

Appendix 3. Transcript annotation and seasonal gene expression in kidney tissue of the Australian pygmy bluetongue skink, *Tiliqua adelaidensis*

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1 Analysis Workflow

Transcript annotation and seasonal gene expression in kidney tissue of the Australian pygmy bluetongue skink, *Tiliqua adelaidensis*

This document outlines the pipeline methods for assembly of poly-a selected transcripts extracted from kidney tissues of three male and five female Australian pygmy bluetongue skinks collected in South Australia during spring (September) and autumn (March or April). Long read transcripts were sequenced from one individual “G6” using Pacific Biosciences’ IsoSeq Sequel platform, and short reads for all eight individuals were sequenced on an Illumina HiSeq. The wet-lab library preparation up to sequencing is outlined in Chapter 3.

Sample ID	Experimental factors
G1	September Female
G2	September Female
G3	March/April Female
G4	March/April Male
G5	September Male
G6	September Male
G7	March/April Female
G8	March/April Female

There are three main sections to this document, which are referenced in three separate chapters of the thesis:

Assembly (Chapter 4): Assembly, cleaning and clustering of transcript isoforms from **both** sequencing formats to form a pseudo-reference for *T. adelaidensis* and to compare these methods.

Annotation (Chapter 5): Annotation and further analysis of the full length transcript isoforms derived from the long read analysis.

Gene expression (Chapter 6): Gene expression analysis using the short read data aligned to the long read pseudo-reference produced in Chapter 4, to assess seasonal variation of gene expression.

Analysis was done using a combination of computing and HPC machines with specifications as below:

- Long read cleaning, read frame prediction and blast searches were run on the Australian National eResearch Collaboration Tools and Resources project (NECTAR) cloud, using an ‘m1.xlarge flavour’ allocation with 8 AMD Opteron 63xx class CPUs, 32GB RAM, 10GB root disk, and 240GB of secondary disk storage.
- The ANGEL program which required a higher memory allocation was run on a Dell PowerEdge server with 40 cores and 512G RAM.
- Short read QC, trimming, initial trinity assembly, and alignments were completed using Flinders University’s high-performance computer “Deep thought” with 16 x 256Gb (4TB RAM), 1024 AMD x86 CPU cores at 2.0GHz across 14 standard compute and 2 data science nodes, and 100 TiB of usable storage via the Dell EMC Network Storage reference architecture.
- Downstream gene expression analysis and manual manipulation of annotation files was completed on a local Intel core i7 machine using RStudio or in-house bash scripts applied in Git Bash for Windows.

All larger scripts included in this document have also been uploaded to Github separately and are linked here at the relevant steps.

All scripts and commands in this pipeline depend on specific directory structure, and may show inconsistencies in this structure between steps where files have been transferred between computing resources. The input and output files of note are highlighted in the text between steps to bridge these gaps.

2 Assembly (Chapter 4)

A genome from a closely related species was unavailable for these data. Gene expression analysis of short read data was initially intended to be carried out using a *de-novo* assembly of this same data as a pseudo-reference for expression counts.

When long read data became available, both datasets have been assembled *de-novo* and these assemblies compared. The long read assembly then provided the reference for expression counts in Chapter 6.

This section is divided into the two methods of assembly; using the long read IsoSeq data, and using the short read HiSeq data. Both these sections contain titles referencing the main program in use at each step.

2.1 Long read data

De-novo assembly of long-read IsoSeq data sequenced from poly-a selected RNA extracted from a single kidney from september male G6. These sequences were be used to annotate genes found in kidney tissue of *T. adelaidensis* and create a set of reference transcripts for gene expression analysis conducted in later chapters.

Sequences were downloaded from Pacific Biosciences & checksums verified.

2.1.1 SMRT Tools - IsoSeq3

SMRT Tools provides various programs for appropriate treatment of Pacific Biosciences sequencing outputs. IsoSeq3 was used to collapse circular sequences, initial polishing, and to characterise full length transcript reads. This analysis was conducted in an environment on the eRSA NECTAR research cloud.

A primer reference file was created manually for reference in the Isoseq3 script using the primers listed in the Clontech SMARTer cDNA Library prep kit which was used during the synthesis of cDNA for these samples.

[primers.fasta](#):

```
>primer_5p
AAGCAGTGGTATCAACGCAGAGTACATGGG
>primer_3p
GTACTCTGCGTTGATACCACTGCTT
```

A single script was used to complete the Pacific Biosciences Isoseq3 pipeline [isoseq3.sh](#):

```
#!/bin/bash
#
# This script is used to characterise full length transcripts de novo using
→ smrttools and bioconda as specified by PacBio workflow
# see: https://github.com/PacificBiosciences/IsoSeq3/blob/master/README\_v3.0.md and
→ follow links for bioconda references
#
# The paths in the script assume that a specific directory structure has been set
→ up.
# This directory structure is for the Nectar cloud VM

# open a screen
# usage from /mnt
```

```

# bash isoseq3.sh <ctrl-a ctrl-d>
#
# Carmel Maher
# April 2019

#-----Installed Programs-----

# smrttools          -> smrttools-release_6.0.0.47835
# help:
#
#   ↪ /pacbio/smartlink/install/smartlink-release_6.0.0.47841/bundles/smarttools/install/
#   ↪ smrttools-release_6.0.0.47835/smrtcmds/bin/isoseq3 -h
#
#   ↪ /pacbio/smartlink/install/smartlink-release_6.0.0.47841/bundles/smarttools/install/
#   ↪ smrttools-release_6.0.0.47835/smrtcmds/bin/isoseq3 --version
# Version: isoseq3 3.0.0 (commit v3.0.0-7-gcc6cddd)

# python 2.7          -> to run miniconda - automatically installed on nectar
# miniconda           -> recommended python 2.7 compatible version by PacBio
#   ↪ workflow. Miniconda2 | VER: 4.6.14
# ccs                  -> installed through conda, inside pbccs package | VER:
#   ↪ pbccs-3.4.1
# lima                 -> installed through conda | VER: lima-1.9.0

#-----adjust these for your run-----

#as per the local installation, the smrttools program directories are (in /mnt).
#   ↪ Notable directories listed in installation:
SMRT_ROOT=/mnt/pacbio/smartlink

# Isoseq3 executable location (from /mnt)
Iso3_DIR=$SMRT_ROOT/install/smartlink-release_6.0.0.47841/bundles/smarttools/install/
#   ↪ smrttools-release_6.0.0.47835/smrtcmds/bin

DATA=/mnt/data
MOVIE="MAH6260A1_m54196_190204_223227"
THREADS=0      #0 for the Iseseq3 tools =autodetection of threads and is the default

#-----

function error_exit
{
    # Exit function due to fatal error
    # Accepts 1 arg:
    # string - descriptive error message

    echo "${PROGNAME}: ${1:-"Unknown error"}" 1>&2
    exit 1
}

```

```

# go to the working data directory
cd $DATA || error_exit "$LINENO: directory error"

# Generate consensus sequences (ccs file) from raw subread data
# NOTE: this automatically runs with default autodetection of threads = 8
ccs $MOVIE.subreads.bam $MOVIE.ccs.bam --noPolish --minPasses 1 || error_exit
↪ "$LINENO: consensus sequence ccs error"

echo " - - - consensus sequences generated"

# The primers.fasta as per the recommended Clontech SMARTer cDNA library prep - no
↪ barcodes used for this sample
# primers.fasta has been uploaded to /mnt/data and does not need to be created
↪ here:
    # primers.fasta
    # >primer_5p
    # AAGCAGTGGTATCAACGCAGAGTACATGGG
    # >primer_3p
    # GTACTCTGCGTTGATACCACTGCTT

# Remove primers and demultiplex:
lima --isoseq --dump-clips --no-pbi $MOVIE.ccs.bam primers.fasta demux.bam ||
↪ error_exit "$LINENO: Primer demultiplex lima error"

echo " - - - primers removed and demultiplexed"

#####
# CHECK # Note: A search using Git bash on the output files for any
↪ of the primers listed for Isoseq by Pacific biosciences returns
↪ no results after this script
#####

# *****
# From here on, execute the following steps for each output BAM file. -- only one
↪ in this case.
# isoseq3 tool usage: isoseq3 <tool>
# due to nectar permissions and installation location, usage: $Iso3_DIR/isoseq3
↪ <tool>
# *****

### cluster Tool: Cluster CCS reads and generate unpolished transcripts.
# recommended to give this step as many cores as possible
# Usage
# isoseq3 cluster [options] input output
# Example
# isoseq3 cluster <two types of potential inputfile> demux.bam <OR>
↪ $MOVIE.consensusreadset.xml unpolished.bam

# Cluster consensus sequences to generate unpolished transcripts:
# note the demux.bam is named after the headers used in primers.fasta
$Iso3_DIR/isoseq3 cluster --verbose -j $THREADS demux.primer_5p--primer_3p.bam
↪ unpolished.bam || error_exit "$LINENO: Isoseq3 cluster error"

```

```

echo " - - - css reads clustered"

### polish Tool: Polish transcripts using subreads.
# Usage
# isoseq3 polish [options] input_1 input_2 output

# Polish transcripts using subreads:
$Iso3_DIR/isoseq3 polish --verbose -j $THREADS unpolished.bam $MOVIE.subreads.bam
→ polished.bam || error_exit "$LINENO: Isoseq3 polish error"

echo " - - - transcripts polished"

### summarize Tool: Create a .csv-format barcode overview from transcripts.
# Usage
# isoseq3 summarize [options] input output
$Iso3_DIR/isoseq3 summarize --verbose polished.bam summary.csv || error_exit
→ "$LINENO: Isoseq3 summary error"

echo " - - - summary file created"
echo " - - - done"

```

All of the above output was moved into a folder ~/data/2019-04-30_isoseq3_out

The output from this script, the polished.hq.fasta.gz file was unzipped and then used as the input for the program COGENT.

2.1.2 Cogent

Transcript clustering and collapse of redundant isoforms. Line by line instructions for running Cogent for clustering of transcript isoforms in a screen on the NECTAR machine as below. Includes instructions for Cupcake and minimap to collapse redundant transcript isoforms.

Some notes and commentary are based on personal communication with Tessa Bradford & Terry Bertozzi at the SA Museum, as well as the [Cogent GitHub Tutorial page](#)

Environments were used for installations (most often achieved with mini conda) on the NEXTAR machine. Activate screen, activate the environment, make Cogent output directory, and go to analysis working directory

```
screen -s cogent
conda activate anaCogent
mkdir /mnt/IsoSeq-analysis/data/Cogent
cd /mnt/IsoSeq-analysis/data/Cogent
```

From the analysis directory the input data directory is ../2019-04-30_out/polished.hq.fasta. The first output will be in this original data directory. Check paths (During installation, all paths were put into .profile)

2.1.2.0.1 Family Finding Running [Family Finding for a small dataset](#)

Create a k-mer profile of the input and calculate pairwise distances

Note: default K-mer size =30

```
python /mnt/Prog/Cogent/Cogent/run_mash.py --cpus=7
↪ /mnt/IsoSeq-analysis/data/2019-04-30_out/polished.hq.fasta
```

The output will be <fasta_filename>.k<sketch_size>.dist

Output written to: /mnt/IsoSeq-analysis/data/2019-04-30_out/polished.hq.fasta.s1000k30.dist

Process the distance file and create the partitions for each gene family

```
process_kmer_to_graph.py ../2019-04-30_out/polished.hq.fasta
↪ ../2019-04-30_out/polished.hq.fasta.s1000k30.dist ./hq hq
```

This generates an output log file hq.partition.txt and for each partition (isoform set), a subdirectory called hq/<partition_number> which contains the subset of fasta sequences belonging to that isoform set. Note that sequences that don't belong to any partition (ones with no similarity with other sequences) will be "unassigned" and noted in the partition log file.

2.1.2.0.2 Coding Genome Reconstruction Each isoform set family must be reconstructed individually for coding region.

Reconstructed contigs will contain the whole coding region. Reconstructed file is called cogent2.renamed.fasta

The script for an individual folder is:

```
reconstruct_contig.py -S T.adelaidensis hq/hq_0
```

The following script was written to loop this procedure and process hundreds of isoform set folders with sequences requiring coding region identification [reconstructContig_1.sh](#):

```
#!/bin/bash
#
## this script is for coding region reconstruction of Cogent gene families as these
↳ must be completed individually.
# This script assumes you are following Running-Cogent.txt and using the same
↳ directory structure
# This script to be used after the process_kmer_to_graph.py step

# script in /mnt/IsoSeq-analysis/src
# usage from /mnt/IsoSeq-analysis/data/Cogent/hq
## bash ../../../../src/reconstructContig.sh

# Carmel Maher
# August 2019

#Terry's error exit
function error_exit
{
    # Exit function due to fatal error
    # Accepts 1 arg:
    # string - descriptive error message
    echo "${PROGNAME}: ${1:-"Unknown error"}" 1>&2
    exit 1
}

cd ~/mnt/IsoSeq-analysis/data/Cogent/hq

for d in */ ; do

    DIR=${d%*}
    echo $DIR

    reconstruct_contig.py -S T.adelaidensis ./${DIR} || error_exit "$LINENO: Error
↳ "$DIR"

done
echo compete
```

An error message prompted further checking of folder completion as below

Number of gene families:

```
ls ./hq/*/cogent2.renamed.fasta | wc -l
4454
```

Finding failed reconstruction jobs (pers. comm. Terry Bertozzi, SA Museum)

Number of hq gene folders:

```
ls hq | wc -l #number of folders
4459
```

Number of /hq folders completed:

```
ls ./hq/*/COGENT.DONE | wc -l
4454
```

This matches the total gene family count so all outputs have been recorded correctly, but does not match the total /hq folder count = 5 folders not completed.

The following will list the folders that have not finished:

```
comm -3 <(find /mnt/IsoSeq-analysis/data/Cogent/hq -iname 'COGENT.DONE' -printf
↪ '%h\n' | sort -u) <(find /mnt/IsoSeq-analysis/data/Cogent/hq -maxdepth 1
↪ -mindepth 1 -type d | sort) | sed -e 's/^.*/hq\\/'

hq_2605
hq_2738
hq_320
hq_6391
hq_9030
```

Some testing was required to create a modified script with a loop for failed folders, in order to include 5 folders which failed to run with kmer length 30 informed by [this issue](#).

Due to how the loop functions inputting kmer size of 27 on execution will actually run all folders at the desired kmer size 30.

```
bash /mnt/IsoSeq-analysis/src/TBreconstructContig-edit.sh
↪ /mnt/IsoSeq-analysis/data/Cogent/hq 27 T.adelaidensis
```

The modified script [TBreconstructContig-edit.sh](#):

```
#!/bin/bash

# This script runs reconstruct_contig.py on every subdirectory contained in the top
↪ directory. reruns with a kmer parameter of +3 (to a max of 99) for failed
↪ directories.
# This script assumes directories and files exist as up until the Coding Genome
↪ Reconstruction step in the Running Cogent GitHub wiki
#
# usage:  bash <path/script> <full directory containing hq_folders> <starting kmer
↪ value> <Species Name>
# e.g.      bash /mnt/IsoSeq-analysis/src/TBreconstructContig-edit.sh
↪ /mnt/IsoSeq-analysis/data/Cogent/hq 27 T.adelaidensis

# DIRNAME=$1
# species=$3

kmerSize=$2

find $1 -mindepth 1 -maxdepth 1 -type d | while read line; do #lists off all the
↪ /hq_* folders within the input directory and passes them through the script one
↪ by one (by line)
    FILE=$line/cogent2.fa
```

```

if [ ! -f $FILE ]; then
    echo "failed no cogent2.fa Increase K-mer size" #Note all jobs will "fail" on
    → first attempt unless you are running this in a previously reconstructed
    → directory. In the latter case only the failed directories will continue
    → to rerun.
    while [ ! -f "$FILE" ];do
        kmerSize=$((kmerSize + 3))
        rerunCMD="reconstruct_contig.py -k ${kmerSize} -S $3 $line"
        → #reconstruction script increasing kmer parameter by 3 -note because
        → all directories fail initially the input kmer value should be 3 below
        → the desired actual value.
        echo ${rerunCMD}
        eval ${rerunCMD}
        if [ -f $FILE ]; then
            touch $1/hq_kmerSize.txt #This text file will give a list of the used
            → kmerSize per hq_* folder
            echo "SUCCESS! The increased Kmer size $kmerSize was successful for
            → $line." >> $1/hq_kmerSize.txt
        fi
        if [ ${kmerSize} -gt 99 ];then
            cp $line/in.fa $line/cogent2.fa # if a folder reruns until 99kmer
            → parameter length is reached the input is simply copied into
            → cogent2.fa
            touch $1/failed-jobs.txt #This text file will remain empty or give a
            → list of all failed folders
            echo "Failed to succeed reconstruction with largest Kmer on ${line}.
            → Copied input transcripts to cogent2.fa as output." >>
            → $1/failed-jobs.txt
        fi
    done
    kmerSize=$2
fi
done

if [ -s $1/failed-jobs.txt ]; then
    echo "failed-jobs.txt not empty. Some jobs failed. Please re-run them."
else
    echo "failed-jobs.txt empty or doesnt exist. All jobs completed."
fi

```

Re-check output.

Number of completed gene families:

```

ls ./hq/*/COGENT.DONE | wc -l
4459

```

OR:

```

ls ./hq/*/cogent2.renamed.fasta | wc -l
4459

```

Finding failed reconstruction jobs.

Number of items in ./hq:

```
ls hq | wc -l
4460
```

The added 1 is the text file reporting the successful kmer size for each “gene” family folder. Therefore all have completed.

List the names of folders that have not finished:

```
comm -3 <(find /mnt/IsoSeq-analysis/data/Cogent/hq -iname 'COGENT.DONE' -printf
→ '%h\n' | sort -u) <(find /mnt/IsoSeq-analysis/data/Cogent/hq -maxdepth 1
→ -mindepth 1 -type d | sort) | sed -e 's/^.*hq\\/'
```

(this command listed no folders, indicating the script has completed all folders successfully)

See failed-jobs.txt and/or hq_kmerSize.txt. Note: all completed at kmer size 30 this time, and filed-jobs.txt does not exist as none failed.

2.1.2.0.3 Using Cogent to collapse redundant transcripts in absence of genome [Creating the “fake genome”](#)

List and number of unassigned sequences

Make sure you are in the correct Cogent directory and can see the file hq.partition.txt:

```
tail -n 1 hq.partition.txt |tr ',' '\n' > unassigned.list
```

Create the unassigned.list file in the same directory:

```
tail -n 1 hq.partition.txt |tr ',' '\n' | wc -l
3193
```

Note: this number did not change with the addition of the five failed reconstruction folders. i.e. they failed completely and did not end up in the unassigned dataset.

The unassigned hq sequences were made into a fasta file.

```
export PATH=$PATH:/mnt/Prog/cDNA_Cupcake/sequence
get_seqs_from_list.py ../2019-04-30_out/polished.hq.fasta unassigned.list >
→ unassigned.fasta
```

Unassigned sequences were concatenated with the Cogent reconstructed contigs by putting the reconstructed genes plus the unassigned single hq isoforms into a single fasta file:

```
mkdir collected
cd collected
cat ../hq/*/cogent2.renamed.fasta ../unassigned.fasta > cogent_fake_genome.fasta
```

Redundant isoforms were collapsed:

Create a SAM alignment with minimap2 (This could also be done with GMAP).

Obtain a final set of unique (non-redundant) transcript Isoforms that can be used as a reference gene set.

As there is natural 5' degradation in RNA some sequences will represent identical isoforms which may not all be identified in clustering.

These parameters are default or suggested in the Cogent tutorial page.

Create an aligned, sorted SAM file:

```
export PATH=$PATH:/mnt/Prog/minimap2
minimap2 -ax splice -t 30 -uf --secondary=no cogent_fake_genome.fasta
↪ ../../2019-04-30_out/polished.hq.fasta > hq.fasta.sam
```

Output:

```
[M::mm_idx_gen::2.392*1.00] collected minimizers
[M::mm_idx_gen::3.526*1.32] sorted minimizers
[M::main::3.528*1.32] loaded/built the index for 9960 target sequence(s)
[M::mm_mapopt_update::3.708*1.30] mid_occ = 31
[M::mm_idx_stat] kmer size: 15; skip: 5; is_hpc: 0; #seq: 9960
[M::mm_idx_stat::3.810*1.29] distinct minimizers: 6267388 (82.12% are singletons);
↪ average occurrences: 1.346; average spacing: 2.846
[M::worker_pipeline::13.901*5.65] mapped 25117 sequences
[M::main] Version: 2.11-r797
[M::main] CMD:
↪ /mnt/Prog/pacbio/smartlink/install/smartlink-release_6.0.0.47841/bundles/smarttools/
↪ install/smarttools-release_6.0.0.47835/private/thirdparty/minimap2/minimap2_2.11/binwrap/../../...
↪ private/thirdparty/minimap2/minimap2_2.11/bin/minimap2 -ax splice -t 30 -uf
↪ --secondary=no cogent_fake_genome.fasta ../../2019-04-30_out/polished.hq.fasta
[M::main] Real time: 13.942 sec; CPU: 78.558 sec
```

Then follow the collapse tutorial from [Cupcake](#)

There is another cupcake page with information but the example scripts are provided on the cogent tutorial page.

Sort the SAM file:

```
sort -k 3,3 -k 4,4n hq.fasta.sam > hq.fasta.sorted.sam
```

Collapse identical isoforms to obtain a list of full length, unique, hq isoforms to use as reference transcripts:

```
cd /mnt/Prog/cDNA_Cupcake/sequence
which collapse_isoforms_by_sam.py
/home/ubuntu/miniconda2/envs/anaCogent/bin/collapse_isoforms_by_sam.py

cd /mnt/IsoSeq-analysis/data/Cogent/collected
```

Usage is:

```
# usage: collapse_isoforms_by_sam.py [-h] [--input INPUT] [--fq] -s SAM -o
# PREFIX [-c MIN_ALN_COVERAGE]
```

```
# [-i MIN_ALN_IDENTITY]
# [--max_fuzzy_junction MAX_FUZZY_JUNCTION]
# [--flnc_coverage FLNC_COVERAGE]
# [--dun-merge-5-shorter]
```

Collapse:

```
collapse_isoforms_by_sam.py --input ../../2019-04-30_out/polished.hq.fasta -s
→ hq.fasta.sorted.sam -c 0.94 -i 0.85 --dun-merge-5-shorter -o hq.fasta.no5merge
```

These parameters taken from pers. comm. Tessa Bradford, SA Museum.

Output is the name 'stem' the collapsed.rep.fa is for annotation.

The output files are <-o>.collapsed.gff, <-o>.collapsed.rep.fq, and <-o>.collapsed.group.txt i.e. hq.fasta.no5merge.collapsed.gff, hq.fasta.no5merge.collapsed.rep.fq, and hq.fasta.no5merge.collapsed.group.txt

The naming system for the post-collapse isoform is PB.<loci_index>.<isoform_index>

More terminal output from the above has been excluded. This is an example:

```
Ignored IDs written to: hq.fasta.no5merge.ignored_ids.txt
Output written to:
hq.fasta.no5merge.collapsed.gff
hq.fasta.no5merge.collapsed.group.txt
hq.fasta.no5merge.collapsed.rep.fa
Namespace(allow_extra_5exon=False, flnc_coverage=-1, fq=False,
→ input='../../2019-04-30_out/polished.hq.fasta', max_3_diff=100,
→ max_5_diff=1000, max_fuzzy_junction=5, min_aln_coverage=0.94,
→ min_aln_identity=0.85, prefix='hq.fasta.no5merge', sam='hq.fasta.sorted.sam')
```

Check outputs:

```
ls

cogent_fake_genome.fasta          hq.fasta.no5merge.collapsed.rep.fa
hq.fasta.no5merge.collapsed.gff   hq.fasta.no5merge.ignored_ids.txt
hq.fasta.no5merge.collapsed.gff.unfuzzy hq.fasta.sam
hq.fasta.no5merge.collapsed.group.txt hq.fasta.sorted.sam
hq.fasta.no5merge.collapsed.group.txt.unfuzzy
```

The file hq.fasta.no5merge.collapsed.group.txt names the isoforms as PB.<loci_index>.<isoform_index> and lists the collapsed identical isoforms.

Each 'locus' consists of a strand-specific locus with isoforms that overlap by at least 1 bp. So PB.11.1 and PB.11.2 means this locus has two isoforms.

Count the 'loci' (transcripts) found:

```
wc -l hq.fasta.no5merge.collapsed.group.txt
15729 hq.fasta.no5merge.collapsed.group.txt
```

Write file statistics:

```

get_abundance_post_collapse.py hq.fasta.no5merge.collapsed
↪ /mnt/IsoSeq-analysis/data/2019-04-30_out/polished.cluster_report.csv

WARNING: isoseq3 format detected. Output `length` column will be `NA`.

Read stat file written to hq.fasta.no5merge.collapsed.read_stat.txt
Abundance file written to hq.fasta.no5merge.collapsed.abundance.txt

```

Note: this is true, hq.fasta.no5merge.collapsed.read_stat.txt has no length information and a whole column of NA values, however the headers of hq.fasta.no5merge.collapsed.rep.fa do have length information. This is how IsoSeq3 format data is processed and does not affect other aspects of this script.

Get count information from the abundance and group .txt files

Add the headers:

```

echo -e "pbid\tcount_fl" > output.collapsed.abundance.txt

```

Add the PB.loci_index.isoform_index information and number of isoforms:

```

paste <(awk '{print $1}' hq.fasta.no5merge.collapsed.group.txt) <(awk -F , '{print
↪ NF}' hq.fasta.no5merge.collapsed.group.txt) >> output.collapsed.abundance.txt

```

All these commands except reconstructContig_1.sh and TBreconstructContig.edit.sh are provided in the one document [Running-Cogent-2.sh](#) on Github.

The longest transcript for each set of isoforms is in hq.fasta.no5merge.collapsed.rep.fa. This file is used in the following step and also provides the full transcripts to be sub-set as a reference based on further clustering in Chapter 5.

