) as thioredoxin-like 1, Singlet777 (HO758701) as F-box protein 11a (fbxo11a), Singlet315 (GR958041) as CoA synthetase family member 2 (acsf2), Singlet1046 (HO762321) as 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide 2 (ywhag2) and Contig92 (HO762389; HO762458) as high-mobility group box 3b.

Likewise annotation of some PTs lead to identification of important proteins such as Contig184 (HO758515; HO758524 HO758557; HO758384; HO758383) as Ependymin, Singlet985 (HO762234) as coiled-coil domain-containing protein, Singlet786 (HO758714) as fibroblast growth factor 8 /androgen-induced growth factor, Singlet1169 (JK492845) as ba2 globin and ba1 globin, Contig105 (HO762218; HO762362; JK492857; JK492849; HO758519; GR958118) as hemoglobin alpha adult-1, Contig163 (HO758295 HO762456 GR463879) as fatty acid-binding protein, Singlet780 (HO758705) as PKR-interacting protein 1, Contig92 (HO762389 HO762458) as high-mobility group. The Contig68 (HO758673; HO762228) showed 529 matches on identities 91% to *D. rerio* NADH dehydrogenase (ubiquinone) Fe-S protein 4. The NADH dehydrogenase is associated with the electron transport chain reaction and involved in the glucose metabolism of spermatogenic cells.

Conclusion:

Out of 1387 putative transcripts, 256 PTs showed alignment with 664 Refseq_RNA, 258 sequences were aligned with 332 homologous transcripts of *D. rerio* UniGene sets and 145 PTs with 1900 proteins of Swiss-Prot based on significant similarity searches. Among these only 92 (5.8%) transcripts (13 contigs and 79 singletons) were aligned with all the databases and thus fully annotated. While a significant fraction of ESTs data could not be identified by similarity searches, the unidentified transcripts are valuable resource and can also be taken up for re sequencing, if found important in expression profiling.

Acknowledgement:

Authors are grateful to National Agricultural Bioinformatics Grid (NABG), under National Agricultural Innovation Project (NAIP), ICAR, New Delhi for providing financial support and the Director, NBFGR, Lucknow for providing necessary facilities and guidance.

References:

[1] Talwar PK & Jhingran AG, *Inland Fishes of India and Adjacent Countries. Oxford & IBH Publishing Co. Pvt. Ltd.* 1991 **1:** 219

[2] Velculescu VE *et al. Cell.* 1997 **88:** 243 [PMID: 9008165]

[3] Adams MD *et al. Science*. 1991 **252:** 1651 [PMID: 2047873]
[4] Kim JM *et al. DNA Res*. 2006 **13:** 275 [PMID: 17213182]
[5] Manickavelu A *et al. DNA Res*. 2010 **17:** 211 [PMID: 20360266]
[6] http://www.fishbase.org/search.php
[7] Li P *et al. BMC Genomics*. 2007 **8:** 177 [PMID: 17577415]
[8] Christoffels A *et al. BMC Bioinformatics*. 2006 **7:** S2 [PMID: 17254304]
[9] Fei Z *et al. Plant Journal*. 2004 **40:** 47 [PMID: 15361140]
[10] Xia JH *et al. DNA Res*. 2011 **18:** 513 [PMID: 22086997]
[11] Harris MA *et al. Nucleic Acids Res*. 2004 **32:** D258 [PMID: 14681407]
[12] Benson DA *et al. Nucleic Acids Res*. 2012 **40:** D48 [PMID: 22144687]
[13] http://compbio.dfci.harvard.edu/tgi/software/
[14] ftp://ftp.ncbi.nlm.nih.gov/pub/UniVec/.
[15] Huang X & Madan A, *Genome Res*. 1999 **9:** 868 [PMID: 10508846]
[16] http://pgrc.ipk-gatersleben.de/misa/misa.html
[17] Sayers EW *et al. Nucleic Acids Res*. 2012 **40:** D13 [PMID: 22140104]
[18] Bairoch A & Apweiler R, *Nucleic Acids Res*. 2000 **28:** 45 [PMID: 10592178]
[19] Altschul SF *et al. J Mol Biol*. 1990 **215:** 403 [PMID: 2231712]
[20] Maglott D *et al. Nucleic Acids Res*. 2011 **39:** D52 [PMID: 21115458]
[21] Knowlton AA & Salfity MJ, *Biosci*. 1996 **21:** 123

Edited by P Kangueane
Citation: Nagpure *et al.* Bioinformation 8(21): 1005-1011 (2012)

Supplementary material:

Table 1: Organ wise distribution of contigs, singletons and total number of ESTs

Tissues	Contigs	EST Clusters	Singletons	Total Accessions
Brain	150	366	926	1292
Testies	18	56	20	76
Liver	-	-	221	221
Spleen	-	-	1	1
Unknown	-	-	35	35
Sum	168		1203	1625