2.1.3 ANGEL

ANGEL: Robust Open Reading Frame prediction

Line by line instructions for running ANGEL in order to predict open reading frame and peptide sequences of transcript Isoforms. Also outputs 5' and 3' untranslated regions and scores transcript completeness.

This analysis was tested on an environment on the eRSA NECTAR research cloud, final data run was completed by Terry Bertozzi on a Dell PowerEdge server with 40 cores and 512G RAM due to memory limitations of the NECTAR allocation.

All steps and script parameters are accurate here, however, this analysis was run on a different HPC machine due to memory constraints. Directories in these commands are consistent with input data and where output was then moved back to, and serve as an example only. All results were immediately placed back in ~IsoSeq-analysis/data/ANGEL

#The python dependencies for ANGEL are:

```
numpy
Biopython
scikit-learn
CD-HIT version 4.8.1
conda install -n anaCogent scikit-learn

mkdir /mnt/IsoSeq-analysis/data/ANGEL
cd /mnt/IsoSeq-analysis/data/ANGEL
```

2.1.3.0.1 Dumb ORF prediction dumb_predict.py takes as input a FASTA file. It outputs the longest ORF in all frames - use to create a top training dataset.

Usage:

```
dumb_predict.py <fasta_filename> <output_prefix>
               [--min_aa_length MIN_AA_LENGTH]
               [--use_firstORF]
               [--use_rev_strand] [--cpus CPUS]
```

By default, only the forward strand is used. This is especially true for PacBio transcriptome sequencing output.

```
dumb_predict.py
↪ /mnt/IsoSeq-analysis/data/Cogent/collected/hq.fasta.no5merge.collapsed.rep.fa
↪ pygmy.dumb --min_aa_length 100 --cpus 24

# -----
13335 finished          7611 clusters
```

2.1.3.0.2 Creating a non-redundant training dataset ANGEL classifier training:

```
angel_make_training_set.py pygmy.dumb.final pygmy.dumb.final.training --random
↪ --cpus 24

angel_train.py pygmy.dumb.final.training.cds pygmy.dumb.final.training.utr
↪ pygmy.dumb.final.classifier.pickle --cpus 12
```

2.1.3.0.3 Robust ORF prediction Based on both the ANGEL Classifier training and the dumb ORF prediction.

Sequences are output as fasta files with whether they are complete, 5' partial, 3' partial, or internal in the header information, which also includes the aa length.

Predictions are tagged as confident, likely, or suspicious, and dumb ORF predictions as dumb.

The proportion of each isoform that is untranslated, as well as the position of the cds sequence is also output.

```
angel_predict.py ../Cogent/collected/hq.fasta.no5merge.collapsed.rep.fa
→ pygmy.dumb.final.classifier.pickle pygmy --output_mode=best
→ --min_angel_aa_length 100 --min_dumb_aa_length 100 --cpus 48
```

Output is written to:

- pygmy.ANGEL.cds
- pygmy.ANGEL.pep
- pygmy.ANGEL.utr

These outputs are used further in Chapter 5 for additional clustering and BLASTx searches.

Note: The reverse strand options within ANGEL are not necessary on Isoseq data.

All these commands are provided in the one document [Running_ANGEL.sh](#) on Github.

2.1.4 CD-HIT

Clustering of transcript isoforms. Notes and output recorded for CD-HIT analysis of peptide sequences output from ANGEL ORF prediction. This analysis was conducted on the [CD-HIT web server](#).

CD-HIT was run by uploading pygmy.ANGEL.pep to the server and selecting a cut-off of 0.99.

CD-HIT notes and recorded output are in [CDHit notes.pep99.bash](#) and below.

```
cd-hit - run on pygmy.ANGEL.pep

separate run cut-off at .99

***
Your job 1598936109 is finished.
Program you ran: cd-hit
You input file is pygmy.ANGEL.pep and we named it as 1598936109.fas.0
Summary information for 1598936109.fas.0 included in 1598936109.fas.0.stat
You required 1 runs for sequence clustering
    1. Fasta file for representative sequences at 99% identity is 1598936109.fas.1
        Summary information for 1598936109.fas.1 included in 1598936109.fas.1.stat
        Corresponding cluster file is 1598936109.fas.1.clstr
        Sorted cluster file by size is 1598936109.fas.1.clstr.sorted
Generated shell script is run-1598936109.sh

faa_stat.pl 1598936109.fas.0
faa_stat.pl 1598936109.fas.1
/data5/data/NGS-ann-project/apps/cd-hit/clstr_sort_by.pl no <
  ↳ 1598936109.fas.1.clstr > 1598936109.fas.1.clstr.sorted
/data5/data/NGS-ann-project/apps/cd-hit/clstr_list.pl 1598936109.fas.1.clstr
  ↳ 1598936109.clstr.dump
gnuplot1.pl < 1598936109.fas.1.clstr > 1598936109.fas.1.clstr.1; gnuplot2.pl
  ↳ 1598936109.fas.1.clstr.1 1598936109.fas.1.clstr.1.png
/data5/data/NGS-ann-project/apps/cd-hit/clstr_list_sort.pl 1598936109.clstr.dump
  ↳ 1598936109.clstr_no.dump
/data5/data/NGS-ann-project/apps/cd-hit/clstr_list_sort.pl 1598936109.clstr.dump
  ↳ 1598936109.clstr_len.dump len
/data5/data/NGS-ann-project/apps/cd-hit/clstr_list_sort.pl 1598936109.clstr.dump
  ↳ 1598936109.clstr_des.dump des
```

The generated CD-HIT script is uploaded to github as [run-1598936109.sh](#)

The full output metadata file is uploaded to github as [1598936109.out](#) and the summary data provided below:

```
=====
Program: CD-HIT, V4.7 (+OpenMP), Sep 06 2018, 09:36:42
Command: /data5/data/NGS-ann-project/apps/cd-hit/cd-hit -i
        1598936109.fas.0 -d 0 -o 1598936109.fas.1 -c 0.99 -n 5
        -G 1 -g 1 -b 20 -l 10 -s 0.0 -aL 0.0 -aS 0.0 -T 4 -M
        32000

Started: Mon Aug 31 21:55:20 2020
=====
                                Output
-----
```

```

total seq: 13882
longest and shortest : 2513 and 99
Total letters: 4952779
Sequences have been sorted

...

13882  finished          9907  clusters

```

The output 1598936109.fas.1.clstr.sorted contains the sorted cluster groups generated by CD-Hit and indicates the longest representative transcript for each cluster.

2.1.5 Reading CD-HIT output in R

dplyr was used to manipulate the CD-Hit output to create a list of unique transcript IDs of the longest representative transcript for each cluster. This list file is required in the following step to subset from the full length transcript file which contains the untranslated regions.

Load libraries and set the working directory

Import 1598936109.fas.1.clstr.sorted, the CD-Hit output file with all transcript IDs assigned to a sorted cluster (note: ./ReferenceClusters/ has been continually included due to setwd errors).

```

clstr <- read.csv("./ReferenceClusters/1598936109.fas.1.clstr.sorted",
  sep = "\t", row.names = NULL, header = FALSE, stringsAsFactors = FALSE)
head(clstr)

```

Initial file had a blank line with ">Cluster #" in between new clusters. Extend the >Cluster # down the column so that all transcripts are directly associated with a cluster number in their row.

```

clstr2 <- clstr
n = nrow(clstr)
x = 0
numbers_only <- function(x) !grepl("\\D", x)
for (row in c(1:n)) {
  if (numbers_only(clstr2[row, 1]) == TRUE) {
    clstr2[row, 1] <- x
  } else {
    NULL
  }
  x <- clstr2[row, 1]
}
head(clstr2, 20)

```

Count the number of transcript members for each cluster.

```

clstr.sums <- data.frame(dplyr::count(clstr2, V1))
write.csv(clstr.sums, "~/@Uni/@Flinders University
↳ PhD/RWorkingDir/Workflow-readthedown/ReferenceClusters/clstr.sums.csv",
  quote = FALSE, row.names = FALSE, col.names = FALSE)
head(clstr.sums)

```

```
##           V1  n
## 1    >Cluster 0 19
## 2    >Cluster 1 16
## 3    >Cluster 10 12
## 4    >Cluster 100 6
## 5    >Cluster 1000 3
## 6    >Cluster 1001 3
```

Remove the additional blank row without a transcript name from between each cluster group.

```
switch <- clstr.sums[1, 2]
clstr3 <- cbind(clstr2[1], clstr)
clstr3[c((switch - 5):(switch + 5)), ]
```

```
clstr4 <- clstr2[-which(clstr2$V2 == ""), ]
clstr4[c(1:5, (switch - 5):(switch + 5)), ]
```

Separate CD-Hit information into columns by delimiters:

- Remove > symbol from transcript names
- Move number of amino acids into a new column by separating “aa”
- Move statistics of % match into a new column by separating “...”
- Give the columns headers

```
library(stringr)
clstr5 <- clstr4
clstr5[] <- lapply(clstr5, gsub, pattern = ">", replacement = "")
clstr5.2 <- data.frame(str_split_fixed(clstr5$V2, "aa, ", 2))
clstr5.3 <- data.frame(str_split_fixed(clstr5.2$X2, "... ", 2))
clstr6 <- cbind(clstr5[1], clstr5.2[1], clstr5.3[1:2])
colnames(clstr6) <- c("cluster", "aa", "TranscriptID", "stat")
head(clstr6)
```

Filter based on rows that have a "*" in the stat column. These indicate the representative sequences for each cluster determined by CD-Hit and will act as the main reference.

```
clstr7 <- filter(clstr6, stat == "*")
head(clstr7, 50)
```

Pull out the column “TranscriptID” and export into a txt file so it can be used as a list to extract these sequences.

```
clstrTranscriptID <- pull(clstr7, var = TranscriptID)

write.csv(clstrTranscriptID, "~/@Uni/@Flinders University
↳ PhD/RWorkingDir/Workflow-readthedown/ReferenceClusters/clstrTranscriptID.csv",
  quote = FALSE, row.names = FALSE, col.names = FALSE)
head(clstrTranscriptID)
```

```
## [1] PB.6088.11|e88f16|path1:164-2792(+)|transcript/22345|m.11362
## [2] PB.2919.1|6c1fb9|path4:13-2574(+)|transcript/10624|m.5342
## [3] PB.535.2|136fa8|path0:1-2775(+)|transcript/4312|m.913
## [4] PB.4689.2|afa3de|path19:22-2096(+)|transcript/11491|m.8666
## [5] PB.6357.1|f27f14|path2:4-2108(+)|transcript/12511|m.11846
## [6] PB.715.4|1b393a|path6:6-8031(+)|transcript/8|m.1216
## 13882 Levels: PB.10.1|004815|path1:5-1713(+)|transcript/17534|m.3 ...
```

Pull out the first segment before the first | from column “TranscriptID” and export into a txt file. this reduced the ID to the initial PB.<loci_index>.<isoform_index> identifier.

```
WholeTranscriptID <- word(clstrTranscriptID, 1, sep = "\\|")
write.csv(WholeTranscriptID, "~/@Uni/@Flinders University
↳ PhD/RWorkingDir/Workflow-readthedown/ReferenceClusters/WholeTranscriptID.csv",
quote = FALSE, row.names = FALSE, col.names = FALSE)
head(WholeTranscriptID)
```

```
## [1] "PB.6088.11" "PB.2919.1" "PB.535.2" "PB.4689.2" "PB.6357.1"
## [6] "PB.715.4"
```

Note there will be “duplicates” if the name is truncated all the way back to the PB.<loci_index>.<isoform_index> identifier due to multiple isoforms that are retained. A longer, but still truncated version of this ID (excluding the information appended by ANGEL), was required to subset from the hq.fasta.no5merge.collapsed.rep.fasta file.

The clstrTranscriptID.csv is now the full list of representative transcripts based on clustering of the translated proteins in predicted read frame. This file was then used in NECTAR to extract a .fasta of all the reference transcripts.

2.1.6 Seqtk

Seqtk was used on the NECTAR machine to manipulate the fasta files and subset based on list files of transcript names.

Installation:

```
#Carmel July 2020

#Used on NECTAR research cloud

#Relies on hard-coded paths & filenames
#run line by line in shell

-----

cd /mnt/Prog
conda install -c bioconda seqtk

#___
The following packages will be downloaded:

package                                | build
-----|-----
```

certifi-2020.6.20		py37hc8dfbb8_0	151 KB	conda-forge
conda-4.8.3		py37hc8dfbb8_1	3.0 MB	conda-forge
openssl-1.1.1g		h516909a_1	2.1 MB	conda-forge
python_abi-3.7		1_cp37m	4 KB	conda-forge
seqtk-1.3		hed695b0_2	39 KB	bioconda

Total:			5.3 MB	

The following NEW packages will be INSTALLED:

```
python_abi      conda-forge/linux-64::python_abi-3.7-1_cp37m
seqtk           bioconda/linux-64::seqtk-1.3-hed695b0_2
#-----
```

Conda packages are located in /mnt/Prog/miniconda3/bin/ if you dont set a path

Manually convert clstrTranscriptID.csv to an clstrTranscriptID.lst file.

Seqtk was used to subset the pygmy.ANGEL.cds fasta file based on the R cluster list outputs: Moved files into /mnt/IsoSeq-analysis/data/Seqtk

```
screen          #to be safe
cd /mnt/IsoSeq-analysis/data/Seqtk
/mnt/Prog/miniconda3/bin/seqtk subseq ../ANGEL/pygmy.ANGEL.cds
↪ clstrTranscriptID.lst > clstrTranscriptID.fa
```

check:

```
wc -l clstrTranscriptID.lst
# 9907
wc -l clstrTranscriptID.fa
# 19814

# 19814/2 = 9907. So it is correct
```

The hq.fasta.no5merge.collapsed.rep.fa file created in the program ANGEL in Chapter 4 is a fasta file where sequence information ran over multiple “lines” within the file.

Seqtk was used to convert this file into a .fasta with sequence on a single line.

```
17/11/2020
# just converting hq.fasta.no5merge.collapsed.rep.fa into a fasta with sequence all
↪ on one line
# command seq is basic fasta/q conversion

cd /mnt/IsoSeq-analysis/data/Seqtk
/mnt/Prog/miniconda3/bin/seqtk seq
↪ ../Cogent/collected/hq.fasta.no5merge.collapsed.rep.fa >
↪ hq.fasta.no5merge.collapsed.rep.1L.fa
```

Checked that the file still has the correct number of sequences:

```
$ grep -c ">" hq.fasta.no5merge.collapsed.rep.fa
15729

grep -c ">" hq.fasta.no5merge.collapsed.rep.1L.fa
15729
```

Seqtk was also used to search for transcripts which returned BLAST results for some genes of interest as data exploration. The reference list files for this were extracted from the BLASTx output were created using find and sort functions in notepad++ and excel, and then applied to the pygmy.ANGEL.cds fasta file to extract sequences.

```
#Usage from /mnt/IsoSeq-analysis/data/ANGEL

cd /mnt/IsoSeq-analysis/data/Seqtk
/mnt/Prog/miniconda3/bin/seqtk subseq ../ANGEL/pygmy.ANGEL.cds AQP.lst > AQP.fa

/mnt/Prog/miniconda3/bin/seqtk subseq ../ANGEL/pygmy.ANGEL.cds HSP.lst > HSP.fa

/mnt/Prog/miniconda3/bin/seqtk subseq ../ANGEL/pygmy.ANGEL.cds SLC.lst > SLC.fa

/mnt/Prog/miniconda3/bin/seqtk subseq ../ANGEL/pygmy.ANGEL.cds MAPK.lst > MAPK.fa
```

2.1.7 Subsetting full length transcripts which represent each cluster

Creation of a subset of the longest representative transcripts for each cluster as per CD-HIT and the previous two to form the reference for counts in the following Chapter 6.

As only the peptide coding sequence of transcripts was used in the BLASTx search and the CD-HIT clustering in order to cluster similarly expressed transcripts, the untranslated regions of transcripts are absent. Our goal is to include these untranslated regions in the reference to be used for gene expression counts.

Rather than attempting to piece pygmy.ANGEL.cds and pygmy.ANGEL.utr back together, a truncated version of the transcript names was used to pull the full length unaltered transcript out of the earlier hq.fasta.no5merge.collapsed.rep.fa file.

Initial checks of clstrTranscriptID.csv were carried out. Files are continually checked for transcript ID or sequence counts to ensure manual manipulation did not remove any data that was not being targeted. Exploration of discrepancies below identified a number of transcripts that with clustering of multiple isoforms, have seeded more than one cluster. This means that there were duplicates in the list file once coding region identifiers were removed and sequences had to be extracted manually and not by applying the clstrTranscriptID list to hq.fasta.no5merge.collapsed.rep.1L.fa in seqtk.

The following was done on a local Intel core i7 machine using RStudio or in-house bash scripts applied in Git Bash for Windows. Some later steps were carried out on the NECTAR allocation.

All files copied to “~/IsoSeq-analysis/data/Seqtk/reference COPIES 2020-11” and contents checked

The following [file](#) contains all of the below:

```
Carmel@CarmelASUS MINGW64 ~
cd "E:/@DATA/@@PBT_KidneyIsoSeq_Working
↪ Data_C.Maher/IsoSeq-analysis/data/Seqtk/reference COPIES 2020-11"

2020-11-17
```



```
cd "C:\Users\Carmel\Desktop\reference COPIES 2020-11"
```

```
19814 clstrTranscriptID.fa          /2 = 9907
9907 clstrTranscriptID.lst
```

```
hq.fasta.no5merge.collapsed.rep.fa
hq.fasta.no5merge.collapsed.rep.1L.fa
pygmy.ANGEL.cds
pygmy.ANGEL.pep
pygmy.ANGEL.utr
```

Sort and filter clstrTranscriptID.lst by unique values to make sure there are no duplicates

```
sort clstrTranscriptID.lst | uniq > clstrUniq.txt
wc -l clstrUniq.txt
9907 clstrUniq.txt
```

List duplicates if there are any found (there were not)

```
sort clstrTranscriptID.lst | uniq -d > clstrUniqD.txt
wc -l clstrUniqD.txt
0 clstrUniqD.txt
```

```
Carmel@CarmelASUS MINGW64 /e/@DATA/@@PBT_KidneyIsoSeq_Working
↪ Data_C.Maher/IsoSeq-analysis/data/Seqtk/reference COPIES 2020-11
```

---> 1. Isoseq HQ output file: hq.fasta.no5merge.collapsed.rep.fa

header format

```
head -n 2 hq.fasta.no5merge.collapsed.rep.fa
>PB.1.1|000643|path0:1-1879(+)|transcript/14742 transcript/14742
↪ full_length_coverage=3;length=1887;num_subreads=52
ATAGAGTACAAGTACTAGAGTTTGTAGTGTGGTGAAGAGAAATCCAAGATCCACCATGGG
```

no of sequences

```
grep -c ">" hq.fasta.no5merge.collapsed.rep.fa
15729
```

--> 2. Angel CDS output from Isoseq HQ file: pygmy.ANGEL.cds

header format

```
head -n 2 pygmy.ANGEL.cds
>PB.2.1|002537|path0:1-1624(+) |transcript/18304|m.1 type:likely-NA len:135 strand:+
  ↳ pos:282-686

  ↳ ATGTCCTACTGCAGGCAAGAAGGAAAGGACAGAATTATATTTGTGACAAAAGAGGACCATGAAACTCCAAGTAGTGCTGAGTTAGTAGCGGAT
```

Note Sequence is on a single line

no of sequences

```
grep -c ">" pygmy.ANGEL.cds
13882
```

--> Angel CDS file clustered with CD-HIT: 1598936109.fas.1.clstr

header format

```
cd cd-hit\ 99
head 1598936109.fas.1.clstr
>Cluster 0
0      2513aa, >PB.715.4|1b393a|path6:6-8031(+) |transcript/8|m.1216... *
1      2250aa, >PB.715.5|1b393a|path6:278-8030(+) |transcript/13|m.1217... at
  ↳ 99.56%
2      2369aa, >PB.715.6|1b393a|path6:524-8029(+) |transcript/20|m.1218... at
  ↳ 99.58%
3      2148aa, >PB.715.7|1b393a|path6:1187-8030(+) |transcript/49|m.1219... at
  ↳ 99.53%
4      1913aa, >PB.715.9|1b393a|path6:1893-8028(+) |transcript/118|m.1221... at
  ↳ 99.79%
5      1688aa, >PB.715.11|1b393a|path6:2596-8054(+) |transcript/279|m.1223... at
  ↳ 99.59%
6      1452aa, >PB.715.14|1b393a|path6:3304-8029(+) |transcript/591|m.1226... at
  ↳ 100.00%
7      1333aa, >PB.715.15|1b393a|path6:3629-8031(+) |transcript/935|m.1227... at
  ↳ 99.77%
8      1314aa, >PB.715.16|1b393a|path6:3916-8078(+) |transcript/962|m.1228... at
  ↳ 100.00%
```

File contains truncated headers from the Angel CDS file, clustered according to similarity. Truncation appears to be at the first whitespace no of sequences

```
grep -c ', >' 1598936109.fas.1.clstr      #search pattern changed to avoid the ">"
  ↳ in front of each cluster label eg >Cluster 0
13882
```

Sequence number matches Angel CDS file

no of clusters

```
grep -c '>C' 1598936109.fas.1.clstr
9907
```

```
grep -c '>C' 1598936109.fas.1.clstr.sorted
9907
```

both match

```
tail 1598936109.fas.1.clstr
>Cluster 9902
0      99aa, >PB.8346.1|transcript/22300:1-1143(+)|transcript/22300|m.14112... *
>Cluster 9903
0      99aa, >PB.8372.1|transcript/22508:1-1103(+)|transcript/22508|m.14137... *
>Cluster 9904
0      99aa, >PB.8489.1|transcript/23315:1-855(+)|transcript/23315|m.14244... *
>Cluster 9905
0      99aa, >PB.8706.1|transcript/25054:1-372(+)|transcript/25054|m.14469... *
>Cluster 9906
0      99aa, >PB.9028.1|transcript/4242:1-2955(+)|transcript/4242|m.14776... *
```

```
tail 1598936109.fas.1.clstr.sorted
>Cluster 9902
0      99aa, >PB.8346.1|transcript/22300:1-1143(+)|transcript/22300|m.14112... *
>Cluster 9903
0      99aa, >PB.8372.1|transcript/22508:1-1103(+)|transcript/22508|m.14137... *
>Cluster 9904
0      99aa, >PB.8489.1|transcript/23315:1-855(+)|transcript/23315|m.14244... *
>Cluster 9905
0      99aa, >PB.8706.1|transcript/25054:1-372(+)|transcript/25054|m.14469... *
>Cluster 9906
0      99aa, >PB.9028.1|transcript/4242:1-2955(+)|transcript/4242|m.14776... *
```

Cluster numbers also match both files: Cluster 9906 + Cluster 0 = 9907

Check if the header changed from Isoseq HQ after processing with Angel?

```
cd ../
grep '>PB.715.4|1b393a|path6:6-8031(+)|transcript/8'
↪ hq.fasta.no5merge.collapsed.rep.fa
>PB.715.4|1b393a|path6:6-8031(+)|transcript/8 transcript/8
↪ full_length_coverage=43;length=7837;num_subreads=60
```

note: the “|m.xxxxx” suffix in the ANGEL output file is added at the first whitespace

---> **3. The longest seed sequence from the CD-HIT output: clstrUniq.txt**

header format

```
head clstrUniq.txt
PB.10.1|004815|path1:5-1713(+) |transcript/17534|m.3
PB.100.1|049515|path5:1-1563(+) |transcript/19243|m.132
PB.1000.1|26d4b2|path0:1-2889(+) |transcript/4521|m.1797
PB.1000.2|26d4b2|path0:1287-2889(+) |transcript/18232|m.1798
PB.1002.1|26ea71|path2:1-2673(+) |transcript/6271|m.1799
PB.1002.2|26ea71|path2:11-2691(+) |transcript/8089|m.1800
PB.1003.1|26fc93|path2:1-3987(+) |transcript/1197|m.1801
PB.1005.1|2718e2|path0:1-5294(+) |transcript/258|m.1804
PB.1006.1|272202|path0:1-2713(+) |transcript/5814|m.1805
PB.1007.1|272202|path1:1-3370(+) |transcript/2554|m.1806
```

Prefix “>” and suffix “... *” have been removed in R Studio

no of strings

```
grep -c 'PB.' clstrUniq.txt
9907
```

matches number of clusters above

strip the “|m.xxxx” from the strings in clstrUniq.txt

```
sed 's/|m.*//' clstrUniq.txt > new_cluster.txt
```

check

```
head new_cluster.txt
PB.10.1|004815|path1:5-1713(+) |transcript/17534
PB.100.1|049515|path5:1-1563(+) |transcript/19243
PB.1000.1|26d4b2|path0:1-2889(+) |transcript/4521
PB.1000.2|26d4b2|path0:1287-2889(+) |transcript/18232
PB.1002.1|26ea71|path2:1-2673(+) |transcript/6271
PB.1002.2|26ea71|path2:11-2691(+) |transcript/8089
PB.1003.1|26fc93|path2:1-3987(+) |transcript/1197
PB.1005.1|2718e2|path0:1-5294(+) |transcript/258
PB.1006.1|272202|path0:1-2713(+) |transcript/5814
PB.1007.1|272202|path1:1-3370(+) |transcript/2554
```

```
grep -c 'PB.' new_cluster.txt
9907
```

How many of these strings are in the original ONE LINE isoseq file?

```
grep -c -Ff new_cluster.txt hq.fasta.no5merge.collapsed.rep.1L.fa
9813
```

94 strings are missing

---> **4. find the missing strings**

convert the isoform hq file into the same format as new_cluster.txt

```

grep '>' hq.fasta.no5merge.collapsed.rep.1L.fa | sed s'/ tra.*//' | sed s'/>/' >
↪ hq_headers
head hq_headers
PB.1.1|000643|path0:1-1879(+)|transcript/14742
PB.2.1|002537|path0:1-1624(+)|transcript/18304
PB.3.2|00361c|path0:43-2240(+)|transcript/10564
PB.4.1|004079|path1:6-745(+)|transcript/23781
PB.4.2|004079|path1:6-612(+)|transcript/24311
PB.5.1|004079|path2:209-642(+)|transcript/24981
PB.6.1|004079|path3:273-616(+)|transcript/25193
PB.7.1|004079|path5:6-664(+)|transcript/24242
PB.8.1|004079|path6:1-607(+)|transcript/24477
PB.9.1|004079|path7:1-725(+)|transcript/23873

```

```

cat hq_headers |wc -l
15729

```

Checking for the same number of matches

```

grep -Ff new_cluster.txt hq_headers > matching_strings
cat matching_strings |wc -l
9813

```

Checking for duplicates

```

cat hq_headers | sort |uniq -cd
0

cat new_cluster.txt | sort |uniq -cd
2 PB.1206.1|2e8181|path5:1-4617(+)|transcript/551
2 PB.1261.1|30ba06|path3:633-1922(+)|transcript/21324
2 PB.1570.2|3bd5a8|path2:20-1934(+)|transcript/13977
2 PB.1719.1|404d72|path0:1-3429(+)|transcript/2407
2 PB.1764.3|429969|path8:44-2062(+)|transcript/12739
2 PB.1813.1|446aed|path0:1-2871(+)|transcript/4320
2 PB.1865.1|46bd4e|path0:1-2536(+)|transcript/6726
2 PB.1869.1|46d663|path2:1-1320(+)|transcript/21375
2 PB.220.1|08884f|path0:1-4120(+)|transcript/991
2 PB.2360.1|5843a6|path32:1-2062(+)|transcript/11326
2 PB.2541.3|5e93d1|path0:5-2495(+)|transcript/7381
2 PB.2560.1|5f09e2|path0:1-1196(+)|transcript/21957
2 PB.2618.1|612418|path1:1-2013(+)|transcript/12200
2 PB.2713.3|65abaa|path0:37-2495(+)|transcript/7693
2 PB.2892.1|6b359a|path12:1-4597(+)|transcript/526
2 PB.3037.2|702e9b|path5:2-3018(+)|transcript/3923
2 PB.3243.1|784f79|path0:1-2683(+)|transcript/5903
2 PB.3259.1|792211|path3:1-5233(+)|transcript/268
2 PB.3286.3|7a2645|path3:103-3087(+)|transcript/3780
2 PB.3405.1|7f2f96|path0:5-2081(+)|transcript/11903
3 PB.3430.1|8055ae|path8:1-3589(+)|transcript/2043
2 PB.3461.1|81f072|path5:1-2492(+)|transcript/7285

```

2 PB.351.4|0cccb8|path5:27-4293(+) |transcript/775
2 PB.3562.1|864d1b|path0:1-3492(+) |transcript/2174
2 PB.3615.1|883e51|path3:1-4044(+) |transcript/1347
2 PB.3836.3|8ff0e9|path0:4-3972(+) |transcript/946
2 PB.3934.1|93b547|path0:1-3353(+) |transcript/2676
2 PB.4042.2|97fba8|path2:34-2096(+) |transcript/13603
2 PB.4219.2|9d8e09|path3:9-3566(+) |transcript/2045
2 PB.4442.1|a6620e|path0:1-2906(+) |transcript/4555
2 PB.4458.1|a6e2a5|path0:1-3673(+) |transcript/1739
2 PB.4822.1|b4950f|path1:1-3315(+) |transcript/2177
2 PB.4822.2|b4950f|path1:1240-3315(+) |transcript/10982
2 PB.4919.1|b7da5b|path0:1-3327(+) |transcript/2652
2 PB.4923.1|b7fed9|path0:1-2394(+) |transcript/8643
2 PB.5080.1|bde542|path3:1-3060(+) |transcript/3777
2 PB.5096.2|be8c26|path4:70-2754(+) |transcript/5782
2 PB.5149.1|c15864|path0:1-1628(+) |transcript/17934
2 PB.5308.1|c8b5b5|path2:35-3805(+) |transcript/1487
2 PB.5395.1|cbd93f|path0:1-1699(+) |transcript/17107
2 PB.5483.2|cf1828|path1:23-2344(+) |transcript/9126
2 PB.5619.1|d4d2ea|path0:1-2677(+) |transcript/6673
2 PB.5663.1|d5e71b|path0:1-5101(+) |transcript/303
2 PB.5939.1|e23f0e|path0:1-2960(+) |transcript/3907
2 PB.5939.2|e23f0e|path0:5-4542(+) |transcript/622
2 PB.613.1|16d05c|path0:1-1558(+) |transcript/18673
2 PB.6133.1|ea032e|path9:1-2052(+) |transcript/12270
2 PB.6276.1|ee992b|path0:1-1487(+) |transcript/19762
2 PB.6283.1|eee48e|path1:1-3454(+) |transcript/2331
2 PB.6286.1|ef0b10|path12:1-2744(+) |transcript/5222
2 PB.6303.1|efc514|path0:1-3222(+) |transcript/2889
2 PB.6378.3|f31894|path9:80-2754(+) |transcript/5975
2 PB.6385.1|f32c31|path0:1-1500(+) |transcript/12840
2 PB.6426.1|f4ded6|path0:1-1796(+) |transcript/16785
2 PB.6576.2|facceb|path2:1-3314(+) |transcript/3157
2 PB.659.1|19437d|path1:1-3483(+) |transcript/2210
2 PB.6763.1|transcript/10186:1-2230(+) |transcript/10186
2 PB.6877.1|transcript/1099:1-4113(+) |transcript/1099
2 PB.6966.1|transcript/11636:1-2103(+) |transcript/11636
2 PB.713.1|1b043a|path0:1-965(+) |transcript/23154
2 PB.7227.1|transcript/138:1-5869(+) |transcript/138
2 PB.7287.1|transcript/14227:1-1898(+) |transcript/14227
3 PB.7342.1|transcript/14710:1-1879(+) |transcript/14710
2 PB.7349.1|transcript/14730:1-1903(+) |transcript/14730
2 PB.7353.1|transcript/14772:1-1860(+) |transcript/14772
2 PB.74.1|02ebe4|path0:1-2606(+) |transcript/6537
2 PB.7501.1|transcript/1596:1-3759(+) |transcript/1596
2 PB.7528.1|transcript/16151:1-1797(+) |transcript/16151
2 PB.7678.1|transcript/1728:1-3672(+) |transcript/1728
2 PB.7707.1|transcript/17496:1-1683(+) |transcript/17496
2 PB.7868.1|transcript/188:1-5558(+) |transcript/188
2 PB.7973.1|transcript/19628:1-1476(+) |transcript/19628
2 PB.8082.1|transcript/20433:1-1412(+) |transcript/20433
2 PB.816.1|1e334e|path4:1-2040(+) |transcript/12316
2 PB.8325.1|transcript/22158:1-1191(+) |transcript/22158

```

2 PB.834.2|1ec273|path2:1291-2750(+)|transcript/19945
2 PB.8389.1|transcript/22625:1-1060(+)|transcript/22625
2 PB.8709.1|transcript/252:1-5299(+)|transcript/252
2 PB.8883.1|transcript/3393:1-3152(+)|transcript/3393
2 PB.8886.1|transcript/3402:1-3146(+)|transcript/3402
2 PB.8920.1|transcript/3588:1-3076(+)|transcript/3588
2 PB.9068.1|transcript/4442:1-2918(+)|transcript/4442
2 PB.9162.1|transcript/4983:1-2815(+)|transcript/4983
2 PB.9209.1|transcript/5238:1-2771(+)|transcript/5238
2 PB.924.1|22d3cf|path3:1-1494(+)|transcript/19025
2 PB.9277.1|transcript/5650:1-2707(+)|transcript/5650
2 PB.944.1|241290|path4:2-2629(+)|transcript/6557
2 PB.9535.1|transcript/7343:1-2508(+)|transcript/7343
2 PB.9581.1|transcript/7647:1-2463(+)|transcript/7647
2 PB.9683.1|transcript/8355:1-2402(+)|transcript/8355
2 PB.9766.1|transcript/9024:1-2324(+)|transcript/9024
2 PB.9784.1|transcript/915:1-4197(+)|transcript/915

```

During the clustering it appears that several of the sequences have been made seed sequences (denoted with a *) for more than one cluster.

The counts above equate to how many strings which is the discrepancy noted in section 3 above.

```

cat new_cluster.txt | sort | uniq -cd | cut -d' ' -f7 | awk '{sum+=$1-1}
↪ END{printf("%d\n",sum)}'
94

```

Both hq.fasta.no5merge.collapsed.rep.fa and hq.fasta.no5merge.collapsed.rep.1L.fa. were checked in this way but the single line file is required in later steps so only those commands have been included.

Final pulling out of transcripts sequences into a .fasta on a single line, also removes the skipped lines that grep outputs as '--'.

note: NOT using seqtk anymore:

```

grep -Ff new_cluster.txt -A 1 hq.fasta.no5merge.collapsed.rep.1L.fa | grep -v ^-- >
↪ reference_transcripts.1Lv.fasta

grep -c ">" reference_transcripts.1Lv.fasta
9813

```

--> next step

Persisting poly-a tails needed to be trimmed from the following file:

```
reference_transcripts.1Lv.fasta
```

Note: The Trim poly-a command in bbdut leaves many tails intact due to single non 'A' bases and does not facilitate hamming distance command at the same time (?), however specifying Ktrim=r literal or "trimming twice" (poly-a command and THEN ktrim) sequences become very short even with different K values.

Terry Bertozzi provided perl script as below to manually trim:

```
# Terry Bertozzi
#command line to remove poly A tails

#for use on /mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.fasta

cd /mnt/IsoSeq-analysis/data/Seqtk

perl -pe 's/(A{5,}.{0,2})+$/gm' reference_transcripts.1L.fasta >
↪ reference_transcripts.1L.clean.fasta

perl -pe 's/(A{5,}.{0,2})+$/gm' reference_transcripts.1Lv.fasta >
↪ reference_transcripts.1Lv.clean.fasta

grep -c ">" reference_transcripts.1Lv.clean.fasta
9813

head reference_transcripts.1Lv.clean.fasta
#also looks good.
```

reference_transcripts.1Lv.clean.fasta - IS THE REFERENCE

2.2 *Short Read data*

De-novo assembly of short-read Transcripts:

Short read transcripts used for gene expression analysis in Chapter 6 were initially intended for de-novo assembly and analysis due to a lack of closely related genomic references for *T. adelaidensis*.

This section outlines the methods for assembly of short read datasets in the absence of a reference or genome for the purposes of comparing these final transcript datasets to the assembly generated from the long reads as above.

Other notes: Initial plans for Chapter 6 did not include the availability of long-read sequencing to supplement a reference transcript list. Therefore, a pooled transcript assembly was created by concatenating reads from all eight samples in order to use this larger dataset as a pseudo-reference for other programs.

This longer assembly is included in the BUSCO analysis below to illustrate assembly completeness and duplication.

Sequences were downloaded from the Australian Genomics Research Facility server & checksums verified. This analysis was conducted on the Flinders University ‘Deep Thought’ HPC.

2.2.1 FASTQC quality assessment

Quality of sequencing output was quantified using the following [script](#) and FastQC v0.11.8 (note there are two commands because there were two sequencing run data folders AGRF_CAGRF13871_HCGYYBCXY and AGRF_CAGRF20022_CDNJBANXX).

The FastQC Reports were then combined for visualisation using [NGSReports](#) The full FastQC output summary .html file of all eight samples PRIOR to trimming is available [here](#).

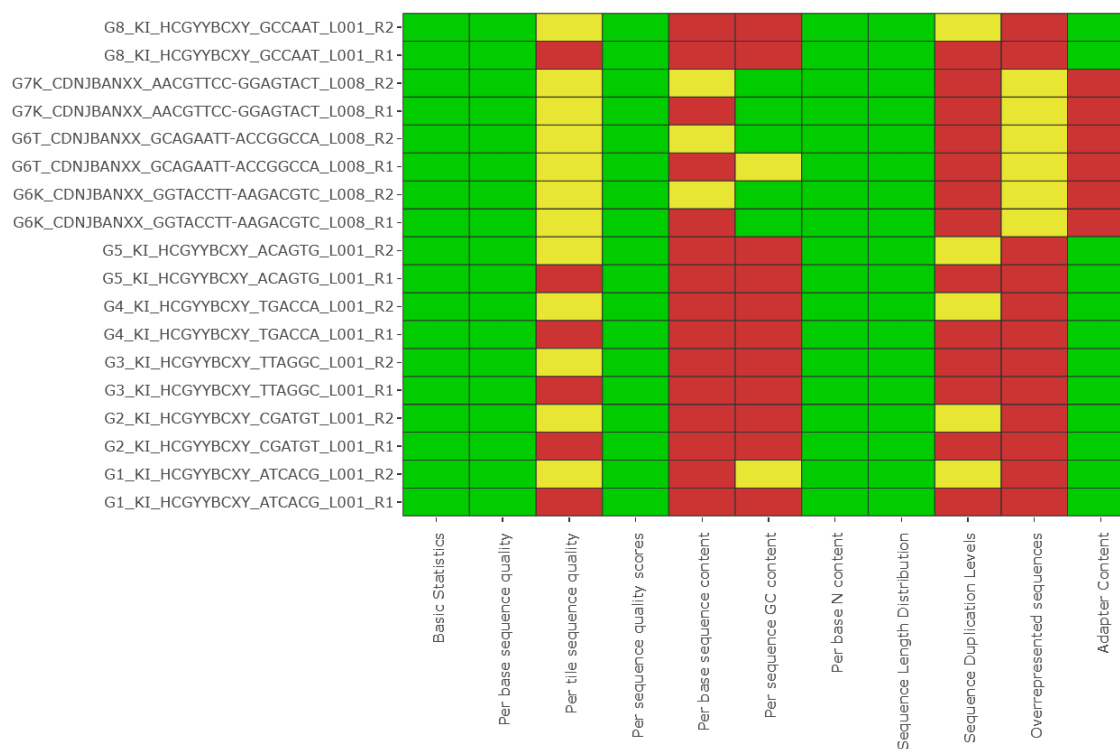


Figure 1: FastQC Summary of scores for paired reads of eight samples prior to any adapter or quality trimming

Samples G6 and G7 were run on a second sequencing run, and have different overall lengths. This is the main source of variation in sequencing scores.

2.2.2 BBduk adapter trimming

Trimming to remove Illumina TruSeq adapters and SMARTer PCR tags as well as trimming based on quality scores was done using bbduk, in in bmap v38.46 tools and the following script [removeAdapters2.sh](#).

```
#!/bin/bash

# November 2019
# Carmel Maher
# This script is to submit a BBduk script to cut adapters and quality on raw reads
→ through Flinders' DeepThought HPC Slurm
# This script therefore assumes submission to Flinders Deepthought with an assumed
→ directory structure and use of available modules

#-----
# Required Modules:
module add bmap #/38.46
module add fastqc #/0.11.8

#adapters location :ref=/cm/shared/apps/bmap/38.46/resources/adapters.fa
```

```
THREADS=8
```

```
#-----
```

```
# Terry's error exit
```

```
function error_exit
```

```
{
```

```
    # Exit function due to fatal error
```

```
    # Accepts 1 arg:
```

```
    # string - descriptive error message
```

```
    echo "${PROGNAME}: ${1:-"Unknown error"}" 1>&2
```

```
    exit 1
```

```
}
```

```
# in = /scratch/user/mahe0050/DE-analysis/0_rawData/
```

```
# out = /scratch/user/mahe0050/DE-analysis/1_trimmedData
```

```
#-----
```

```
#change directories into raw sequencing directory 1
```

```
cd /scratch/user/mahe0050/DE-analysis/0_rawData/AGRF_CAGRF13871_HCGYYBCXY ||
```

```
↪ error_exit "$LINENO: directory error 1"
```

```
mkdir ../../1_trimmedData/temp
```

```
for file in *R1.fastq.gz
```

```
do
```

```
    echo $file
```

```
    FILESTEM=${file%_*}
```

```
    echo $FILESTEM
```

```
    FILESTEM2=`echo $file | cut -d "_" -f1,2 --output-delimiter="_"`
```

```
    echo $FILESTEM2
```

```
#remove Illumina adapters from all paired data using bbduk
```

```
bbduk.sh in=$file in2=$FILESTEM"_R2.fastq.gz"
```

```
↪ out=../../1_trimmedData/temp/$FILESTEM2"_R1_Iclean.fq.gz"
```

```
↪ out2=../../1_trimmedData/temp/$FILESTEM2"_R2_Iclean.fq.gz"
```

```
↪ outs=../../1_trimmedData/temp/$FILESTEM2"_singletons_Iclean.fq.gz"
```

```
↪ literal=GATCGGAAGAGCACACA,AGATCGGAAGAGCGT ktrim=r k=15 mink=15 hdist=1
```

```
↪ threads=$THREADS || error_exit "$LINENO: Error removing Illumina adapters
```

```
↪ from R1 or R2 - directory 1"
```

```
#remove SMARTer PCR tags from all paired data using bbduk
```

```
bbduk.sh in=../../1_trimmedData/temp/$FILESTEM2"_R1_Iclean.fq.gz"
```

```
↪ in2=../../1_trimmedData/temp/$FILESTEM2"_R2_Iclean.fq.gz"
```

```
↪ out=../../1_trimmedData/$FILESTEM2"_R1_clean.fq.gz"
```

```
↪ out2=../../1_trimmedData/$FILESTEM2"_R2_clean.fq.gz"
```

```
↪ outs=../../1_trimmedData/$FILESTEM2"_singletons_clean.fq.gz"
```

```
↪ literal=AAGCAGTGGTATCAACGCAGAGTACNNNNN,GTACTCTGCGTTGATACCACTGCTT ktrim=r k=15
```

```
↪ mink=15 hdist=1 qtrim=r trimq=10 minlength=30 threads=$THREADS || error_exit
```

```
↪ "$LINENO: Error removing SMARTer adapters from R1 or R2 - directory 1"
```

```

#remove SMARTer PCR tags from the singleton file using bbduk
bbduk.sh in=../../1_trimmedData/temp/$FILESTEM2"_singletons_Iclean.fq.gz"
↳ out=../../1_trimmedData/$FILESTEM2"_singletons1.fq.gz"
↳ literal=AAGCAGTGGTATCAACGCAGAGTACNNNNN,GTACTCTGCGTTGATACCACTGCTT ktrim=r k=15
↳ mink=15 hdist=1 qtrim=r trimq=20 minlength=30 threads=$THREADS || error_exit
↳ "$LINENO: Error removing SMARTer adapters from singletons - directory 1"

#concatenate the singleton files
cat ../../1_trimmedData/$FILESTEM2"_singletons1.fq.gz" >>
↳ ../../1_trimmedData/$FILESTEM2"_singletons_clean.fq.gz" || error_exit
↳ "$LINENO: Error concatenating singletons - directory 1"

#clean up: i.e. remove temporary directories
rm ../../1_trimmedData/$FILESTEM2"_singletons1.fq.gz" || error_exit "$LINENO:
↳ Error deleting singleton intermediate - directory 1"

done

#change directories into raw sequencing directory 2 and do it again for these
↳ samples (output to same trimmed directory for further analysis together)
cd /scratch/user/mahe0050/DE-analysis/0_rawData/AGRF_CAGRF20022_CDNJBANXX ||
↳ error_exit "$LINENO: directory error 2"

for file in *R1.fastq.gz
do
    echo $file
    FILESTEM=${file%_*}
    echo $FILESTEM
    FILESTEM2=`echo $file | cut -d "_" -f1,2 --output-delimiter="_"`
    echo $FILESTEM2

#(repeat of the above but edit directories & names: same output folder as all
↳ samples have unique names)
#remove Illumina adapters from all paired data using bbduk
bbduk.sh in=$file in2=$FILESTEM"_R2.fastq.gz"
↳ out=../../1_trimmedData/temp/$FILESTEM2"_R1_Iclean.fq.gz"
↳ out2=../../1_trimmedData/temp/$FILESTEM2"_R2_Iclean.fq.gz"
↳ outs=../../1_trimmedData/temp/$FILESTEM2"_singletons_Iclean.fq.gz"
↳ literal=GATCGGAAGAGCACACA,AGATCGGAAGAGCGT ktrim=r k=15 mink=15 hdist=1
↳ threads=$THREADS || error_exit "$LINENO: Error removing Illumina adapters
↳ from R1 or R2 - directory 2"

#remove SMARTer PCR tags from all paired data using bbduk
bbduk.sh in=../../1_trimmedData/temp/$FILESTEM2"_R1_Iclean.fq.gz"
↳ in2=../../1_trimmedData/temp/$FILESTEM2"_R2_Iclean.fq.gz"
↳ out=../../1_trimmedData/$FILESTEM2"_R1_clean.fq.gz"
↳ out2=../../1_trimmedData/$FILESTEM2"_R2_clean.fq.gz"
↳ outs=../../1_trimmedData/$FILESTEM2"_singletons_clean.fq.gz"
↳ literal=AAGCAGTGGTATCAACGCAGAGTACNNNNN,GTACTCTGCGTTGATACCACTGCTT ktrim=r k=15
↳ mink=15 hdist=1 qtrim=r trimq=10 minlength=30 threads=$THREADS || error_exit
↳ "$LINENO: Error removing SMARTer adapters from R1 or R2 - directory 2"

```

```

#remove SMARTer PCR tags from the singleton file using bbdut
bbduk.sh in=../../1_trimmedData/temp/$FILESTEM2"_singletons_1clean.fq.gz"
↳ out=../../1_trimmedData/$FILESTEM2"_singletons1.fq.gz"
↳ literal=AAGCAGTGGTATCAACGCAGAGTACNNNNN,GTACTCTGCGTTGATACCACTGCTT ktrim=r k=15
↳ mink=15 hdist=1 qtrim=r trimq=20 minlength=30 threads=$THREADS || error_exit
↳ "$LINENO: Error removing SMARTer adapters from singletons - directory 2"

#concatenate the singleton files
cat ../../1_trimmedData/$FILESTEM2"_singletons1.fq.gz" >>
↳ ../../1_trimmedData/$FILESTEM2"_singletons_clean.fq.gz" || error_exit
↳ "$LINENO: Error concatenating singletons - directory 2"

#clean up: i.e. remove temporary directories
rm ../../1_trimmedData/$FILESTEM2"_singletons1.fq.gz" || error_exit "$LINENO:
↳ Error deleting singleton intermediate - directory 2"
done

cd /scratch/user/mahe0050/DE-analysis/1_trimmedData
rm -rf temp || error_exit "$LINENO: Error removing temp directory"

```

Quality of trimmed data was assessed again using FastQC v0.11.8.

```

#run cleaned data through fastqc
mkdir -p /scratch/user/mahe0050/DE-analysis/1_trimmedData/FastQC-clean

for file in *R1_clean.fq.gz
do
  echo $file
  FILESTEM2=`echo $file | cut -d "_" -f1,2 --output-delimiter="_"`
  echo "FILESTEM2" $FILESTEM2

  fastqc --noextract --threads $THREADS -o ./FastQC-clean
  ↳ ./FILESTEM2"_R1_clean.fq.gz" ./FILESTEM2"_R2_clean.fq.gz"
  ↳ ./FILESTEM2"_singletons_clean.fq.gz" || error_exit "$LINENO: Error with
  ↳ FastQC at "FILESTEM2""
done

#primers check
#zcat G1_KI_HCGYYBCXY_ATCACG_L001_R1.fastq.gz | grep "AGATCGGAAGAGCAC" -m10
#zcat G1_KI_HCGYYBCXY_ATCACG_L001_R1.fastq.gz | grep "GATCGGAAGAGCACA" -m10
#zcat G1_KI_HCGYYBCXY_ATCACG_L001_R2.fastq.gz | grep "AGATCGGAAGAGCGT" -m10

```

The output html file of all eight samples FastQC reports AFTER trimming is available [here](#).

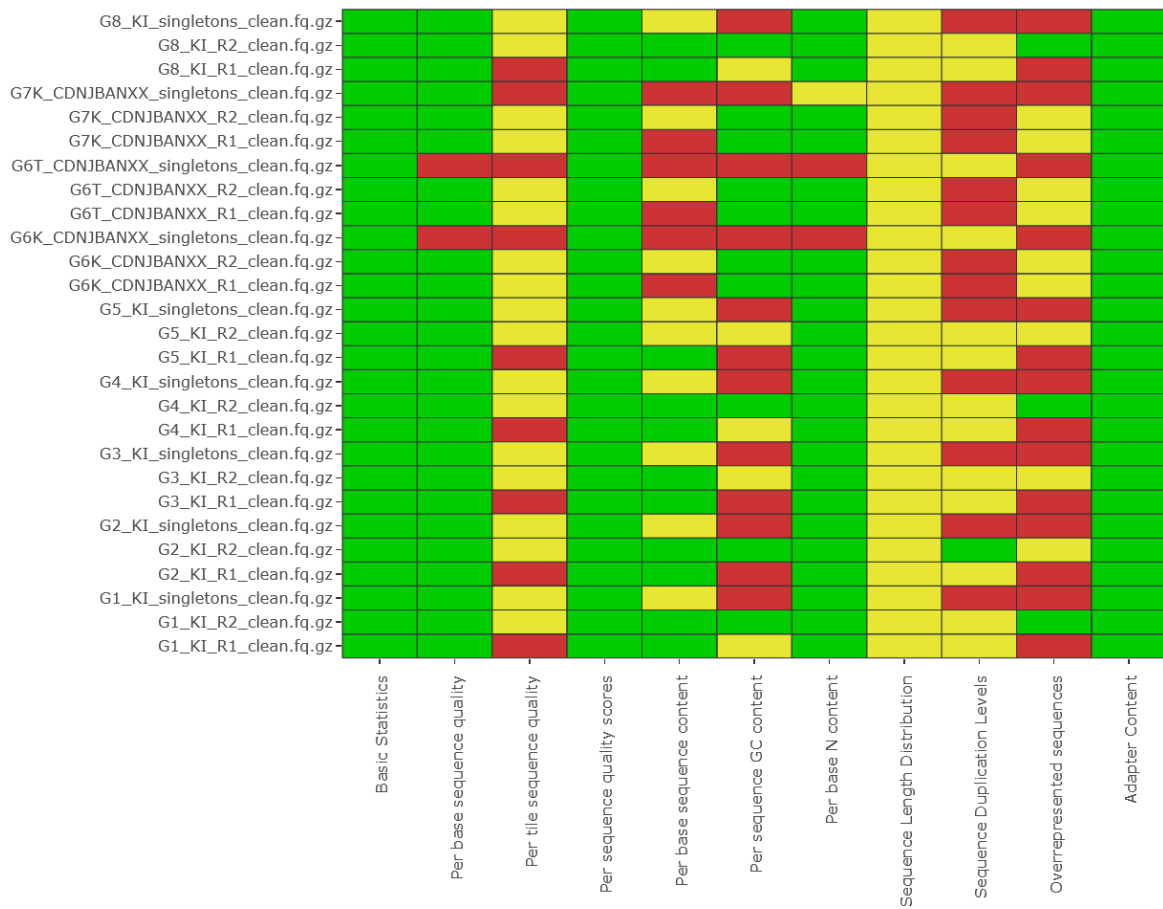


Figure 2: FastQC Summary of scores for paired reads of eight samples and resulting 'singleton' files for trimmed reads with no pair, after adapter and quality trimming in BBduk

2.2.3 De-novo assembly with TRINITY

De-novo assembly of transcripts.

Short reads were assembled *de novo* using the program TRINITY v2.8.4.

Sequencing run AGRF_CAGRF13871_HCGYYBCXY involved the synthesis of a non-stranded cDNA library prior to sequencing, while sequencing run AGRF_CAGRF20022_CDNJBANXX was completed with a different kit. All samples were treated as non stranded for consistency. Singleton reads (where a paired read was eliminated in quality trimming) were included for all TRINITY assembly runs.

Assembly was run on each sample individually as well as on a pooled (concatenated) file of all reads.

The script [trinity2019.sh](#)

Single samples were run through Trinity as per the linked script above using the following:

```
THREADS=16
MEM="64G"

cd /scratch/user/mahe0050/DE-analysis/1_trimmedData
```

To run trinity separately on each sample

```
mkdir -p ./trinity-sep
mkdir -p ./trinity-sep/temp

for file in *R1_clean.fq.gz
do
    FILESTEM=${file%_*}
    #this FILESTEM only cuts to _clean, the _R1 is included
    FILESTEM=${FILESTEM/R1/}
    #removes R1 from FILESTEM (FILESTEM ends in _ therefore not needed in "text"
    ↪ names)
```

concatenate relevant singleton file to R1 as library is not stranded?

```
cat ./file ./FILESTEM"singletons_clean.fq.gz" >
↪ ./trinity-sep/$FILESTEM"concat_R1.fq.gz" || error_exit "$LINENO: Error
↪ concatenating R1 and singletons"
```

Run TRINITY to assemble transcripts from a single paired sample file. Note trinity requires output directory with "trinity" in name.

```
Trinity --seqType fq --max_memory $MEM --bflyCalculateCPU --left
↪ ./trinity-sep/$FILESTEM"concat_R1.fq.gz" --right ./FILESTEM"R2_clean.fq.gz"
↪ --CPU $THREADS --output ./trinity-sep/$FILESTEM"trinity"/trinity ||
↪ error_exit "$LINENO: Error running trinity-sep at $FILESTEM"
```

clean up

```
rm -rf ./trinity-sep/$FILESTEM"concat_R1.fq.gz" || error_exit "$LINENO: Error
↪ removing trinity-sep concat at $FILESTEM"

done
echo "Trinity separate samples complete"
```

All kidney samples were run through Trinity together for a single large assembly as per the linked script above using the following:

Sample files to be concatenated and named appropriately

```
# create temp & working dir
mkdir -p ./trinity-all
mkdir -p ./trinity-all/temp
```

concatenate relevant sample files for one transcriptome - add singletons to R1 as its not stranded

```
cat /*_R1_clean.fq.gz /*_singletons_clean.fq.gz >>
↳ ./trinity-all/temp/PBTKI_cat_R1.fq.gz || error_exit "$LINENO: Error
↳ concatenating R1 and singletons"
cat /*_R2_clean.fq.gz >> ./trinity-all/temp/PBTKI_cat_R2.fq.gz || error_exit
↳ "$LINENO: Error concatenating R2"
```

Run TRINITY

```
Trinity --seqType fq --max_memory $MEM --bflyCalculateCPU --left
↳ ./trinity-all/temp/PBTKI_cat_R1.fq.gz --right
↳ ./trinity-all/temp/PBTKI_cat_R2.fq.gz --CPU $THREADS --output
↳ ./trinity-all/trinity || error_exit "$LINENO: Error running Trinity-all"
```

clean up

```
rm -rf ./trinity-all/temp || error_exit "$LINENO: Error removing trinity-all temp
↳ directory"
echo "Trinity concatenated kidney samples complete"
```

TRINITY outputs each samples assembly as Trinity.fasta, within a /trinity output folder, within the folder assigned with a sample name. In order to compare different Trinity.fasta results in one directory the following script was used to copy and rename these final files and place them in a common folder [TrinityName.sh](#).

This renaming was done using a script to avoid any errors in manual handling of files and it is these moved files which are used later in a BUSCO comparison of assemblies.

2.3 Summary / Quantification

2.3.1 Long read sequences data summary

15,729 total transcript isoforms retained in initial cleaning and clustering of redundant isoforms.

13,882 sequences in a predicted open reading frame.

9,907 clusters identified based on translated proteins of predicted open reading frame.

9,813 full length transcripts were subset into a reference file as the longest representatives of one or more transcript clusters.

2.3.2 Short read sequences data summary

Total Transcript count of each TRINITY generated dataset was simply conducted using shell commands. Total transcript count for long read files are included in the BUSCO usage file below as headers required manipulation for input into BUSCO and totals were checked at this step.

Sample ID	Experimental factors	File name	Total transcripts/sequences assembled
G1	September Female	G1_KI_trinity	46538
G2	September Female	G2_KI_trinity	43283
G3	March/April Female	G3_KI_trinity	35991
G4	March/April Male	G4_KI_trinity	39239
G5	September Male	G5_KI_trinity	37379
G6	September Male	G6K_CDNJBANXX_trinity	214799
G7	March/April Female	G7K_CDNJBANXX_trinity	266539
G8	March/April Female	G8_KI_trinity	43050
All Samples combined	n/a	trinity-all	373287

2.4 BUSCO

Completeness of assemblies were assessed using Benchmarking Universal Single-Copy Orthologs BUSCO version 5.0 ([BUSCO](#)) and the dataset vertebrata_odb10.

Moving finalised assembly files into the BUSCO Directory:

TRINITY automatically names the final outputs “trinity.fasta” within folders which have the sample ID. Therefore for all these files to be moved into one directory for BUSCO to reference, these sequence IDs from the folders must be appended to the file names.

Conda Version 4.8.3 has been used for all analyses up to this point. Updated conda as version 4.8.4 or higher is recommended for BUSCO. This analysis was conducted on eRSA’s NECTAR research cloud

Working directory of full (single line) hq transcript Isoseq dataset, and trimmed, non-redundant file of transcripts identified as cluster representatives.

```
/mnt/IsoSeq-analysis/data/Seqtk/hq.fasta.no5merge.collapsed.rep.1L.fa  
/mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.clean.fasta
```

Working directory of assembled (using TRINITY) short read transcript files

```
/mnt/DE-analysis/2_alignedData/trinity
```

concatenated short reads all assembled using trinity into largest transcript file /mnt/DE-analysis/2_alignedData/trinity/trinity-all/trinity

```
/mnt/DE-analysis/2_alignedData/trinity/trinity-all/trinity
# Then under
# /trinity-sep/<sample_ID_trinity>/trinity

# Sample IDs folders:
#   G1_KI_trinity
#   G2_KI_trinity
#   G3_KI_trinity
#   G4_KI_trinity
#   G5_KI_trinity
#   G6K_CDNJBANXX_trinity
#   G7K_CDNJBANXX_trinity
#   G8_KI_trinity
```

This was done using the script [output-BUSCO_Name2020.sh](#) which also moved the long-read assemblies into the correct path:

```
#!/bin/bash
#
#
# This script is used to rename and copy trinity output files with their input file
↳ "filestem" into the /BUSCO directory so all files have similar naming
↳ convention for input into the BUSCO script.
#
# The paths in the script assume that a specific directory structure has been set
↳ up.
#
# Modules required: none
#
# usage:  bash Output-BUSCO_Name2020.sh
# location:          cd /scratch/user/mahe0050/BUSCO/

# Carmel Maher
# August 2020

#-----adjust these for your run-----

#-----

function error_exit
{
    # Exit function due to fatal error
    # Accepts 1 arg:
    # string - descriptive error message
```

```

    echo "${PROGNAME}: ${1:-"Unknown error"}" 1>&2
    exit 1
}

# go to the working directory - working from /clean
cd /scratch/user/mahe0050/DE-analysis/1_trimmedData || error_exit "$LINENO:
↳ Directory Error 1"

for file in *R1_clean.fq.gz
do

    FILESTEM=${file%_*}
    #this FILESTEM only cuts to _clean, the _R1 is included in stem
    FILESTEM=${FILESTEM/R1/}
    #removes R1 from FILESTEM (FILESTEM ends in _ therefore not needed in "text"
    ↳ names)

    cp -i /scratch/user/mahe0050/DE-analysis/2_alignedData/trinity-sep/
    ↳ $FILESTEM"trinity"/trinity/Trinity.fasta
    ↳ /scratch/user/mahe0050/BUSCO/$FILESTEM"t_assembled".fasta || error_exit
    ↳ "$LINENO: Error copying separate trinity outputs"

    # -> all files should end up in /scratch/user/mahe0050/BUSCO named
    ↳ G#_XX.t_assembled.fasta

done

echo "Trinity-sep done"

cp -i /scratch/user/mahe0050/DE-analysis/2_alignedData/trinity-all/trinity/
↳ Trinity.fasta /scratch/user/mahe0050/BUSCO/trinity-all_assembled.fasta ||
↳ error_exit "$LINENO: Error copying concatenated trinity-all output"

# -> one ANGEL file should end up in /scratch/user/mahe0050/BUSCO named
↳ trinity-all_assembled.fasta

echo "Trinity-all done"

cp -i /scratch/user/mahe0050/IsoSeq-analysis/data/Cogent/collected/
↳ hq.fasta.no5merge.collapsed.rep.fa
↳ /scratch/user/mahe0050/BUSCO/hq.fasta.no5merge.collapsed.rep_assembled.fasta
↳ || error_exit "$LINENO: Error copying Cogent output"

# -> one Cogent file should end up in /scratch/user/mahe0050/BUSCO named
↳ hq.fasta.no5merge.collapsed.rep_assembled.fasta

echo "Cogent done"

cp -i /scratch/user/mahe0050/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds
↳ /scratch/user/mahe0050/BUSCO/pygmy.ANGEL_assembled.fasta || error_exit
↳ "$LINENO: Error copying ANGEL output"

```

```

# -> one ANGEL file should end up in /scratch/user/mahe0050/BUSCO named
# -> pygmy.ANGEL_assembled.fasta

echo "ANGEL done"

# all file sizes checked after run = matches
# Trinity outputs removed from scratch 20/12/2020 to save space

```

Editing .fasta headers so that BUSCO can input the files

This [BUSCO_all_v5.0.sh](#) document contains notes and single lines run to run BUSCO 5.0 installed using the conda package on all short read and long read assembled transcript files. It outlines how to format the input files in preparation for BUSCO and the usage of the BUSCO script.

Sample format compatibility

```

cd /mnt/IsoSeq-analysis/BUSCO/

# NOTE the Isoseq files have symbols in fasta headers before transcript IDs which
# -> need to be removed for BUSCO

```

Header formats:

```

head /mnt/IsoSeq-analysis/data/Seqtk/hq.fasta.no5merge.collapsed.rep.1L.fa
>PB.4.2|004079|path1:6-612(+) |transcript/24311 transcript/24311
# -> full_length_coverage=2;length=640;num_subreads=60

head /mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.clean.fasta
>PB.11.2|004815|path2:1047-3035(+) |transcript/13401 transcript/13401
# -> full_length_coverage=2;length=1995;num_subreads=8

head /mnt/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds
>PB.11.2|004815|path2:1047-3035(+) |transcript/13401|m.5 type:dumb-complete len:311
# -> strand:+ pos:214-1146

```

Total number of sequences:

```

grep -c ">" /mnt/IsoSeq-analysis/data/Seqtk/hq.fasta.no5merge.collapsed.rep.1L.fa
15729
grep -c ">" /mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.clean.fasta
9813
grep -c ">" /mnt/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds
13882

```

after sed to remove all “/” resulted in an error at metaeuk headers “ValueError: could not convert string to float: ‘+’”, after sed to remove all “+” same error.

Goal is a %score of alignments to BUSCOs and which transcripts match is no explored further. Therefore all headers were grossly simplified. The files created here are ONLY used for BUSCO.

```
cut -d '|' -f1
→ /mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.clean.fasta >
→ reference_transcripts.1Lv.clean_.fasta

cut -d '|' -f1 /mnt/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds > pygmy.ANGEL_.cds

cut -d '|' -f1
→ /mnt/IsoSeq-analysis/data/Seqtk/hq.fasta.no5merge.collapsed.rep.1L.fa >
→ hq.fasta.no5merge.collapsed.rep.1L_.fa
```

Total number of sequences retained as counted above

```
grep -c ">" reference_transcripts.1Lv.clean_.fasta
9813
grep -c ">" pygmy.ANGEL_.cds
13882
grep -c ">" hq.fasta.no5merge.collapsed.rep.1L_.fa
15729
```

Two of these files will have “duplicate” headers in this simplified format. Numbers were simply appended as below so that all values are unique. These files are for use in BUSCO *only*:

```
awk '/^>/{ $0=$0"_(++i)}1' pygmy.ANGEL_.cds > pygmy.ANGEL__.cds
awk '/^>/{ $0=$0"_(++i)}1' hq.fasta.no5merge.collapsed.rep.1L_.fa >
→ hq.fasta.no5merge.collapsed.rep.1L__.fa

grep -c ">" pygmy.ANGEL__.cds
13882
grep -c ">" hq.fasta.no5merge.collapsed.rep.1L__.fa
15729
```

BUSCO Installation & usage:

The script [run-conda-BUSCO.bash](#) was used to run the program BUSCO on all samples on the NECTAR machine.

```
cd /mnt/IsoSeq-analysis/BUSCO

#Create a conda environment for the BUSCO Install

conda create -n conda-BUSCO -c bioconda -c conda-forge busco=5.0.0
conda activate conda-BUSCO
```

The basic repeated usage of BUSCO for all samples listed below will be:

```
busco -i [SEQUENCE_FILE] -l [LINEAGE] -o [OUTPUT_NAME] -m transcriptome

# note: E-value cut-off for BLAST searches Default: 1e-03

# busco --list-datasets
```

```

#check $LINEAGE in script file
# - vertebrata_odb10
# may also --auto-lineage-euk

# script currently set to threads (-c) = 12

```

Run BUSCO -> usage

```

# location of usage doesnt matter as paths are specified within script.
# use from within /mnt/IsoSeq-analysis/BUSCO/ in case of log files.
# Usage:
    conda activate conda-BUSCO
    screen
    bash run-conda-BUSCO.bash 2>&1 | tee BUSCO-out.txt

```

run-conda-BUSCO.bash:

```

#!/bin/bash
#
#
# This script is used to assess transcriptome outputs against BUSCO lineages
# The paths in the script assume that a specific directory structure has been set
→ up.
# Analysis run on eRSA NECTAR machine
#
#Usage:
#          conda activate conda-BUSCO
#          screen
#          bash run-conda-BUSCO.bash 2>&1 | tee BUSCO-out.txt

# Carmel Maher
# February 2021

# See notes file associated with usage: BUSCO_all_v5.0.sh
# Inputs include:
#   Short-read Trinity outputs for all 8 Kidney samples assembled from short
→ reads
#   Short-read Trinity output for one file of all 8 kidney sequencing
→ concatenated and assembled into a single transcript file
#   Isoseq3 long-read de novo assembly full list of hq transcripts
#   Isoseq3 long-read de novo assembly list of non-redundant representative
→ transcripts of peptide clusters, with additional poly-a tail trimming

#-----adjust these for your run-----

LINEAGE="vertebrata_odb10"

#-----

function error_exit

```

```

{
    echo "${PROGNAME}: ${1:-"Unknown error"}" 1>&2
    exit 1
}

# (commented out because it failed after "done" - rerun with # )

#go to the directory containing trimmed files to pull ID names
cd /mnt/DE-analysis/1_trimmedData

for file in *R1_clean.fq.gz
do
    FILESTEM=${file%_*}
    #this FILESTEM only cuts to _clean, the _R1 is included in stem
    FILESTEM=${FILESTEM/R1/}
    #removes R1 from FILESTEM (FILESTEM ends in _ therefore not needed in "text"
    ↪ names)

    mkdir /mnt/IsoSeq-analysis/BUSCO/${FILESTEM}BUSCOout || error_exit "$LINENO:
    ↪ Error creating trinity-sep output directory at $FILESTEM"

    cd /mnt/IsoSeq-analysis/BUSCO/

    busco -i
    ↪ /mnt/DE-analysis/2_alignedData/trinity/trinity-sep/${FILESTEM}trinity"/
    ↪ trinity/Trinity.fasta -l $LINEAGE -f -c 12 -o ${FILESTEM}BUSCOout -m
    ↪ transcriptome || error_exit "$LINENO: Error running BUSCO at $FILESTEM"

    cd /mnt/DE-analysis/1_trimmedData
done

#

cd /mnt/IsoSeq-analysis/BUSCO/

mkdir ./trinity-all_BUSCOout || error_exit "$LINENO: directory error at
↪ trinity-all"

busco -i /mnt/DE-analysis/2_alignedData/trinity/trinity-all/trinity/Trinity.fasta
↪ -l $LINEAGE -f -c 12 -o trinity-all_BUSCOout -m transcriptome || error_exit
↪ "$LINENO: Error running BUSCO at trinity-all"

#

cd /mnt/IsoSeq-analysis/BUSCO/

mkdir ./reference-transcripts_BUSCOout || error_exit "$LINENO: directory error at
↪ reference-transcripts"

busco -i reference_transcripts.1Lv.clean_.fasta -l $LINEAGE -f -c 12 -o
↪ reference-transcripts_BUSCOout -m transcriptome || error_exit "$LINENO: Error
↪ running BUSCO at reference-transcripts"

```

```

#

mkdir ./ANGEL.cds_BUSCOout || error_exit "$LINENO: directory error at
↳ reference-transcripts"

busco -i pygmy.ANGEL__.cds -l $LINEAGE -f -c 12 -o ANGEL.cds_BUSCOout -m
↳ transcriptome || error_exit "$LINENO: Error running BUSCO at ANGEL.cds"

#

mkdir ./hq-fasta_BUSCOout || error_exit "$LINENO: directory error at hq-fasta"

busco -i hq.fasta.no5merge.collapsed.rep.1L__.fa -l $LINEAGE -f -c 12 -o
↳ hq-fasta_BUSCOout -m transcriptome || error_exit "$LINENO: Error running BUSCO
↳ at hq-fasta"

#

echo "all BUSCOs complete"

```

Visualisation

BUSCO outputs a file named short_summary.specific.vertebrata_odb10.<-o>.txt. These are used to plot a summary figure of all samples.

generate plot.py and r script of all sample BUSCO summaries

```

mkdir BUSCO_summaries

cp G1_KI_BUSCOout/short_summary.*_odb10.G1_KI_BUSCOout.txt BUSCO_summaries/.
cp G2_KI_BUSCOout/short_summary.*_odb10.G2_KI_BUSCOout.txt BUSCO_summaries/.
cp G3_KI_BUSCOout/short_summary.*_odb10.G3_KI_BUSCOout.txt BUSCO_summaries/.
cp G4_KI_BUSCOout/short_summary.*_odb10.G4_KI_BUSCOout.txt BUSCO_summaries/.
cp G5_KI_BUSCOout/short_summary.*_odb10.G5_KI_BUSCOout.txt BUSCO_summaries/.
cp G6K_CDNJBANXX_BUSCOout/short_summary.*_odb10.G6K_CDNJBANXX_BUSCOout.txt
↳ BUSCO_summaries/.
cp G7K_CDNJBANXX_BUSCOout/short_summary.*_odb10.G7K_CDNJBANXX_BUSCOout.txt
↳ BUSCO_summaries/.
cp G8_KI_BUSCOout/short_summary.*_odb10.G8_KI_BUSCOout.txt BUSCO_summaries/.
cp reference-transcripts_BUSCOout/
↳ short_summary.*_odb10.reference-transcripts_BUSCOout.txt BUSCO_summaries/.
cp ANGEL.cds_BUSCOout/short_summary.*_odb10.ANGEL.cds_BUSCOout.txt
↳ BUSCO_summaries/.
cp hq-fasta_BUSCOout/short_summary.*_odb10.hq-fasta_BUSCOout.txt BUSCO_summaries/.
cp trinity-all_BUSCOout/short_summary.*_odb10.trinity-all_BUSCOout.txt
↳ BUSCO_summaries/.

#(The path below is where the config files for conda-BUSCO environment are located)

python3 /mnt/Prog/miniconda3/envs/conda-BUSCO/bin/generate_plot.py -wd
↳ /mnt/IsoSeq-analysis/BUSCO/BUSCO_summaries

```

Short Read summaries:


```

mkdir BUSCO_SR_summaries

cp G1_KI_BUSCOout/short_summary.*_odb10.G1_KI_BUSCOout.txt BUSCO_SR_summaries/.
cp G2_KI_BUSCOout/short_summary.*_odb10.G2_KI_BUSCOout.txt BUSCO_SR_summaries/.
cp G3_KI_BUSCOout/short_summary.*_odb10.G3_KI_BUSCOout.txt BUSCO_SR_summaries/.
cp G4_KI_BUSCOout/short_summary.*_odb10.G4_KI_BUSCOout.txt BUSCO_SR_summaries/.
cp G5_KI_BUSCOout/short_summary.*_odb10.G5_KI_BUSCOout.txt BUSCO_SR_summaries/.
cp G6K_CDNJBANXX_BUSCOout/short_summary.*_odb10.G6K_CDNJBANXX_SR_BUSCOout.txt
↪ BUSCO_summaries/.
cp G7K_CDNJBANXX_BUSCOout/short_summary.*_odb10.G7K_CDNJBANXX_SR_BUSCOout.txt
↪ BUSCO_summaries/.
cp G8_KI_BUSCOout/short_summary.*_odb10.G8_KI_BUSCOout.txt BUSCO_SR_summaries/.
cp trinity-all_BUSCOout/short_summary.*_odb10.trinity-all_BUSCOout.txt
↪ BUSCO_SR_summaries/.

python3 /mnt/Prog/miniconda3/envs/conda-BUSCO/bin/generate_plot.py -wd
↪ /mnt/IsoSeq-analysis/BUSCO/BUSCO_SR_summaries

```

Long Read summaries:

```

mkdir BUSCO_LR_summaries

cp reference-transcripts_BUSCOout/
↪ short_summary.*_odb10.reference-transcripts_BUSCOout.txt BUSCO_LR_summaries/.
cp ANGEL.cds_BUSCOout/short_summary.*_odb10.ANGEL.cds_BUSCOout.txt
↪ BUSCO_LR_summaries/.
cp hq-fasta_BUSCOout/short_summary.*_odb10.hq-fasta_BUSCOout.txt
↪ BUSCO_LR_summaries/.

python3 /mnt/Prog/miniconda3/envs/conda-BUSCO/bin/generate_plot.py -wd
↪ /mnt/IsoSeq-analysis/BUSCO/BUSCO_LR_summaries

```

These files for each sample were also moved into an R working directory to allow the generated figure to be edited.

The [R script](#) produced by the python command above was edited to allow for local directory structure and streamline the labelling and order of sample names.

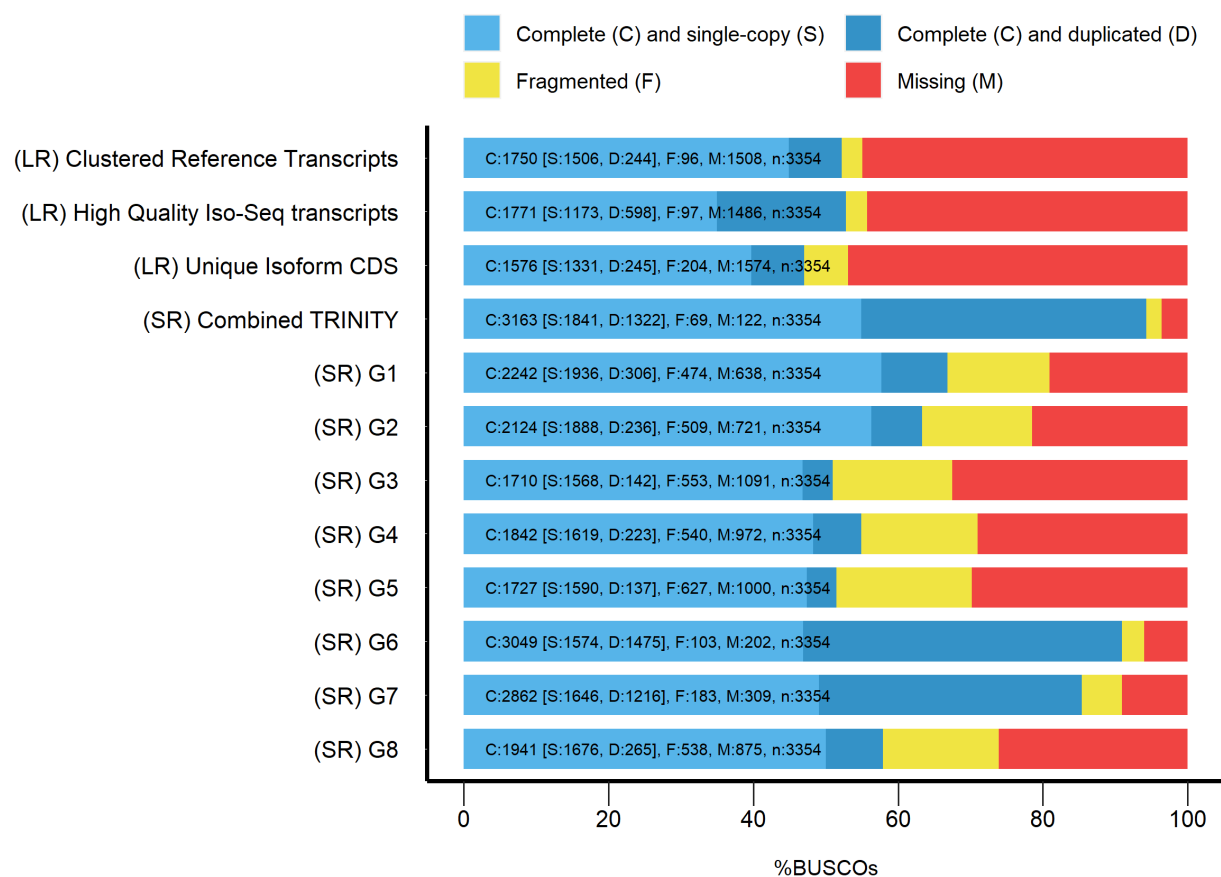


Figure 3: Busco Summary Statistics (BUSCO v5.0.0 run on database vertebrata_odb10. LR indicates transcript databases at different assembly steps, assembled from long read sequencing data. SR indicates short read sequencing sample data assembled with TRINITY)

3 Annotation (Chapter 5)

Completed on the long read data from Chapter 4

- `pygmy.ANGEL.cds`
- `pygmy.ANGEL.pep`
- `pygmy.ANGEL.utr`

3.1 BLASTx

BLASTx searches were conducted on the dataset at this stage to include isoforms that are collapsed later on.

BLASTx was run on the `pygmy.ANGEL.cds` file as it contains the predicted open reading frame sequence of nucleotides for translatable proteins. This was completed on the NECTAR machine.

```
# Package: blast 2.9.0, build Mar 11 2019 15:20:05
# Uniprot sprot & trembl databases downloaded: 28/5/19      (note: trembl database
↳ not indexed or used)
# Anolis .pep fasta files downloaded 12/11/19
  Anolis_carolinensis.AnoCar2.0.pep.all.fa
  Anolis_carolinensis.AnoCar2.0.pep.abinitio.fa

# To format a database: #
# makeblastdb -in mydb.fsa -dbtype nucl -parse_seqids
```

UniProt SwissProt database:

```
/mnt/Prog/blast/ncbi-blast-2.9.0+/bin/makeblastdb -in uniprot_sprot.fasta
↳ -parse_seqids -blastdb_version 5 -title "sprot" -dbtype prot -out sprot
```

Anolis carolinensis proteins database:

```
#AnoCar2.0.pep.all
/mnt/Prog/blast/ncbi-blast-2.9.0+/bin/makeblastdb -in
↳ Anolis_carolinensis.AnoCar2.0.pep.all.fa -parse_seqids -blastdb_version 5 -title
↳ "sprot" -dbtype prot -out AnoCar2.0.pep.all
```

Anolis Carolinensis database *ab initio*:

```
#AnoCar2.0.pep.abinitio
/mnt/Prog/blast/ncbi-blast-2.9.0+/bin/makeblastdb -in
↳ Anolis_carolinensis.AnoCar2.0.pep.abinitio.fa -parse_seqids -blastdb_version 5
↳ -title "sprot" -dbtype prot -out AnoCar2.0.pep.abinitio
```

Various searches were run in a screen to examine the effects of parameters and databases. Final searches are as listed below.

Searches included all databases listed above and variations of the parameters: `-max_target_seqs 5` or `-max_target_seqs 1` `-max_hsps 5` or `-max_hsps 1` `-evalue 0.00001` or `-evalue 1e-10`

The NCBI Blast results most often referenced in the thesis are final parameters `blastx -fmt6 -maxtarget1 -maxhsps1 -eval0.00001` against the UniProt SwissProt database.

```
# uniprot sprot
/mnt/Prog/blast/ncbi-blast-2.9.0+/bin/blastx -db
↪ /mnt/Prog/blast/blastdb/sprot/sprot -query
↪ /mnt/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds -out
↪ pygmy.ANGEL_blastx_sprot-maxtarg1-maxhsp1.out -outfmt 6 -max_target_seqs 1
↪ -max_hsps 1 -evaluate 0.00001 -num_threads 3
```

```
#AnoCar2.0.pep.all
/mnt/Prog/blast/ncbi-blast-2.9.0+/bin/blastx -db
↪ /mnt/Prog/blast/blastdb/AcarProt/AnoCar2.0.pep.all/AnoCar2.0.pep.all -query
↪ /mnt/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds -out
↪ pygmy.ANGEL_blastx_AnoCar.pep.all-maxtarg1-maxhsp1.out -outfmt 6
↪ -max_target_seqs 1 -max_hsps 1 -evaluate 0.00001 -num_threads 3
```

All searches except the below were output in tab separated -outfmt 6. Due to import requirements an -outfmt 5 an .xml file was required for results to be visualised in the program BLAST2GO (-max_target_seqs 5 -max_hsps 5 -evaluate 0.00001).

```
/mnt/Prog/blast/ncbi-blast-2.9.0+/bin/blastx -db
↪ /mnt/Prog/blast/blastdb/AcarProt/AnoCar2.0.pep.all/AnoCar2.0.pep.all -query
↪ /mnt/IsoSeq-analysis/data/ANGEL/pygmy.ANGEL.cds -out
↪ pygmy.ANGEL_blastx_AnoCar.pep.all-BLAST2GO -outfmt 5 -max_target_seqs 5
↪ -max_hsps 5 -evaluate 0.00001 -num_threads 4
```

The above and other trial searches are all listed in the file [IsoSeq analysis post-ANGEL blast notes.sh](#)

3.2 Gene Name IDs assigned to Transcript ID

The ANGEL predicted open reading frame transcripts (Section 2.1.3) were searched against the UniProt swiss prot database using BLASTx with parameters: `-max_target_seqs 1 -max_hsps 1 -evalue 0.00001`) (Section 3.1 above).

The BLASTx results are uploaded here (**).

Protein IDs from the BLASTx results were uploaded to the Uni Prot [Retrieve/ID mapping tool](#) and mapped to Gene names. This produced a list (**) of unique UniProt swiss prot identifiers and their corresponding gene name ID.

3.2.1 Full transcript putative gene reference file

Using dplyr the BLAST results and these gene ID mappings were joined and filtered to create a reference file of transcript IDs assigned both a Uni Prot Swiss prot database protein ID, and a gene name.

The BLASTx results were imported:

```
setwd("~/@Uni/@Flinders University
↳ PhD/RWorkingDir/Workflow-readthedown/ReferenceClusters")

Unisprot.BLASTx.out <- read.csv("pygmy.ANGEL_blastx_sprot-maxtarg1-maxhsp1.out",
  sep = "\t", header = FALSE)
head(Unisprot.BLASTx.out)
```

```
##                               V1      V2      V3  V4  V5
## 1    PB.2.1|002537|path0:1-1624(+)|transcript/18304|m.1 Q6PBC3 95.000 100  5
## 2    PB.3.2|00361c|path0:43-2240(+)|transcript/10564|m.2 P55209 88.995 209 22
## 3    PB.11.1|004815|path2:1-3039(+)|transcript/3426|m.4 P36633 61.892 719 258
## 4 PB.11.2|004815|path2:1047-3035(+)|transcript/13401|m.5 Q8JZQ5 67.010 194  63
## 5    PB.12.1|004815|path5:1-1665(+)|transcript/17167|m.6 Q8JZQ5 61.712 222  80
## 6    PB.13.1|004c10|path2:1-2811(+)|transcript/4993|m.7 Q9H063 83.984 256  38
##  V6  V7  V8  V9 V10      V11 V12
## 1  0   1  300   1 100  2.49e-69 207
## 2  1   1  627 184 391  5.86e-61 198
## 3  8  46 2175  32 743  0.00e+00 855
## 4  1 196  777 513 705  3.11e-92 246
## 5  3   1  666 532 748  3.96e-77 249
## 6  1   1  759   1 256  4.61e-156 437
```

Column Headers Renamed to match BLAST output format 6:

```
Unisprot.BLASTx.out <- Unisprot.BLASTx.out %>%
  rename(TranscriptID = V1, UniprotID = V2, pident = V3, length = V4,
    mismatch = V5, gapopen = V6, qstart = V7, qend = V8,
    sstart = V9, send = V10, evalue = V11, bitscore = V12)
colnames(Unisprot.BLASTx.out)
```

```
## [1] "TranscriptID" "UniprotID"    "pident"       "length"       "mismatch"
## [6] "gapopen"      "qstart"       "qend"         "sstart"       "send"
## [11] "evalue"       "bitscore"
```

```
head(Unisprot.BLASTx.out)
```

```
##                                TranscriptID UniprotID pident
## 1    PB.2.1|002537|path0:1-1624(+)|transcript/18304|m.1    Q6PBC3 95.000
## 2    PB.3.2|00361c|path0:43-2240(+)|transcript/10564|m.2    P55209 88.995
## 3    PB.11.1|004815|path2:1-3039(+)|transcript/3426|m.4     P36633 61.892
## 4    PB.11.2|004815|path2:1047-3035(+)|transcript/13401|m.5  Q8JZQ5 67.010
## 5    PB.12.1|004815|path5:1-1665(+)|transcript/17167|m.6    Q8JZQ5 61.712
## 6    PB.13.1|004c10|path2:1-2811(+)|transcript/4993|m.7    Q9H063 83.984
##  length mismatch gapopen qstart qend sstart send    evalue bitscore
## 1    100         5        0      1  300      1  100  2.49e-69    207
## 2    209        22        1      1  627     184 391  5.86e-61    198
## 3    719       258        8     46 2175     32 743  0.00e+00    855
## 4    194        63        1    196  777     513 705  3.11e-92    246
## 5    222        80        3      1  666     532 748  3.96e-77    249
## 6    256        38        1      1  759      1 256  4.61e-156    437
```

Gene ID mapping list for Uniprot IDs were imported:

```
BLASTx.to.gene <- read.csv("~/@Uni/@Flinders University
↪ Phd/RWorkingDir/Workflow-readthedown/ReferenceClusters/pygmy.ANGEL_blastx_sprot-maxtarg1-maxhsp1==G
↪ Name IDs.txt",
  sep = "\t", header = TRUE)

head(BLASTx.to.gene)
```

```
##      From      To
## 1 Q6PBC3 chchd4
## 2 P55209 NAP1L1
## 3 P36633 Aoc1
## 4 Q8JZQ5 Aoc1
## 5 Q9H063 MAF1
## 6 A1XBS5 FAM92A
```

Column Headers were renamed:

```
BLASTx.to.gene <- BLASTx.to.gene %>%
  rename(UniprotID = From, Gene = To)

colnames(BLASTx.to.gene)
```

```
## [1] "UniprotID" "Gene"
```

```
head(BLASTx.to.gene)
```

```
##  UniprotID  Gene
## 1   Q6PBC3 chchd4
## 2   P55209 NAP1L1
## 3   P36633 Aoc1
## 4   Q8JZQ5 Aoc1
## 5   Q9H063 MAF1
## 6   A1XBS5 FAM92A
```

```
BLASTx.gene.join <- left_join(Unisprot.BLASTx.out, BLASTx.to.gene,
  by = "UniprotID", copy = FALSE)
head(BLASTx.gene.join)
```

```
##                                TranscriptID UniprotID pident
## 1    PB.2.1|002537|path0:1-1624(+)|transcript/18304|m.1    Q6PBC3 95.000
## 2    PB.3.2|00361c|path0:43-2240(+)|transcript/10564|m.2    P55209 88.995
## 3    PB.11.1|004815|path2:1-3039(+)|transcript/3426|m.4     P36633 61.892
## 4 PB.11.2|004815|path2:1047-3035(+)|transcript/13401|m.5    Q8JZQ5 67.010
## 5    PB.12.1|004815|path5:1-1665(+)|transcript/17167|m.6    Q8JZQ5 61.712
## 6    PB.13.1|004c10|path2:1-2811(+)|transcript/4993|m.7     Q9H063 83.984
##  length mismatch gapopen qstart qend sstart send    evalue bitscore  Gene
## 1    100         5        0        1  300        1  100  2.49e-69    207  chchd4
## 2    209        22        1        1  627       184  391  5.86e-61    198  NAP1L1
## 3    719       258        8        46 2175        32  743  0.00e+00    855  Aoc1
## 4    194        63        1       196  777       513  705  3.11e-92    246  Aoc1
## 5    222        80        3        1  666       532  748  3.96e-77    249  Aoc1
## 6    256        38        1        1  759        1  256  4.61e-156   437  MAF1
```

```
BLASTx.gene.join <- relocate(BLASTx.gene.join, Gene, .before = pident)
head(BLASTx.gene.join)
```

```
##                                TranscriptID UniprotID  Gene
## 1    PB.2.1|002537|path0:1-1624(+)|transcript/18304|m.1    Q6PBC3  chchd4
## 2    PB.3.2|00361c|path0:43-2240(+)|transcript/10564|m.2    P55209  NAP1L1
## 3    PB.11.1|004815|path2:1-3039(+)|transcript/3426|m.4     P36633  Aoc1
## 4 PB.11.2|004815|path2:1047-3035(+)|transcript/13401|m.5    Q8JZQ5  Aoc1
## 5    PB.12.1|004815|path5:1-1665(+)|transcript/17167|m.6    Q8JZQ5  Aoc1
## 6    PB.13.1|004c10|path2:1-2811(+)|transcript/4993|m.7     Q9H063  MAF1
##  pident length mismatch gapopen qstart qend sstart send    evalue bitscore
## 1 95.000    100         5        0        1  300        1  100  2.49e-69    207
## 2 88.995    209        22        1        1  627       184  391  5.86e-61    198
## 3 61.892    719       258        8        46 2175        32  743  0.00e+00    855
## 4 67.010    194        63        1       196  777       513  705  3.11e-92    246
## 5 61.712    222        80        3        1  666       532  748  3.96e-77    249
## 6 83.984    256        38        1        1  759        1  256  4.61e-156   437
```

Write the output to .csv

```
write.csv(BLASTx.gene.join, "~/@Uni/@Flinders University
  ↳ PhD/RWorkingDir/Workflow-readthedown/ReferenceClusters/BLASTx.gene.join.csv",
  quote = FALSE, row.names = FALSE, col.names = TRUE)
```

3.2.2 Putative genes for clustered transcripts used as the gene expression analysis reference

Import the list of unique clustered transcripts that were used as the reference for gene expression analysis

```
# clstrUniq <- read.csv("~/@Uni/@Flinders University
# PhD/RWorkingDir/Workflow-readthedown/ReferenceClusters/clstrUniq.txt",
# sep = '\t', header = TRUE) head(clstrUniq.txt)
```

Filter the BLASTx results with gene name based on the transcript references used for the expression analysis

How many transcripts were used as a reference for gene expression?

How many of these returned a BLASTx result?

3.2.3 Presence of identified “genes of interest” in the *T. adelaidensis* transcript set

Note: summary counts of genes IDs assigned to transcripts was initially conducted in excel (Appendix)

Import the reference “genes of interest” list built from an NCBI database search (Chapter 5)

Filter the reference transcript

(***) note the below has already been done in excel. Its not ideal but such a waste of time to do again

Counts of how many transcripts from the BLASTx search match a gene of interest. Also, how many from the clustered file match genes of interest counts.

3.3 BLAST 2 GO

BLAST2GO (Within [OmicsBox – Bioinformatics Made Easy](#), [BioBam Bioinformatics](#), March 3, 2019) was used to perform a gene ontology analysis of the above BLASTx results.

Götz S., Garcia-Gomez JM., Terol J., Williams TD., Nagaraj SH., Nueda MJ., Robles M., Talon M., Dopazo J. and Conesa A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic acids research*, 36(10), 3420-35.

For import into the program a BLASTx search with the output set to .xml was performed using the fasta file containing the predicted open reading frame compared to the *Anolis carolinensis* proteins database (-max_target_seqs 5 -max_hsps 5 -evalue 0.00001).

Comparison to GO databases and visualisation was all completed within a Windows x64 v1.2.4 build of the OmicsBox program and figures were exported as below.

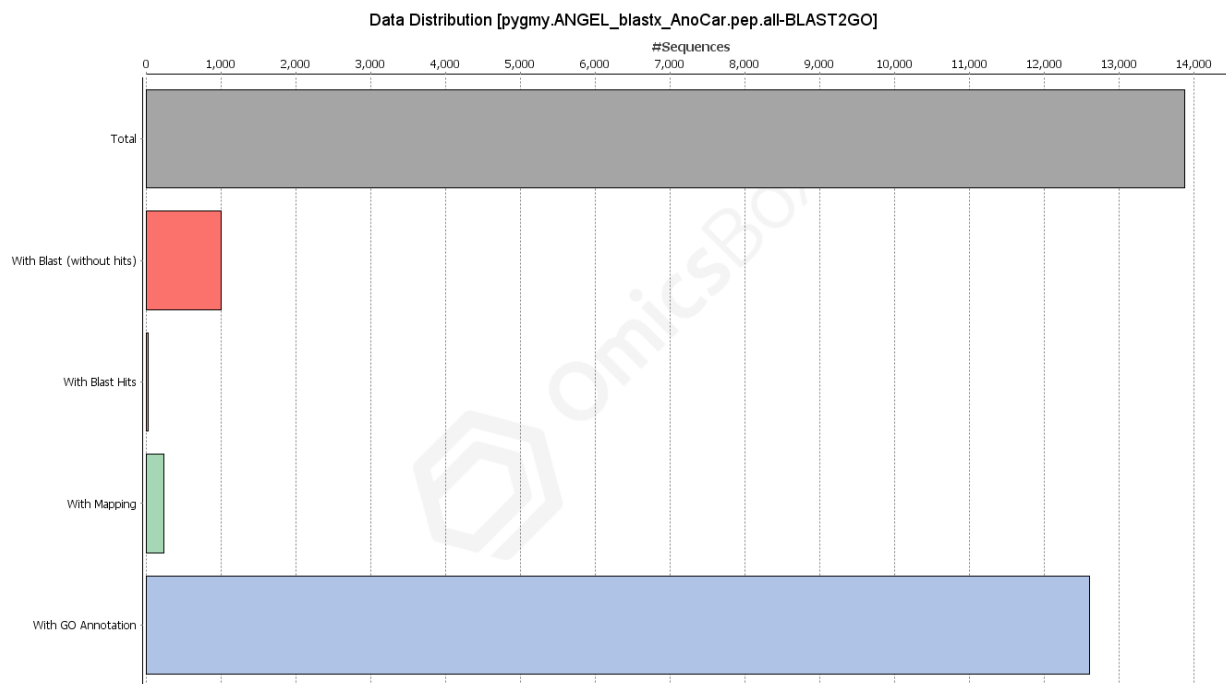


Figure 4: Summary data distribution. pygmy.ANGEL contains 13,882 unique sequences representing predicted open reading frame of transcripts: 12,602 of these sequences produced >1 BLASTx hit with Mapping and GO Annotation, 243 produced a BLASTx hit and mapping only, 32 produced a BLAST hit only, and 1005 did not produce BLASTx hits

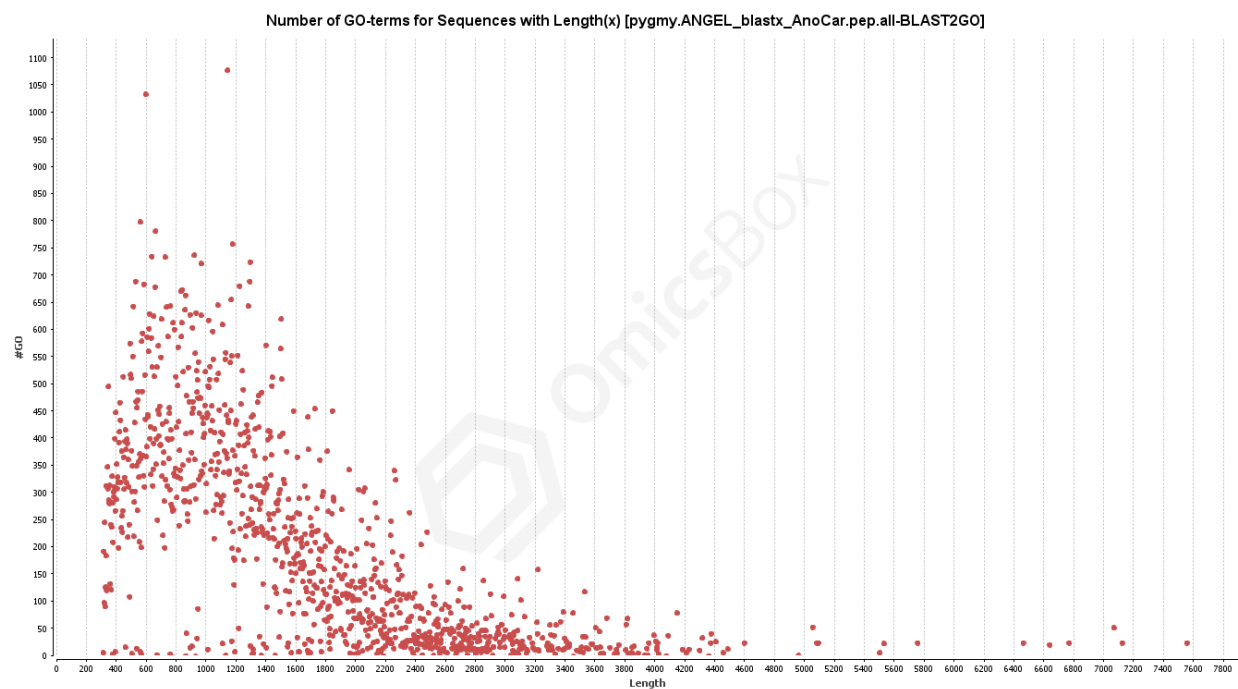


Figure 5: Number of GO-terms identified for sequences with length (x). Length is based on nucleotide sequence input into the BLASTx search

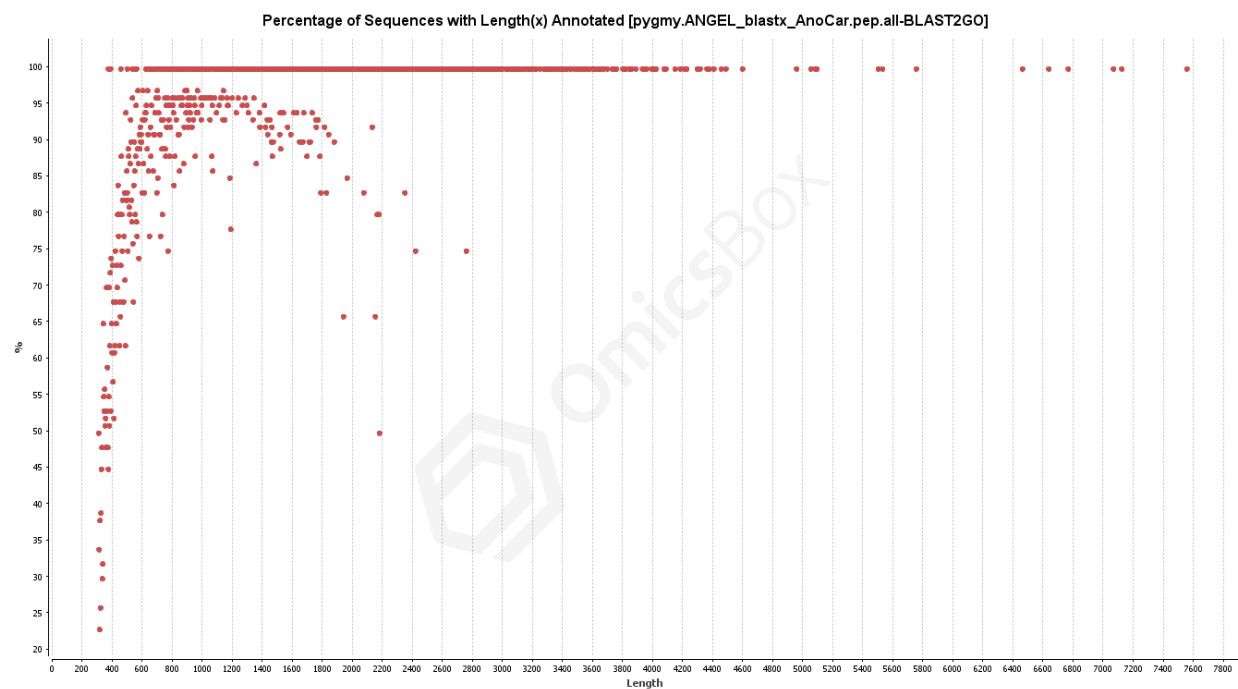


Figure 6: Percentage of sequences with length (x) that were annotated

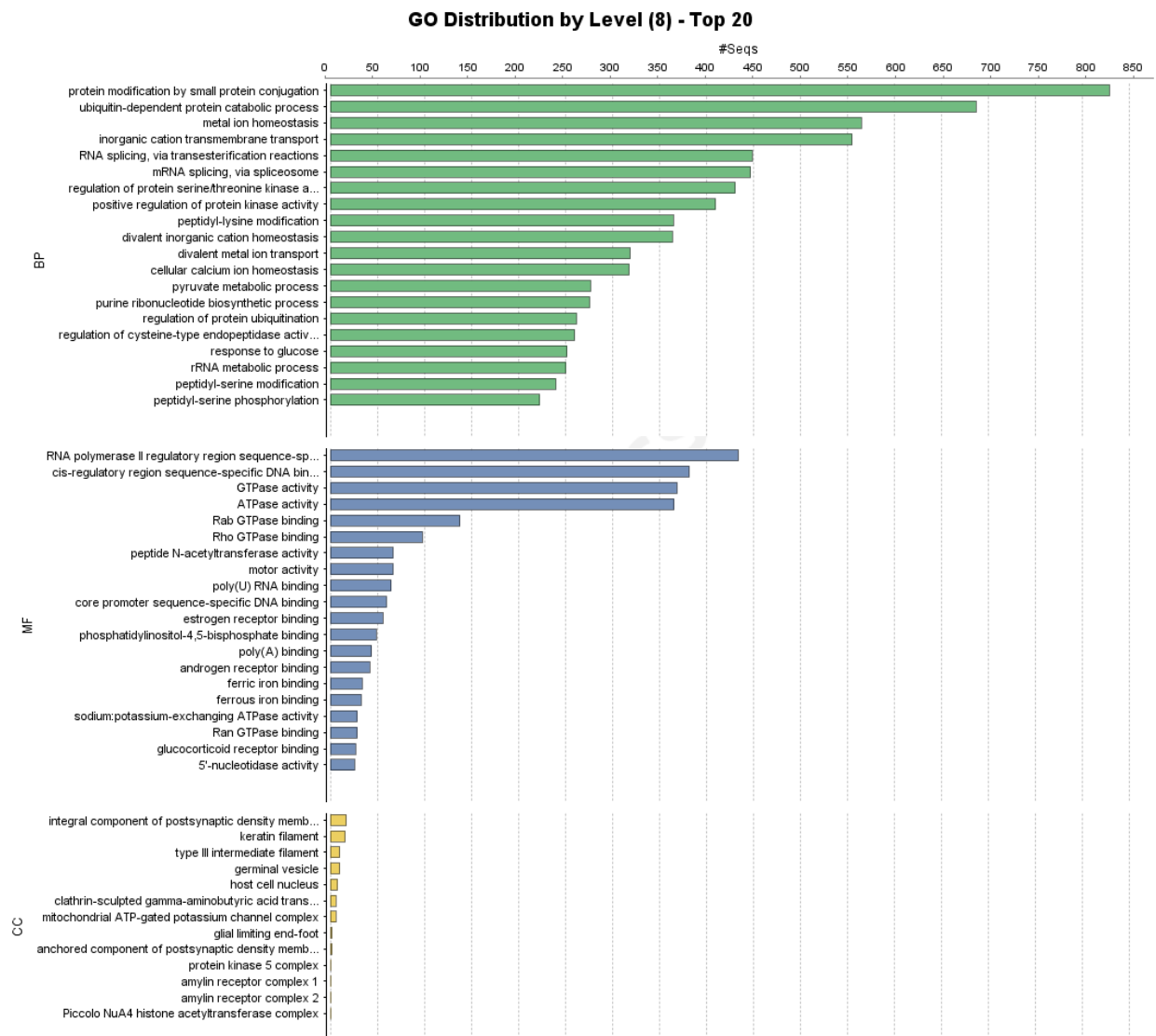


Figure 7: Top 20 annotation results (at level 8) for each category BP - Biological Process, MF - Molecular Function, and CC - Cellular Component

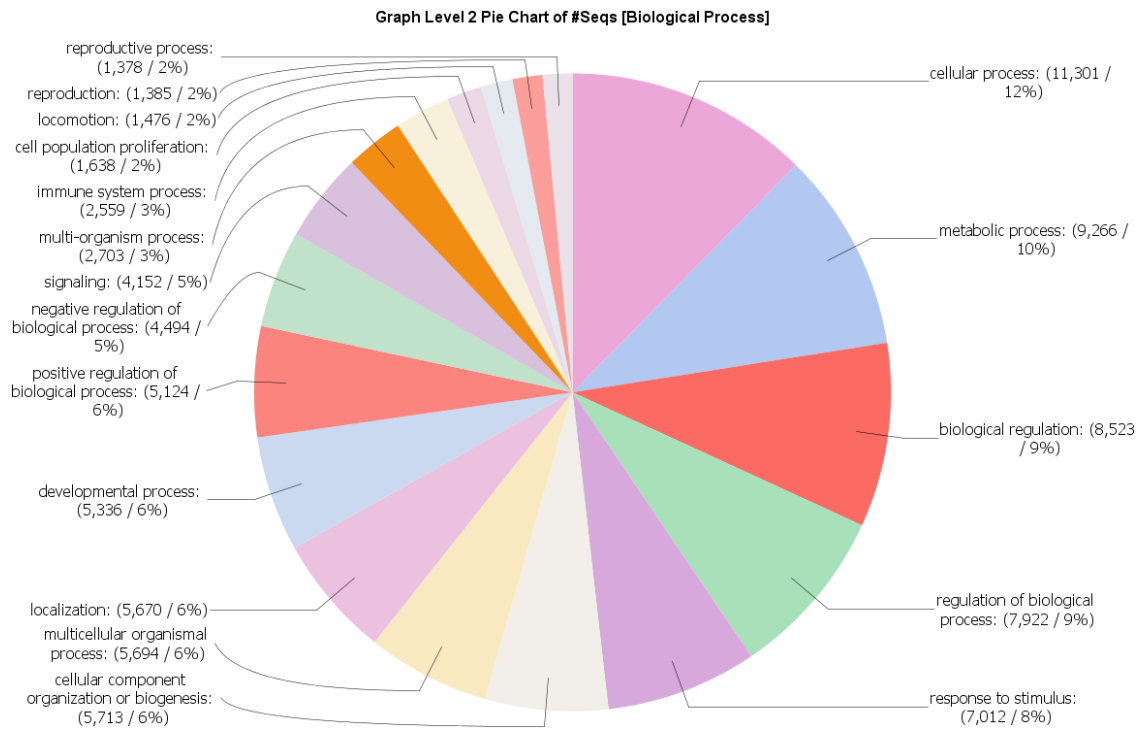


Figure 8: Pie chart of proportion of sequences annotated to Level 2 of the Biological Process category

3.4 Summary

15,729 Total transcript isoforms retained in initial cleaning and clustering of redundant isoforms.

13,882 sequences in a predicted open reading frame.

9,907 clusters identified based on translated proteins of predicted open reading frame.

9,813 full length transcripts have been subset into a reference file as the longest representatives of one or more transcript clusters.

12,602 of the 13,882 sequences in a predicted open reading frame were successfully annotated to a BLASTx result, protein ID, and gene ontology category.

4 Gene expression analysis (Chapter 6)

4.0.0.0.1 Long reads (reference index) - Previous analysis:

- Isoseq3 pipe
- Collapsing of isoforms & generating fasta of longest transcripts in predicted ORFs (Cogent/ANGEL)
- BLASTx search of coding sequence file for the longest transcripts in predicted ORFs
- Grouping of transcript clusters/putative gene families (CD-HIT)
- Longest representative transcript collapsed into a reference .fasta (R & Shell scripts)
- As this longest transcript was taken from earlier in the pipeline, persisting poly-a tails were trimmed from the reference .fasta file (Perl script)

4.0.0.0.2 Short reads - Previous analysis:

- FASTQC quality assessment
- BBduk trimming
- FASTQC quality assessment (note: TRINITY assembly not used for counts)

4.0.0.0.3 Both datasets are combined in this analysis:

- Transcript counts per million estimated using Kallisto
 - Clean short reads (above) counted against reference index generated from long read .fasta (above)
- Kallisto output imported into R for analysis using EdgeR

4.1 Kallisto

Short reads have already been trimmed using BBDuk as per Chapter 4. These cleaned sequences were imported directly into Kallisto.

As TRINITY was run on the Flinders' HPC Deep Thought that is where trimming was completed, Kallisto however was run on the eRSA NECTAR cloud machine and files were moved accordingly, keeping directories relative locations intact.

The file `reference_transcripts.1Lv.clean.fasta` created in the subsetting section of Chapter 4's methods was used as the index.

All of this was achieved using the following [kallisto-clstr-clean.sh](#) script summarised below.

create the index the below contains the representative transcript for clustered isoforms with (most) of the poly-a tails trimmed:

```
kallisto index -i reference_transcripts.1Lv.clean.idx
↪ /mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.clean.fasta
```

Output:

```
# [build] loading fasta file
↪ /mnt/IsoSeq-analysis/data/Seqtk/reference_transcripts.1Lv.clean.fasta
# [build] k-mer length: 31
# [build] counting k-mers ... done.
# [build] building target de Bruijn graph ... done
# [build] creating equivalence classes ... done
# [build] target de Bruijn graph has 59186 contigs and contains 17770125 k-mers
```

Run kallisto:

```
KallistoDIR=/mnt/DE-analysis/2_alignedData/kallisto-clstr-clean
ReadDIR=/mnt/DE-analysis/1_trimmedData #contains trimmed R1, R2, and singletons

# need to be in the input directory for for file
cd $ReadDIR

for file in *R1_clean.fq.gz
do
  FILESTEM=${file%_*}
  #this FILESTEM only cuts to _clean, the _R1 is included
  FILESTEM=${FILESTEM/R1/}
  #removes R1 from FILESTEM (FILESTEM ends in _ therefore not needed in "text"
  ↪ names)

  echo $FILESTEM

  kallisto quant -i $KallistoDIR/reference_transcripts.1Lv.clean.idx -o
  ↪ $KallistoDIR/$FILESTEM"kallisto-out" -b, --bootstrap-samples=100 --threads=8
  ↪ --pseudobam $file $FILESTEM"R2_clean.fq.gz" || error_exit "$LINENO: kallisto
  ↪ error at $FILESTEM"
```

The full kallisto terminal output is here [kallisto-clstr-clean-log.txt](#).

4.2 EdgeR Expression Analysis

Preliminary data exploration for these data has been included twice for different groupings. The first analysis below includes all original eight samples.

Samples G6 and G7 have ultimately been removed due to batch effects caused by sequencing runs.

4.3 Eight *T. adelaidensis* individuals collected between two seasonal periods.

Exploration of all Eight samples by each group factor:

Import the data:

```
# create paths
setwd("~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr/kallisto-clstr/")
DIR8 <- ("~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr/kallisto-clstr/")
head(DIR8)
```

```
## [1] "~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr/kallisto-clstr/"
```

```
paths8 <- list.dirs(path = DIR8, full.names = FALSE, recursive = FALSE)
# the working dir only includes kallisto output for the
# kidneys at this time View(paths)
head(paths8)
```



```
## [1] "G1_SepF" "G2_SepF" "G3_MarF" "G4_MarM" "G5_SepM" "G6_SepM"
```

```
Kcaught8 <- catchKallisto(paths8, verbose = TRUE)
```

```
## Reading G1_SepF, 9813 transcripts, 100 bootstraps
## Reading G2_SepF, 9813 transcripts, 100 bootstraps
## Reading G3_MarF, 9813 transcripts, 100 bootstraps
## Reading G4_MarM, 9813 transcripts, 100 bootstraps
## Reading G5_SepM, 9813 transcripts, 100 bootstraps
## Reading G6_SepM, 9813 transcripts, 100 bootstraps
## Reading G7_MarF, 9813 transcripts, 100 bootstraps
## Reading G8_MarF, 9813 transcripts, 100 bootstraps
```

```
setwd("~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr")
# View(Kcaught)
```

Set the group factors. Input Sample files are listed G1-G8 Group factors are applied to the DGElist object and are the point of comparison for expression analyses.

4.3.0.1 Season of collection: 1 = March, 2 = Sep. For the purposes of consistent labelling per group factors some April collections are referred to in the March group. Accurate collection information and dates are outlined in the methods chapter.

```
# these factors correlate to season of collection. 1 =
# March, 2 = Sep.
season8_group <- factor(c(2, 2, 1, 1, 2, 2, 1, 1))

# these factors correlate to individuals sex. 1 = Female, 2
# = Male
sex8_group <- factor(c(1, 1, 1, 2, 2, 2, 1, 1))
```

4.3.0.2 Individuals sex: 1 = Female, 2 = Male.

4.3.1 Group Factor: Season

Create the EdgeR DGE list

```
dge_Season8 <- DGEList(counts = Kcaught8$counts/Kcaught8$annotation$Overdispersion,
  genes = Kcaught8$annotation, group = season8_group)
# View(dge_Season8)
names(dge_Season8)
```

```
## [1] "counts" "samples" "genes"
```

dge_Season8

```
## An object of class "DGEList"
## $counts
##
##          G1_SepF    G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 4.158820e-07 1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
##
##          G3_MarF    G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.5527639 1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 11.2812462 4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.000000
##
##          G5_SepM    G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 47.04729
##
##          G7_MarF    G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 139.72439 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 34.68487 0.00000
## 9808 more rows ...
##
## $samples
##      group lib.size norm.factors
## G1_SepF    2  5568916           1
## G2_SepF    2  4333716           1
## G3_MarF    1  3738942           1
## G4_MarM    1  4341981           1
## G5_SepM    2  3387520           1
## G6_SepM    2 41354721           1
## G7_MarF    1 43500763           1
## G8_MarF    1  4747833           1
##
## $genes
##
##          Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1530 1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 2065 1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 1612 1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2999 2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1965 1632.281
##
##          Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 2.880729
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 1.188489
```

```
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401      3.028806
## 9808 more rows ...
```

Obtain Counts Per Million

```
myTPM_Season8 <- dge_Season8$counts
# head(myTPM_Season8)
```

4.3.2 Initial Data Exploration

```
# Which values in myCPM are greater than 0.5?
thresh_Season8 <- myTPM_Season8 > 0.5
# This produces a logical matrix with TRUEs and FALSEs
head(thresh_Season8)
```

```
##                               G1_SepF G2_SepF G3_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534     FALSE      TRUE      TRUE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   FALSE     FALSE     FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       TRUE      TRUE      TRUE
##                               G4_MarM G5_SepM G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      TRUE     FALSE      TRUE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   FALSE     FALSE      TRUE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       TRUE      TRUE      TRUE
##                               G7_MarF G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      TRUE     FALSE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   TRUE     FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       TRUE      TRUE
```

Insert colour palette for following visualisations consistency

```
library(RColorBrewer)
myPalette8 <- c("#999999", "#F0E442", "#56B4E9", "#009E73", "#D55E00",
               "#0072B2", "#E69F00", "#CC79A7")
```

Plot CPM distribution with logged CPM

```
# export the plot as png
png("plotDensities_Season8.png", width = 600)
unfilteredExpr_Season8 <- cpm(dge_Season8, log = T)
plotDensities(unfilteredExpr_Season8, col = myPalette8, legend = TRUE)
```

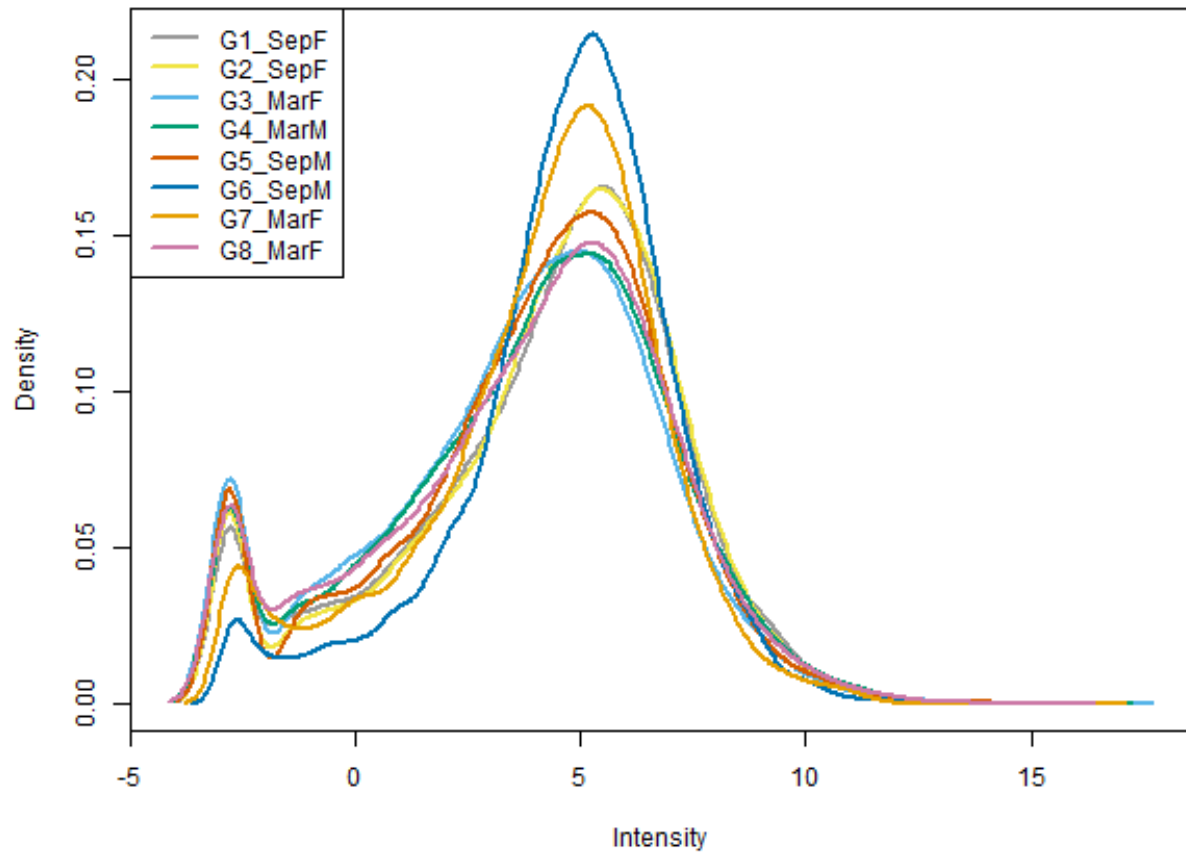


Figure 9: Log Counts per million Densities, for poly-a selected mRNA expression data from eight *T. adalaidensis* kidney samples

Visualise library sizes per sample

```
# The names argument tells the barplot to use the sample
# names on the x-axis The las argument rotates the axis
# names export the plot as png
png("Barplot.LibSize_Season8.png", width = 600)
barplot(dge_Season8$samples$lib.size, names = colnames(dge_Season8),
        las = 2, col = myPalette8)
# title('Barplot of library sizes')
```

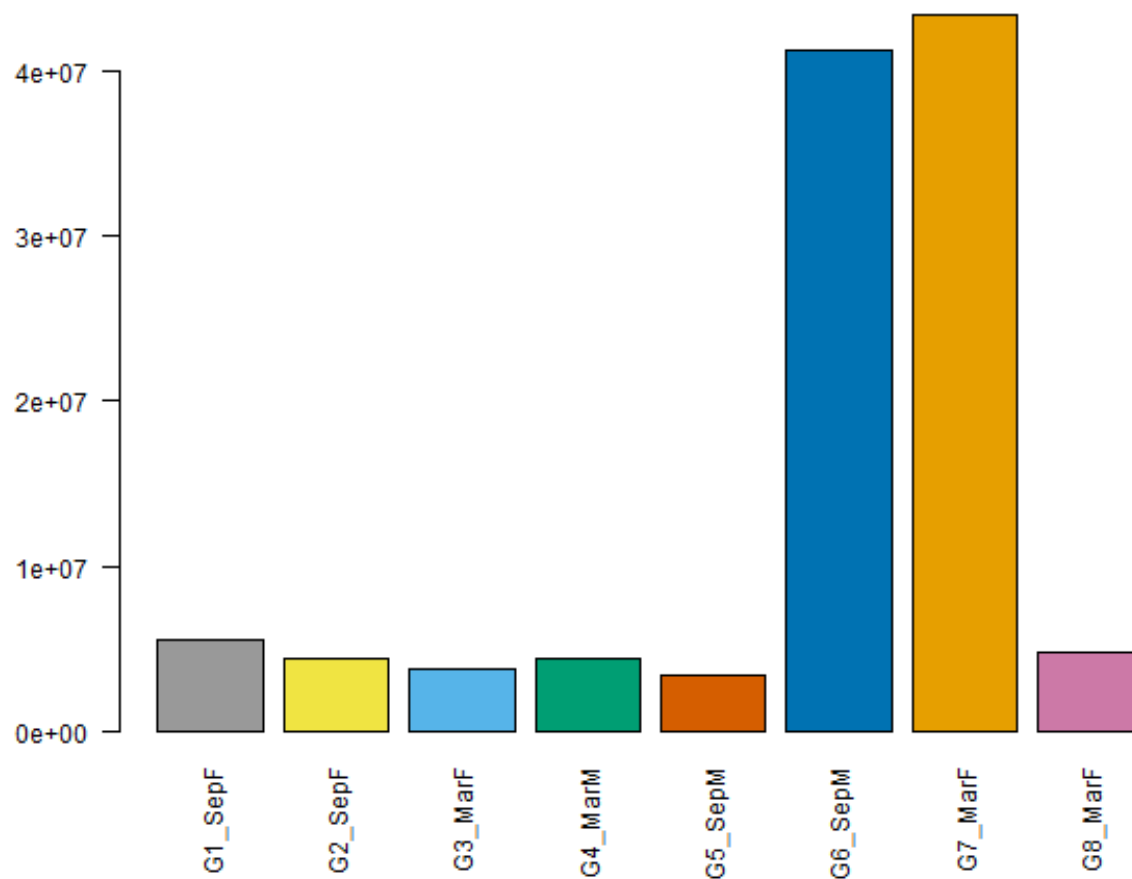


Figure 10: Library size of poly-a selected mRNA sequenced from kidney tissue of eight *T. adelaidensis* individuals

Visualise transcript total estimated counts per sample

```
# export the plot as png
png("Boxplot.Counts_Season8.png", width = 600)
boxplot(dge_Season8$counts, col = myPalette8, las = 2, legend = TRUE)
# title('Boxplot of total unfiltered Counts')
```

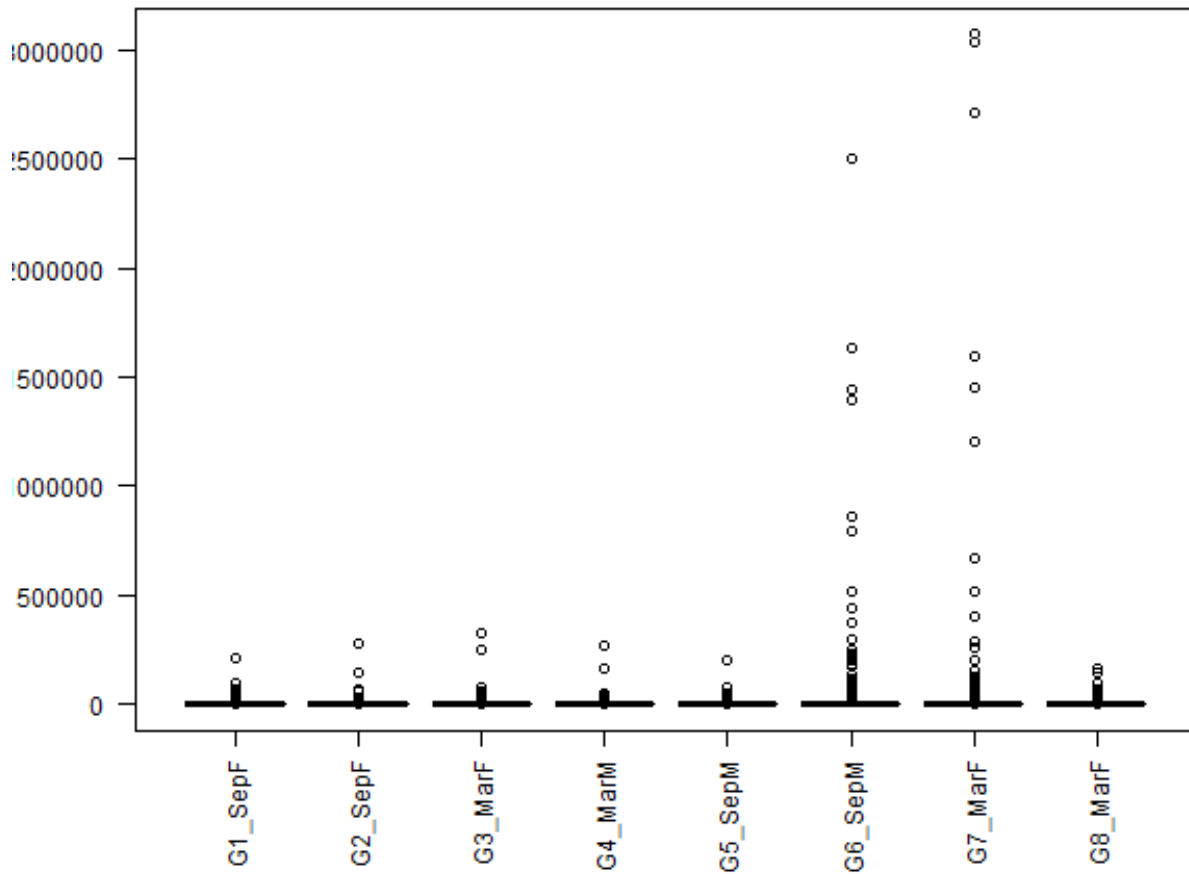


Figure 11: Total estimated counts, for poly-a selected mRNA expression data from eight *T. adelaidensis* kidney samples

```

# Get log2 counts per million
logcounts_Season8 <- cpm(dge_Season8, log = TRUE)
# Check distributions of samples using boxplots export the
# plot as png
png("boxplot.CPM_Season8.png", width = 600)
boxplot(logcounts_Season8, xlab = "", ylab = "Log2 counts per million",
        las = 2, col = myPalette8, legend = TRUE)
# Let's add a blue horizontal line that corresponds to the
# median logCPM
abline(h = median(logcounts_Season8), col = myPalette8, legend = TRUE)
# title('Boxplots of log Counts Per Million')

```

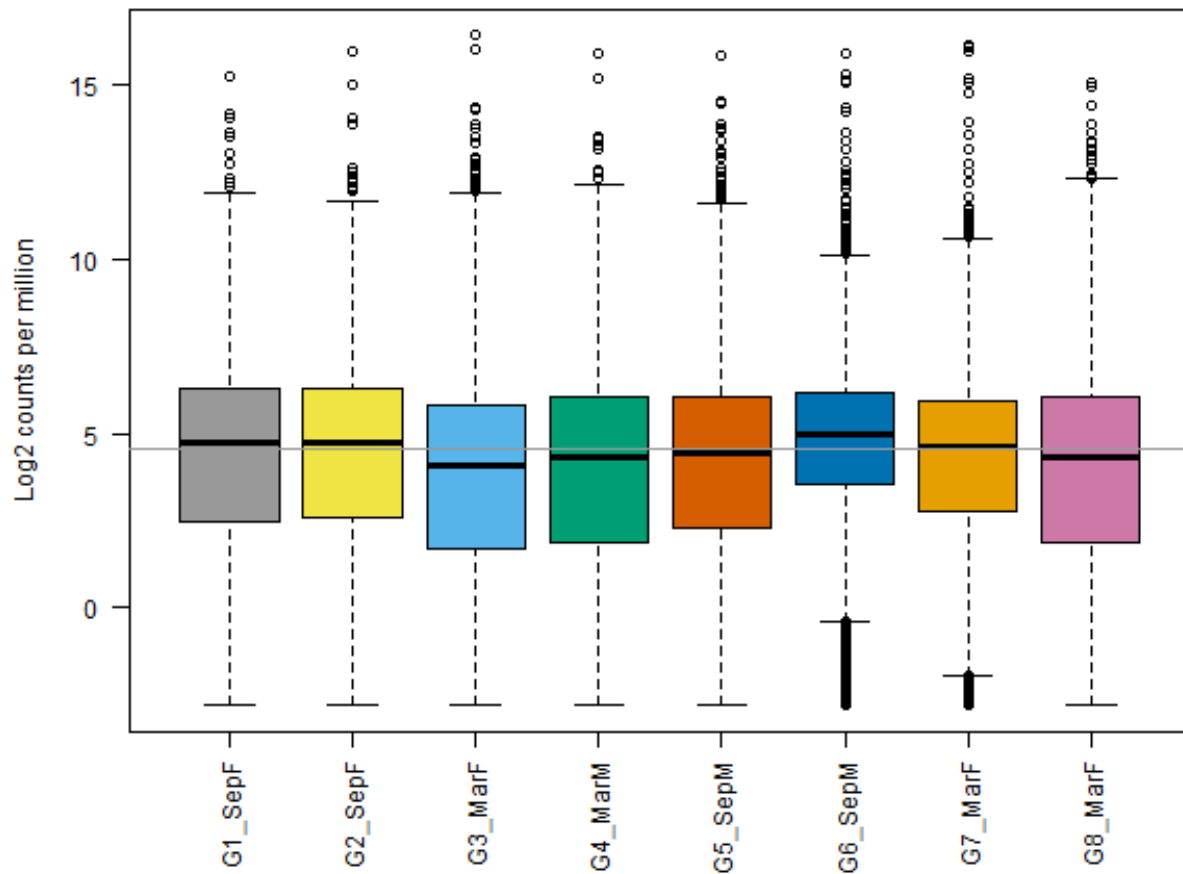


Figure 12: Log2 of estimated counts per million, for poly-a selected mRNA expression data from eight *T. adelaidensis* kidney samples

Visualise sample variation using an MDS plot

```
# export the plot as png
png("MDS_Season8.png", width = 600)
plotMDS(dge_Season8, col = myPalette8)
# title('MDS plot')
```

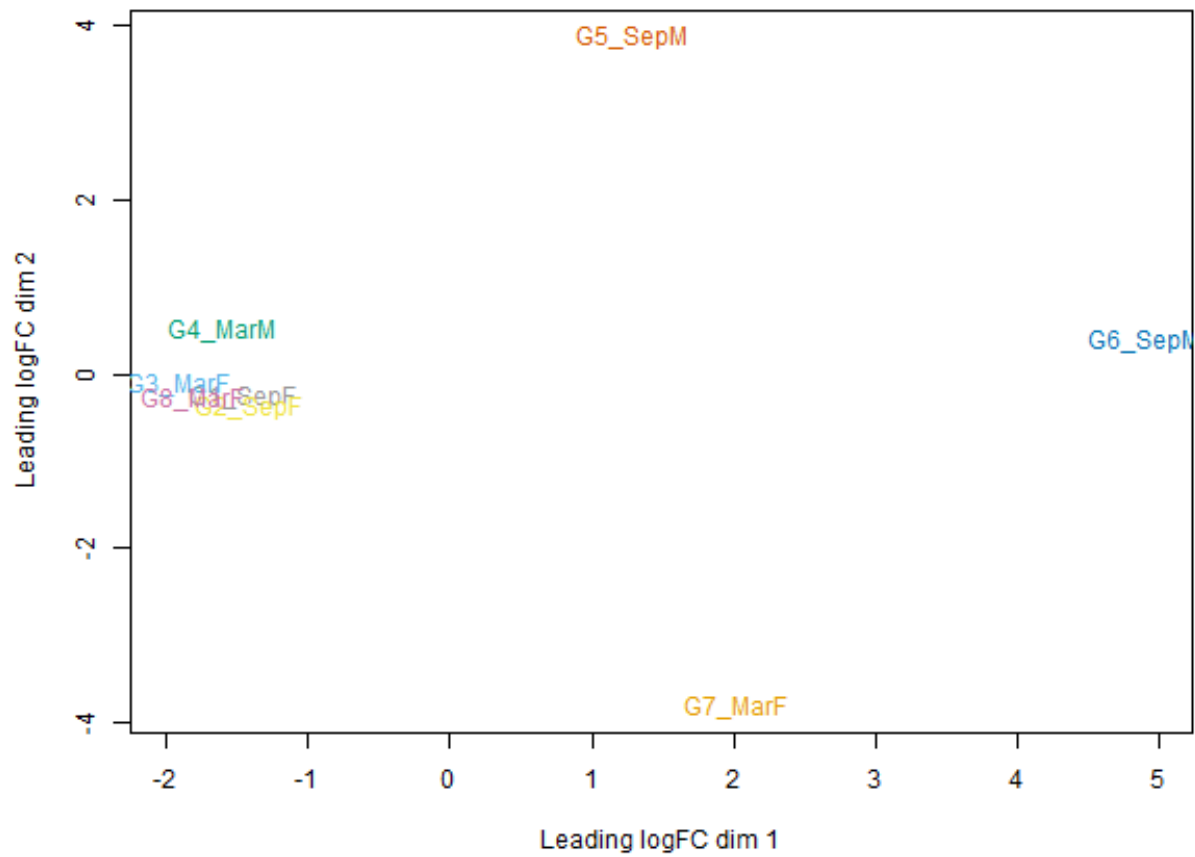


Figure 13: MDS plot of variation among eight *T. adelaidensis* individuals

4.3.3 Calculations

```
# Data with actual counts, not TPM = 'dge'
```

```
head(dge_Season8)
```

```
## An object of class "DGEList"
```

```
## $counts
```

```
##
##               G1_SepF   G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 4.158820e-07 1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 7.600000e+01 35.000000
##
##               G3_MarF   G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.0000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.5527639 1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 11.2812462 4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.0000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 11.0000000 13.0000000
##
##               G5_SepM   G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 47.04729
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 11.00000 2959.00000
##
##               G7_MarF   G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 139.72439 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 34.68487 0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 4547.00000 27.00000
```

```
##
```

```
## $samples
```

```
##      group lib.size norm.factors
## G1_SepF    2  5568916           1
## G2_SepF    2  4333716           1
## G3_MarF    1  3738942           1
## G4_MarM    1  4341981           1
## G5_SepM    2  3387520           1
## G6_SepM    2 41354721           1
## G7_MarF    1 43500763           1
## G8_MarF    1  4747833           1
```

```
##
```

```
## $genes
```

```
##
##      Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1530 1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 2065 1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 1612 1279.281
```

```
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1965      1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      2714      2381.281
##
## Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      2.880729
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      1.188489
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   3.028806
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      1.000000
```

```
head(myTPM_Season8)
```

```
##
## G1_SepF G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      4.158820e-07  1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.000000e+00  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      7.600000e+01 35.000000
##
## G3_MarF G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      49.0000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     645.0000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.5527639  1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      11.2812462  4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.0000000  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      11.0000000 13.000000
##
## G5_SepM G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      77.00000  793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.00000  136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.00000  47.04729
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      11.00000 2959.00000
##
## G7_MarF G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564    12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534     139.72439  0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426     3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   34.68487  0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993     4547.00000 27.00000
```

filter out genes with less than 5 reads in 2 samples for each transcript

```
filter_Season8 <- apply(dge_Season8, 1, function(x) length(x[x >
5]) >= 2)
filtered_Season8 <- dge_Season8[filter_Season8, ]
head(filtered_Season8)
```

```
## An object of class "DGEList"
## $counts
##
## G1_SepF G2_SepF
```

```

## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      4.158820e-07  1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  0.000000e+00  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       7.600000e+01 35.000000
##
##          G3_MarF    G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      49.000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     645.000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.5527639  1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      11.2812462  4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.0000000  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      11.0000000 13.000000
##
##          G5_SepM    G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      77.00000  793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.00000  136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.00000  47.04729
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      11.00000 2959.00000
##
##          G7_MarF    G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564    12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534     139.72439  0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426     3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   34.68487  0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993     4547.00000 27.00000
##
## $samples
##      group lib.size norm.factors
## G1_SepF    2  5568916          1
## G2_SepF    2  4333716          1
## G3_MarF    1  3738942          1
## G4_MarM    1  4341981          1
## G5_SepM    2  3387520          1
## G6_SepM    2 41354721          1
## G7_MarF    1 43500763          1
## G8_MarF    1  4747833          1
##
## $genes
##
##          Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      2065      1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      1612      1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1965      1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       2714      2381.281
##
##          Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      2.880729
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      1.188489
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   3.028806
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      1.000000

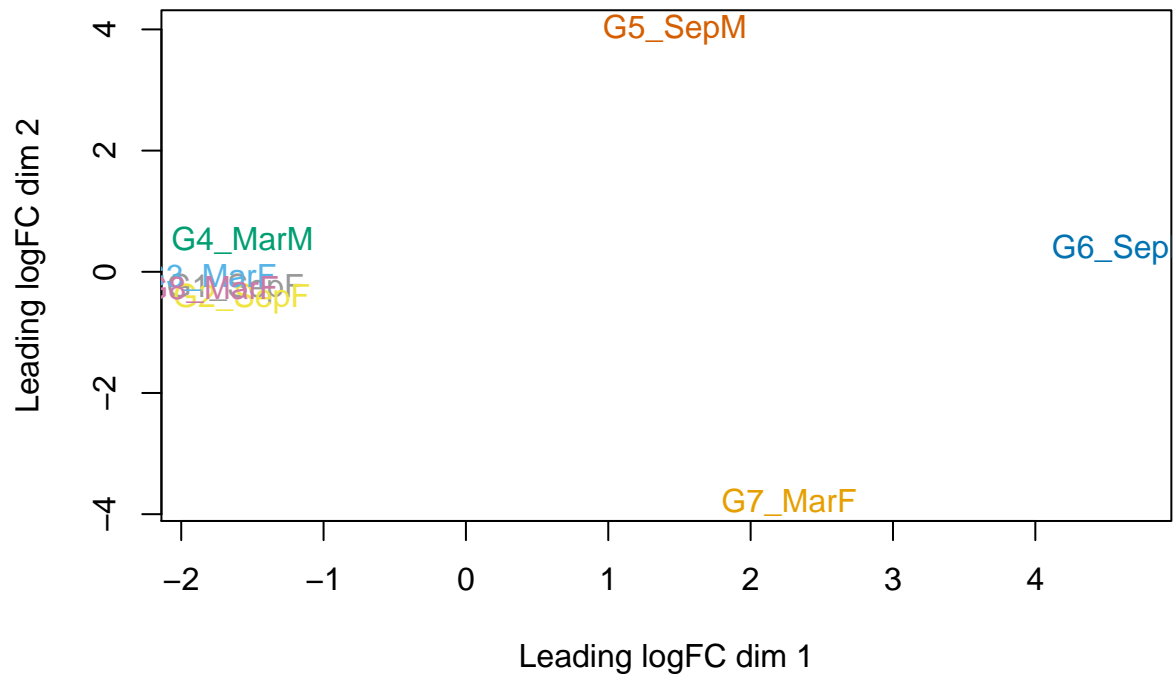
```

Run calculations

```
dge_Season8 <- calcNormFactors(dge_Season8)
dge_Season8 <- estimateCommonDisp(dge_Season8)
dge_Season8 <- estimateTagwiseDisp(dge_Season8)
```

Note that filtering did not change the appearance of the MDS plot

```
plotMDS(dge_Season8, col = myPalette8)
```



4.3.4 Differentially expressed genes:

View the top ten 'differentially expressed genes'

```
results_Season8 <- exactTest(dge_Season8)
topTags(results_Season8)
```

```
## Comparison of groups: 2-1
```

```
##
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418    2000    1667.2807
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227    1771    1438.2807
## PB.147.1|0620ba|path0:1-1756(+)|transcript/23387      701     368.6542
## PB.3353.1|7cf053|path5:1-7730(+)|transcript/10       7701    7368.2807
```

	1227	894.2807
## PB.621.2 172e3f path6:3-1224(+) transcript/21583	1227	894.2807
## PB.6088.12 e88f16 path1:164-4329(+) transcript/5912	2488	2155.2807
## PB.1449.1 36bd5c path2:1-1532(+) transcript/18562	1341	1008.2807
## PB.985.1 261589 path8:1-2545(+) transcript/6665	2510	2177.2807
## PB.6065.1 e74bdb path2:1-2471(+) transcript/15776	1754	1421.2807
## PB.1854.1 466cde path0:1-1088(+) transcript/22568	908	575.3308
##	Overdispersion	logFC
## PB.499.11 124cf9 path4:2108-4109(+) transcript/7418	1.856008	3.444092
## PB.4788.1 b34d56 path10:2-1859(+) transcript/15227	1.293367	11.758742
## PB.147.1 0620ba path0:1-1756(+) transcript/23387	1.055680	14.177786
## PB.3353.1 7cf053 path5:1-7730(+) transcript/10	2.287311	7.525344
## PB.621.2 172e3f path6:3-1224(+) transcript/21583	1.000000	14.179990
## PB.6088.12 e88f16 path1:164-4329(+) transcript/5912	1.577810	8.743147
## PB.1449.1 36bd5c path2:1-1532(+) transcript/18562	2.304372	7.882813
## PB.985.1 261589 path8:1-2545(+) transcript/6665	1.000000	12.154915
## PB.6065.1 e74bdb path2:1-2471(+) transcript/15776	1.360280	9.388137
## PB.1854.1 466cde path0:1-1088(+) transcript/22568	1.000000	8.436555
##	logCPM	PValue
## PB.499.11 124cf9 path4:2108-4109(+) transcript/7418	5.702989	3.114653e-08
## PB.4788.1 b34d56 path10:2-1859(+) transcript/15227	8.735038	1.079896e-07
## PB.147.1 0620ba path0:1-1756(+) transcript/23387	6.322527	2.504299e-07
## PB.3353.1 7cf053 path5:1-7730(+) transcript/10	6.704730	6.235719e-07
## PB.621.2 172e3f path6:3-1224(+) transcript/21583	6.324077	8.295346e-07
## PB.6088.12 e88f16 path1:164-4329(+) transcript/5912	10.781344	9.758992e-07
## PB.1449.1 36bd5c path2:1-1532(+) transcript/18562	6.902254	1.430707e-06
## PB.985.1 261589 path8:1-2545(+) transcript/6665	6.383915	1.518649e-06
## PB.6065.1 e74bdb path2:1-2471(+) transcript/15776	5.982169	1.530897e-06
## PB.1854.1 466cde path0:1-1088(+) transcript/22568	6.356782	1.552334e-06
##	FDR	
## PB.499.11 124cf9 path4:2108-4109(+) transcript/7418	0.0003056409	
## PB.4788.1 b34d56 path10:2-1859(+) transcript/15227	0.0005298509	
## PB.147.1 0620ba path0:1-1756(+) transcript/23387	0.0008191561	
## PB.3353.1 7cf053 path5:1-7730(+) transcript/10	0.0011003949	
## PB.621.2 172e3f path6:3-1224(+) transcript/21583	0.0011003949	
## PB.6088.12 e88f16 path1:164-4329(+) transcript/5912	0.0011003949	
## PB.1449.1 36bd5c path2:1-1532(+) transcript/18562	0.0011003949	
## PB.985.1 261589 path8:1-2545(+) transcript/6665	0.0011003949	
## PB.6065.1 e74bdb path2:1-2471(+) transcript/15776	0.0011003949	
## PB.1854.1 466cde path0:1-1088(+) transcript/22568	0.0011003949	

Output summary expression data

```
dim(results_Season8)
```

```
## [1] 9813      3
```

```
summary(de_Season8 <- decideTests(results_Season8))
```

```
##          2-1
## Down      6
## NotSig 9620
## Up       187
```

```
detags_Season8 <- rownames(dge_Season8)[as.logical(de_Season8)]
```

Visualise with a plotsmear

```
detags_Season8 <- rownames(dge_Season8)[as.logical(de_Season8)]  
# export the plot as png  
png("plotSmear_Season8.png", width = 600)  
plotSmear(results_Season8, de.tags = detags_Season8)
```

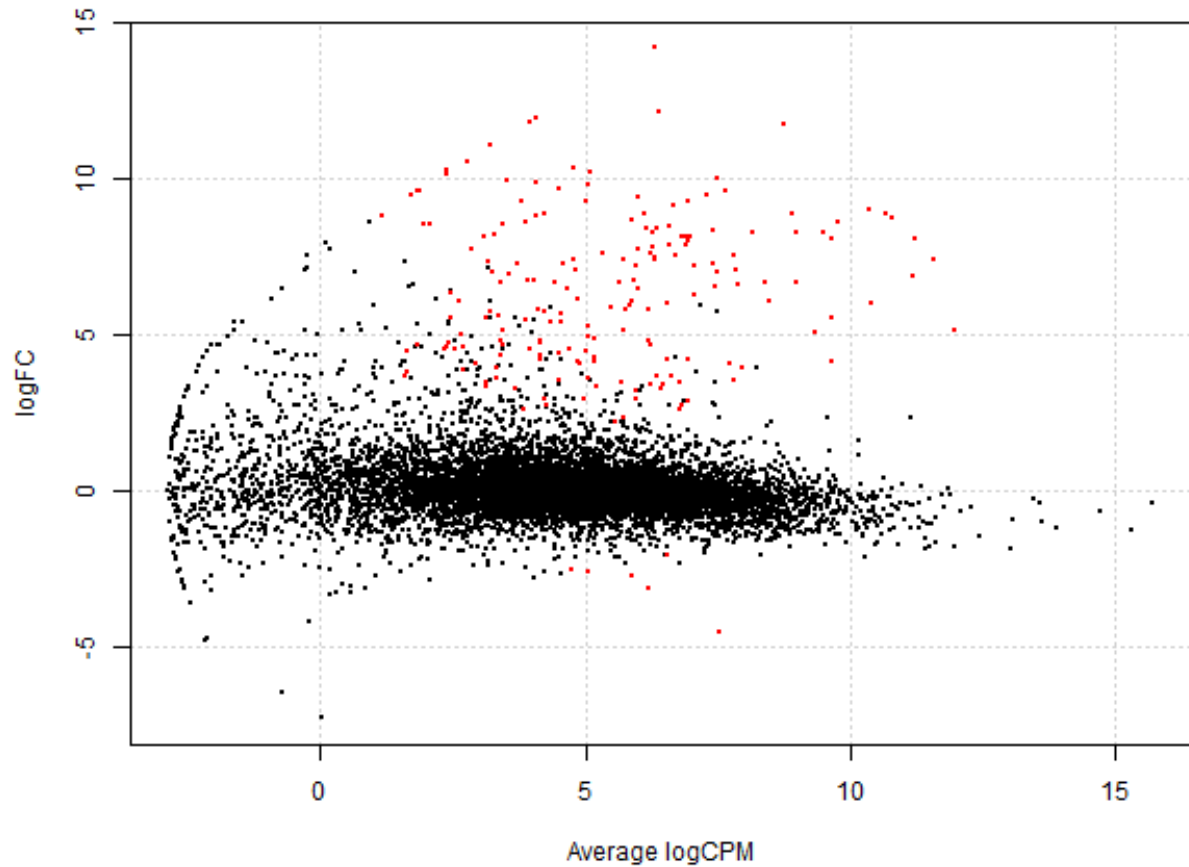


Figure 14: Log Fold Change vs average Log counts per million for expression level comparing Season 2 (September collection) to Season 1 (April/March collection) among eight *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values < 0.05.

Visualise with a volcano plot

```
# export the plot as png
png("volcanoData_Season8.png", width = 600)
volcanoData_Season8 <- cbind(results_Season8$table$logFC,
  ↪ -log10(results_Season8$table$PValue))
colnames(volcanoData_Season8) <- c("logFC", "negLogPval")
DEGs_Season8 <- results_Season8$table$PValue < 0.05 & abs(results_Season8$table$logFC) >
  2
point.col_Season8 <- ifelse(DEGs_Season8, "red", "black")
plot(volcanoData_Season8, pch = 16, col = point.col_Season8,
  cex = 0.5)
```

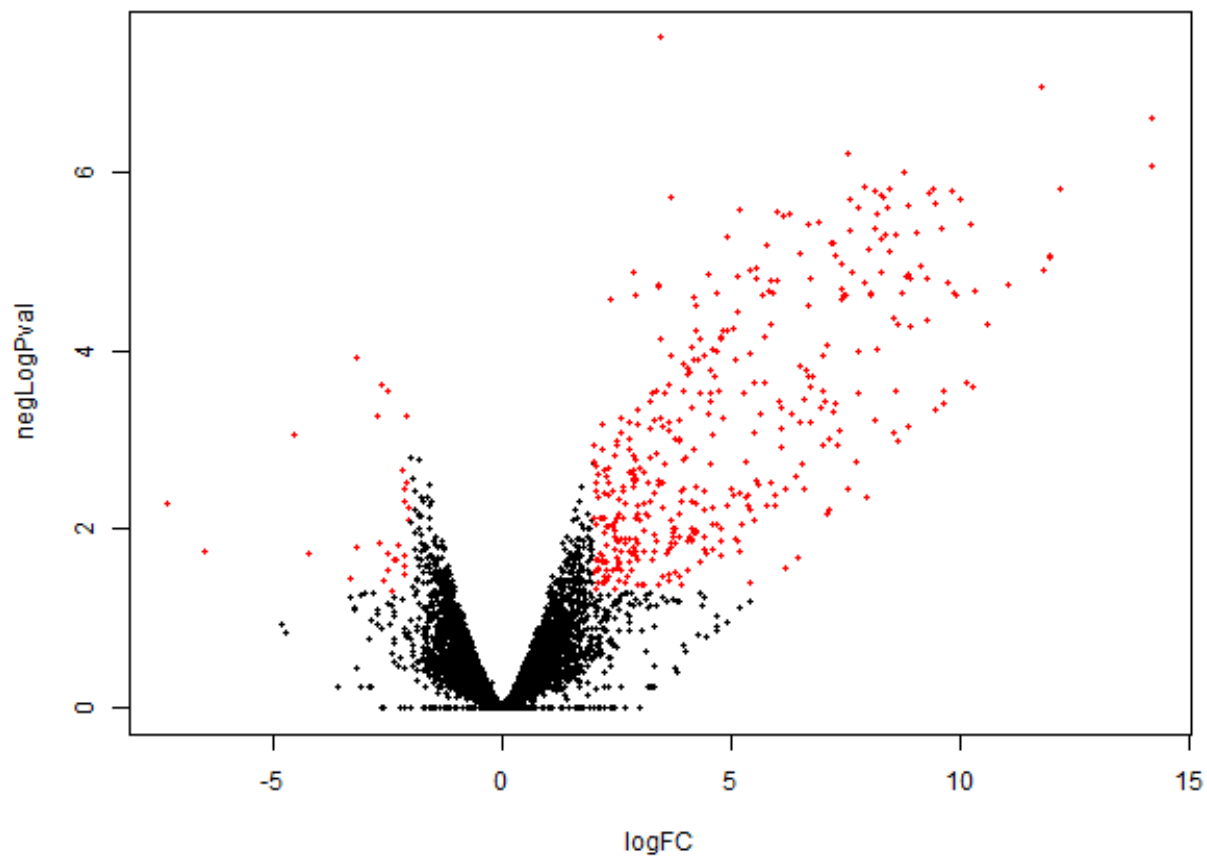


Figure 15: Negative Log of the p-value against Log Fold Change in expression level comparing Season 2 (September collection) to Season 1 (April/March collection) among eight *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values <0.05 and *also* a log fold change >2.

4.3.5 Group Factor: Sex

Create the EdgeR DGE list

```
dge_Sex8 <- DGEList(counts = Kcaught8$counts/Kcaught8$annotation$Overdispersion,
  genes = Kcaught8$annotation, group = sex8_group)
# View(dge_Sex8)
names(dge_Sex8)
```

```
## [1] "counts" "samples" "genes"
```

```
dge_Sex8
```

```
## An object of class "DGEList"
## $counts
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 4.158820e-07 1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.5527639 1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 11.2812462 4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.000000
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 47.04729
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 139.72439 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 34.68487 0.00000
## 9808 more rows ...
##
## $samples
## group lib.size norm.factors
## G1_SepF 1 5568916 1
## G2_SepF 1 4333716 1
## G3_MarF 1 3738942 1
## G4_MarM 2 4341981 1
## G5_SepM 2 3387520 1
## G6_SepM 2 41354721 1
## G7_MarF 1 43500763 1
## G8_MarF 1 4747833 1
##
```

```
## $genes
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      2065      1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      1612      1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1965      1632.281
##
## Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      2.880729
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      1.188489
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   3.028806
## 9808 more rows ...
```

```
# Obtain Counts Per Million
myTPM_Sex8 <- dge_Sex8$counts
# Have a look at the output
head(myTPM_Sex8)
```

```
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      4.158820e-07  1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.000000e+00  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      7.600000e+01 35.000000
##
## G3_MarF      G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      49.0000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     645.0000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.5527639  1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      11.2812462  4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.0000000  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      11.0000000 13.000000
##
## G5_SepM      G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      77.00000  793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.00000  136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.00000  47.04729
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      11.00000 2959.00000
##
## G7_MarF      G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564    12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      139.72439  0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426     3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   34.68487  0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      4547.00000 27.00000
```

4.3.6 Initial Data Exploration

```
# Which values in myCPM are greater than 0.5?
thresh_Sex8 <- myTPM_Sex8 > 0.5
# This produces a logical matrix with TRUEs and FALSEs
head(thresh_Sex8)
```

```
##                                G1_SepF G2_SepF G3_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534     FALSE      TRUE      TRUE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  FALSE     FALSE     FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       TRUE      TRUE      TRUE
##                                G4_MarM G5_SepM G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      TRUE     FALSE      TRUE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  FALSE     FALSE      TRUE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       TRUE      TRUE      TRUE
##                                G7_MarF G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      TRUE     FALSE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  TRUE     FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       TRUE      TRUE
```

Raw Counts per million and library sizes will be consistent with the Season comparison above. There is no need to plot them again however scripts are included to create separate R objects for downstream application.

CPM distribution with logged CPM

```
# export the plot as png
png("plotDensities_Sex8.png", width = 600)
unfilteredExpr_Sex8 <- cpm(dge_Sex8, log = T)
plotDensities(unfilteredExpr_Sex8, col = myPalette8, legend = TRUE)
```

Library sizes per sample

```
# The names argument tells the barplot to use the sample
# names on the x-axis The las argument rotates the axis
# names export the plot as png
png("Barplot.LibSize_Sex8.png", width = 600)
barplot(dge_Sex8$samples$lib.size, names = colnames(dge_Sex8),
        las = 2, col = myPalette8)
# title('Barplot of library sizes')
```

Transcript total estimated counts per sample

```
# export the plot as png
png("Boxplot.Counts_Sex8.png", width = 600)
boxplot(dge_Sex8$counts, col = myPalette8, las = 2, legend = TRUE)
# title('Boxplot of total unfiltered Counts')
```

Logged total estimated transcript counts per sample

```
# Get log2 counts per million
logcounts_Sex8 <- cpm(dge_Sex8, log = TRUE)
# Check distributions of samples using boxplots export the
# plot as png
png("boxplot.CPM_Sex8.png", width = 600)
boxplot(logcounts_Sex8, xlab = "", ylab = "Log2 counts per million",
        las = 2, col = myPalette8, legend = TRUE)
# Let's add a blue horizontal line that corresponds to the
# median logCPM
abline(h = median(logcounts_Sex8), col = myPalette8, legend = TRUE)
# title('Boxplots of log Counts Per Million')
```

4.3.7 Calculations

```
# Data with actual counts, not TPM = 'dge'
head(dge_Sex8)
```

```
## An object of class "DGEList"
## $counts
##
```

	G1_SepF	G2_SepF
## PB.2.1 002537 path0:1-1624(+) transcript/18304	1.790000e+02	118.000000
## PB.3.2 00361c path0:43-2240(+) transcript/10564	8.410000e+02	746.000000
## PB.10.1 004815 path1:5-1713(+) transcript/17534	4.158820e-07	1.539241
## PB.11.1 004815 path2:1-3039(+) transcript/3426	2.860775e+01	30.766683
## PB.11.2 004815 path2:1047-3035(+) transcript/13401	0.000000e+00	0.000000
## PB.13.1 004c10 path2:1-2811(+) transcript/4993	7.600000e+01	35.000000

```
##
```

	G3_MarF	G4_MarM
## PB.2.1 002537 path0:1-1624(+) transcript/18304	49.00000000	113.000000
## PB.3.2 00361c path0:43-2240(+) transcript/10564	645.00000000	997.000000
## PB.10.1 004815 path1:5-1713(+) transcript/17534	0.5527639	1.114611
## PB.11.1 004815 path2:1-3039(+) transcript/3426	11.2812462	4.870982
## PB.11.2 004815 path2:1047-3035(+) transcript/13401	0.00000000	0.000000
## PB.13.1 004c10 path2:1-2811(+) transcript/4993	11.00000000	13.000000

```
##
```

	G5_SepM	G6_SepM
## PB.2.1 002537 path0:1-1624(+) transcript/18304	77.000000	793.000000
## PB.3.2 00361c path0:43-2240(+) transcript/10564	415.000000	9814.000000
## PB.10.1 004815 path1:5-1713(+) transcript/17534	0.000000	136.63002
## PB.11.1 004815 path2:1-3039(+) transcript/3426	31.97337	7657.54555
## PB.11.2 004815 path2:1047-3035(+) transcript/13401	0.000000	47.04729
## PB.13.1 004c10 path2:1-2811(+) transcript/4993	11.000000	2959.000000

```
##
```

	G7_MarF	G8_MarF
## PB.2.1 002537 path0:1-1624(+) transcript/18304	564.000000	147.000000
## PB.3.2 00361c path0:43-2240(+) transcript/10564	12448.000000	1577.000000

```
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      139.72439      0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      3505.65982     13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   34.68487       0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      4547.00000     27.00000
##
## $samples
##      group lib.size norm.factors
## G1_SepF    1  5568916          1
## G2_SepF    1  4333716          1
## G3_MarF    1  3738942          1
## G4_MarM    2  4341981          1
## G5_SepM    2  3387520          1
## G6_SepM    2 41354721          1
## G7_MarF    1 43500763          1
## G8_MarF    1  4747833          1
##
## $genes
##                                     Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     2065      1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534     1612      1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426     2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  1965      1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993     2714      2381.281
##
##                                     Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      2.880729
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      1.188489
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   3.028806
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      1.000000
```

```
head(myTPM_Sex8)
```

```
##
##      G1_SepF      G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      4.158820e-07  1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  0.000000e+00  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      7.600000e+01 35.000000
##
##      G3_MarF      G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      49.0000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     645.0000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.5527639  1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426     11.2812462  4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   0.0000000  0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993     11.0000000 13.000000
##
##      G5_SepM      G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      77.00000  793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564     415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      0.00000  136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426     31.97337 7657.54555
```

```
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 47.04729
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 11.00000 2959.00000
## G7_MarF G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 139.72439 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 34.68487 0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 4547.00000 27.00000
```

filter out genes with less than 5 reads in 2 samples for each transcript

```
filter_Sex8 <- apply(dge_Sex8, 1, function(x) length(x[x > 5]) >=
2)
filtered_Sex8 <- dge_Sex8[filter_Sex8, ]
head(filtered_Sex8)
```

```
## An object of class "DGEList"
## $counts
## G1_SepF G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 4.158820e-07 1.539241
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.860775e+01 30.766683
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 7.600000e+01 35.000000
## G3_MarF G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.5527639 1.114611
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 11.2812462 4.870982
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 11.0000000 13.000000
## G5_SepM G6_SepM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 793.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 9814.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 136.63002
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 31.97337 7657.54555
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 47.04729
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 11.00000 2959.00000
## G7_MarF G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 564.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 12448.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 139.72439 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 3505.65982 13.46247
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 34.68487 0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 4547.00000 27.00000
## $samples
## group lib.size norm.factors
## G1_SepF 1 5568916 1
## G2_SepF 1 4333716 1
## G3_MarF 1 3738942 1
```

```
## G4_MarM      2  4341981      1
## G5_SepM      2  3387520      1
## G6_SepM      2  41354721     1
## G7_MarF      1  43500763      1
## G8_MarF      1  4747833       1
##
## $genes
##                                     Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      2065      1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      1612      1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1965      1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       2714      2381.281
##                                     Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      2.880729
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       1.188489
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   3.028806
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       1.000000
```

Run calculations

```
dge_Sex8 <- calcNormFactors(dge_Sex8)
dge_Sex8 <- estimateCommonDisp(dge_Sex8)
dge_Sex8 <- estimateTagwiseDisp(dge_Sex8)
```

4.3.8 Differentially expressed genes:

View the top ten ‘differentially expressed genes’

```
results_Sex8 <- exactTest(dge_Sex8)
topTags(results_Sex8)
```

```
## Comparison of groups: 2-1
##                                     Length EffectiveLength
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195    1136      803.2807
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021    1103      770.2807
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860      905      572.3308
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575      3066      2733.2807
## PB.602.1|167374|path2:1-1363(+)|transcript/21155     1304      971.2807
## PB.3895.1|91ba83|path6:1-1594(+)|transcript/18563    1376      1043.2807
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901      896      563.3308
## PB.5140.3|c12866|path2:10-2402(+)|transcript/8294    2359      2026.2807
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823   1337      1004.2807
## PB.419.2|0f691f|path1:48-1473(+)|transcript/20262    1261      928.2807
##                                     Overdispersion      logFC
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195    1.381179  8.978789
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021    3.333104 10.106744
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860      1.020264  8.208384
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575       1.000000  9.770416
```



```
## PB.602.1|167374|path2:1-1363(+)|transcript/21155      1.150674  8.495981
## PB.3895.1|91ba83|path6:1-1594(+)|transcript/18563     3.245530  9.669816
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901      1.733808  8.200739
## PB.5140.3|c12866|path2:10-2402(+)|transcript/8294     1.000000  8.957352
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823   6.388942 10.964074
## PB.419.2|0f691f|path1:48-1473(+)|transcript/20262    3.986170  9.411064
##
##                                     logCPM      PValue
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195   9.608930 6.213295e-32
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021   5.693299 6.559852e-32
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860     7.447866 5.678994e-20
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575      4.970157 7.054220e-19
## PB.602.1|167374|path2:1-1363(+)|transcript/21155     4.468639 1.521816e-17
## PB.3895.1|91ba83|path6:1-1594(+)|transcript/18563    7.501882 5.359428e-17
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901     6.700558 8.047662e-17
## PB.5140.3|c12866|path2:10-2402(+)|transcript/8294     5.885019 3.358521e-16
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823   7.178685 1.527940e-15
## PB.419.2|0f691f|path1:48-1473(+)|transcript/20262    6.238564 2.419542e-15
##
##                                     FDR
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195   3.218591e-28
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021   3.218591e-28
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860     1.857599e-16
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575      1.730577e-15
## PB.602.1|167374|path2:1-1363(+)|transcript/21155     2.986716e-14
## PB.3895.1|91ba83|path6:1-1594(+)|transcript/18563    8.765344e-14
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901     1.128167e-13
## PB.5140.3|c12866|path2:10-2402(+)|transcript/8294     4.119646e-13
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823   1.665964e-12
## PB.419.2|0f691f|path1:48-1473(+)|transcript/20262    2.374296e-12
```

Output summary expression data

```
dim(results_Sex8)
```

```
## [1] 9813    3
```

```
summary(de_Sex8 <- decideTests(results_Sex8))
```

```
##          2-1
## Down      3
## NotSig 9520
## Up       290
```

```
detags_Sex8 <- rownames(dge_Sex8)[as.logical(de_Sex8)]
```


Visualise with a `plotsmear`

```
# export the plot as png
png("plotSmear_Sex8.png", width = 600)
plotSmear(results_Sex8, de.tags = detags_Sex8)
```

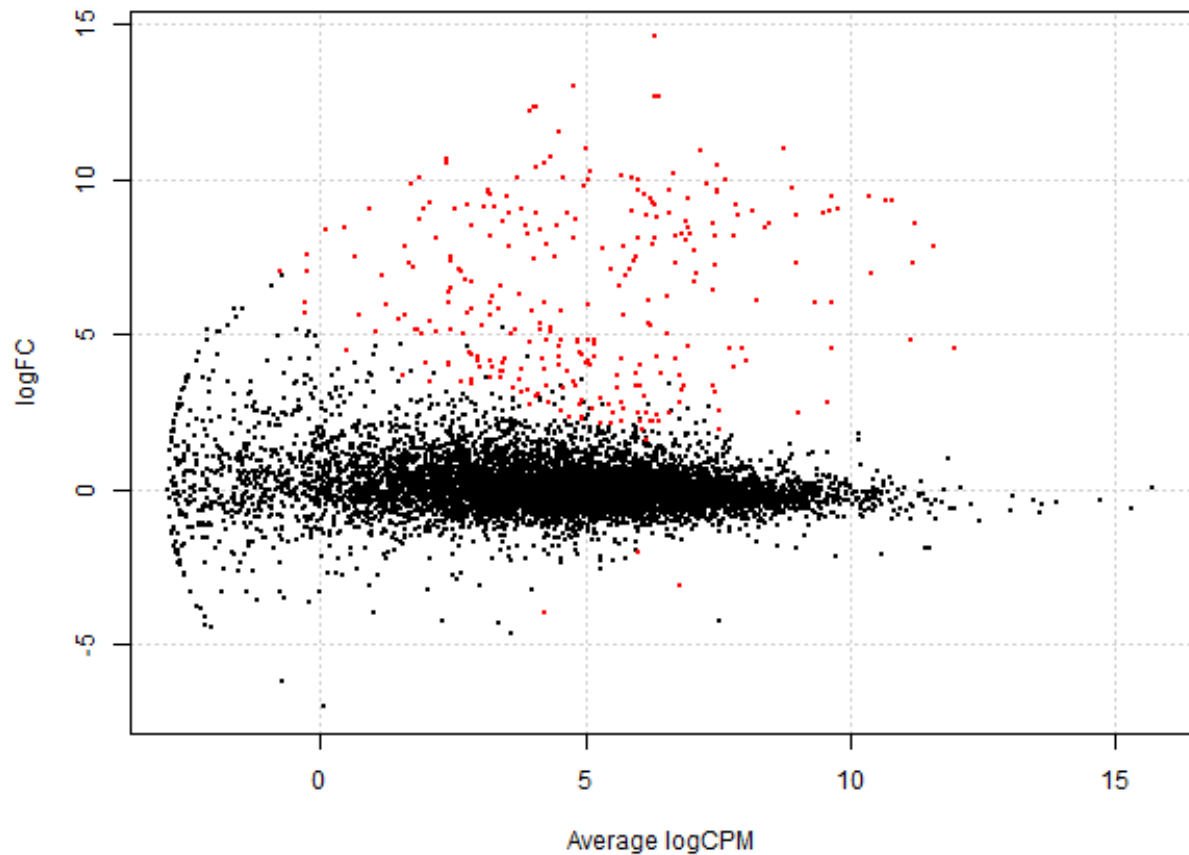


Figure 16: Log Fold Change vs average Log counts per million for expression level comparing Male to Female as variables among eight *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values < 0.05.

Visualise with a volcano plot

```
# export the plot as png
png("volcanoData_Sex8.png", width = 600)
volcanoData_Sex8 <- cbind(results_Sex8$table$logFC, -log10(results_Sex8$table$PValue))
colnames(volcanoData_Sex8) <- c("logFC", "negLogPval")
DEGs_Sex8 <- results_Sex8$table$PValue < 0.05 & abs(results_Sex8$table$logFC) >
  2
point.col_Sex8 <- ifelse(DEGs_Sex8, "red", "black")
plot(volcanoData_Sex8, pch = 16, col = point.col_Sex8, cex = 0.5)
```

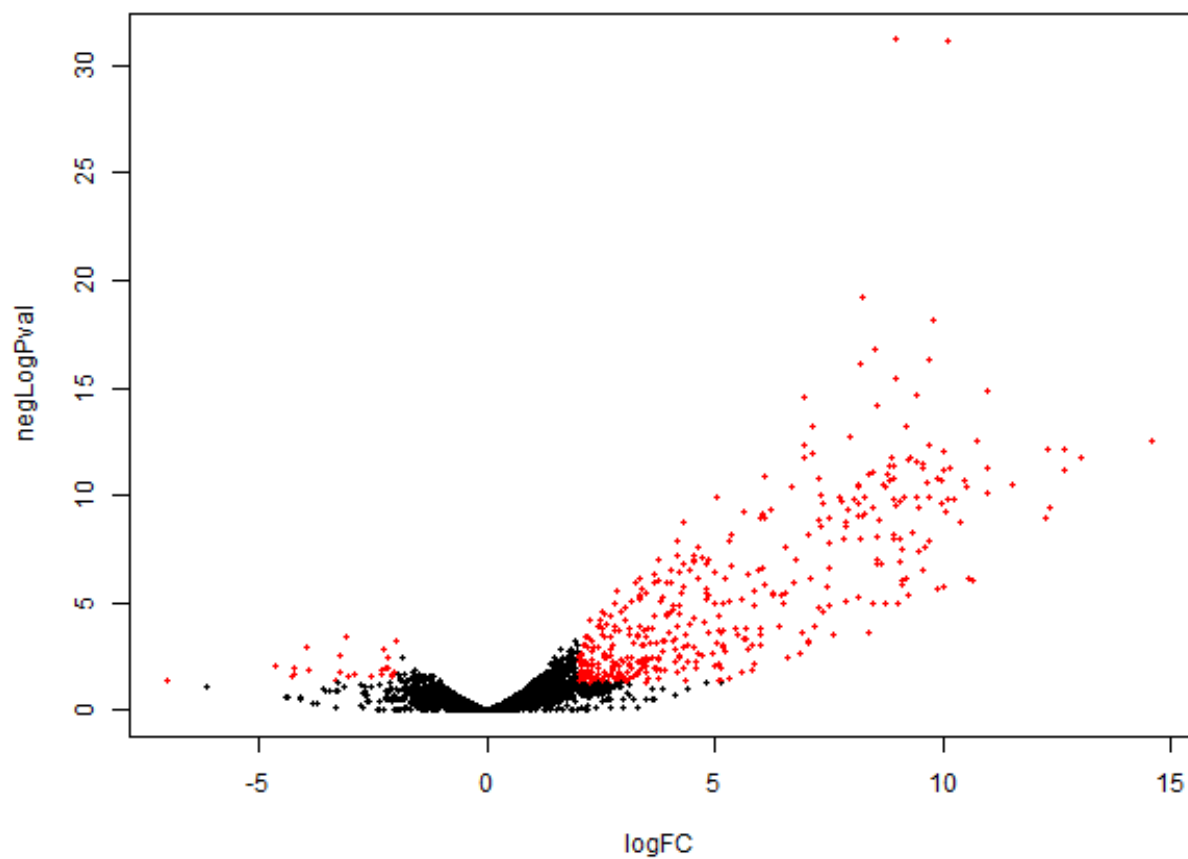


Figure 17: Negative Log of the p-value against Log Fold Change in expression level comparing Male to Female as variables among eight *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values <0.05 and also a log fold change >2.

4.4 Six *T. adelaidensis* individuals collected between two seasonal periods.

(These methods exclude samples G6 and G7).

This document separates samples by Seasonal group factors AND Sex Group Factors to explore the effects of sample G5 on the data.

Import the data:

```
# create paths
setwd("~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr-6&7/kallisto-clstr/")
DIR <- ("~/@Uni/@Flinders University
↳ PhD/RWorkingDir/R-kallisto-clstr-6&7/kallisto-clstr/")

head(DIR)
```

```
## [1] "~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr-6&7/kallisto-clstr/"
```

```
paths <- list.dirs(path = DIR, full.names = FALSE, recursive = FALSE)
# the working dir only includes kallisto output for the
# kidneys at this time
head(paths)
```

```
## [1] "G1_SepF" "G2_SepF" "G3_MarF" "G4_MarM" "G5_SepM" "G8_MarF"
```

```
Kcaught <- catchKallisto(paths, verbose = TRUE)
```

```
## Reading G1_SepF, 9813 transcripts, 100 bootstraps
## Reading G2_SepF, 9813 transcripts, 100 bootstraps
## Reading G3_MarF, 9813 transcripts, 100 bootstraps
## Reading G4_MarM, 9813 transcripts, 100 bootstraps
## Reading G5_SepM, 9813 transcripts, 100 bootstraps
## Reading G8_MarF, 9813 transcripts, 100 bootstraps
```

```
setwd("~/@Uni/@Flinders University PhD/RWorkingDir/R-kallisto-clstr-6&7")
# View(Kcaught)
```

Set the group factors. Input Sample files are listed in number order G1-G8 excluding G6 and G7. Group factors are applied to the DGElist object and are the point of comparison for expression analyses.

4.4.0.1 Season of collection: 1 = March, 2 = Sep. For the purposes of consistent labelling per group factors some April collections are referred to in the March group. Accurate collection information and dates are outlined in the methods chapter.

```
# these factors correlate to season of collection. 1 =
# March, 2 = Sep.
season_group <- factor(c(2, 2, 1, 1, 2, 1))
```

```
# these factors correlate to individuals sex. 1 = Female, 2
# = Male
sex_group <- factor(c(1, 1, 1, 2, 2, 1))
```

4.4.0.2 Individuals sex: 1 = Female, 2 = Male.

4.4.1 Group Factor: Season

Create the EdgeR DGE list for use in subsequent analyses

```
dge_Season <- DGEList(counts = Kcaught$counts/Kcaught$annotation$Overdispersion,
  genes = Kcaught$annotation, group = season_group)
# View(dge_Season) names(dge_Season)
dge_Season
```

```
## An object of class "DGEList"
## $counts
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.682740e-07 1.363036
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.639676e+01 28.388835
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.0000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.4894863 0.9870162
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 10.4093586 4.4945206
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.0000000
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 147.000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 1577.000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 0.000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 29.50226 12.422
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 0.000
## 9808 more rows ...
##
## $samples
## group lib.size norm.factors
## G1_SepF 2 5796184 1
## G2_SepF 2 4480604 1
## G3_MarF 1 3867272 1
## G4_MarM 1 4517543 1
## G5_SepM 2 3518940 1
## G8_MarF 1 4920453 1
##
## $genes
## Length EffectiveLength
```

```
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      2065      1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      1612      1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1965      1632.281
##
##                               Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      3.253130
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      1.288037
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1.000000
## 9808 more rows ...
```

Obtain Counts Per Million to standardise count data comparison

```
myTPM_Season <- dge_Season$counts
# head(myTPM_Season)
```

4.4.2 Initial Data Exploration

Which values in myCPM are greater than 0.5? This produces a logical matrix with TRUEs and FALSEs. TRUE values are samples with > 0.5 counts in that sample per million

```
thresh_Season <- myTPM_Season > 0.5
# This produces a logical matrix with TRUEs and FALSEs
head(thresh_Season)
```

```
##                               G1_SepF G2_SepF G3_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      FALSE      TRUE      FALSE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   FALSE      FALSE      FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      TRUE      TRUE      TRUE
##
##                               G4_MarM G5_SepM G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      TRUE      FALSE      FALSE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   FALSE      FALSE      FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      TRUE      TRUE      TRUE
```

Insert colour palette for following visualisations consistency

```
library(RColorBrewer)
myPalette <- c("#999999", "#F0E442", "#56B4E9", "#009E73", "#D55E00",
               "#CC79A7")
```

Plot CPM distribution with logged CPM

```
# export the plot as png
png("plotDensities_Season.png", width = 600)
unfilteredExpr_Season <- cpm(dge_Season, log = T)
plotDensities(unfilteredExpr_Season, col = myPalette, legend = TRUE)
```

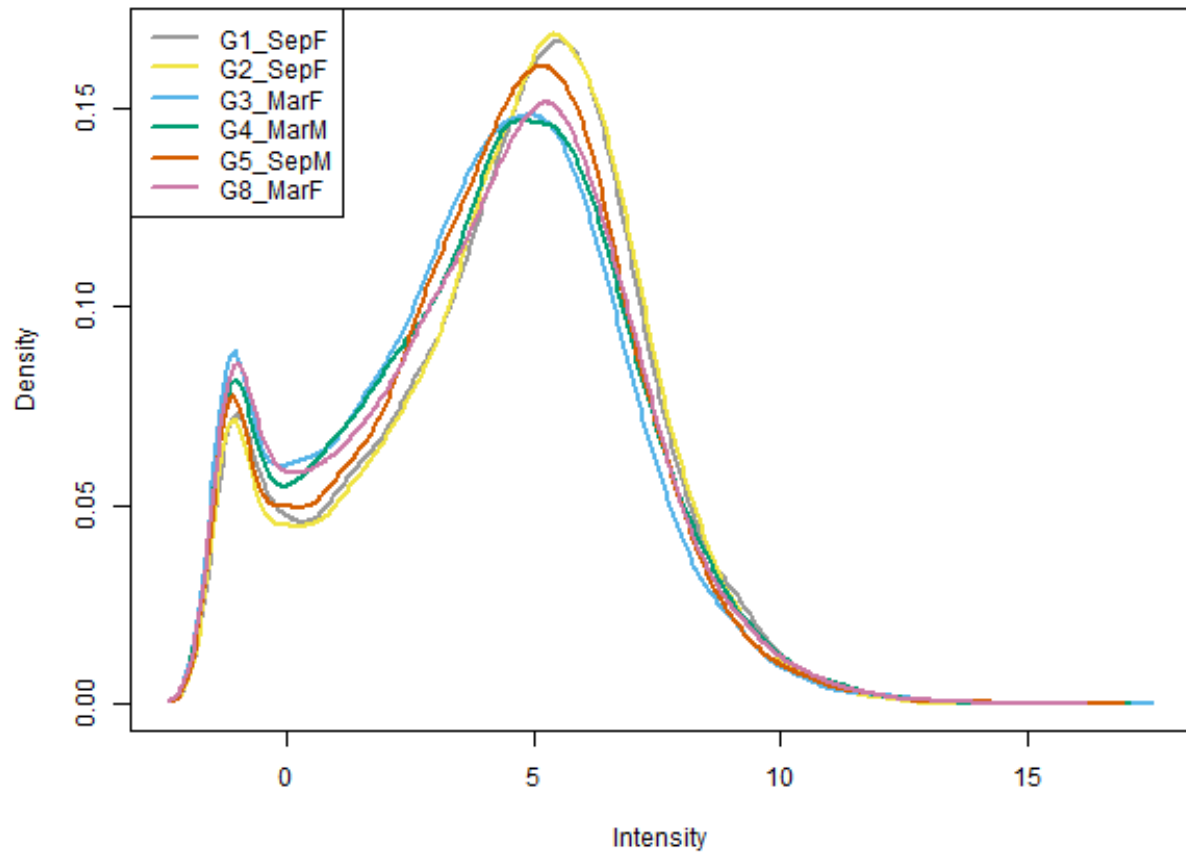


Figure 18: Log Counts per million Densities, for poly-a selected mRNA expression data from six *T. adeni* kidney samples

Visualise library sizes per sample

```
# export the plot as png
png("LibSize_Season.png", width = 600)
# The names argument tells the barplot to use the sample
# names on the x-axis The last argument rotates the axis
# names
barplot(dge_Season$samples$lib.size, names = colnames(dge_Season),
        las = 2, col = myPalette, legend = TRUE)
# title('Library size per sample')
```

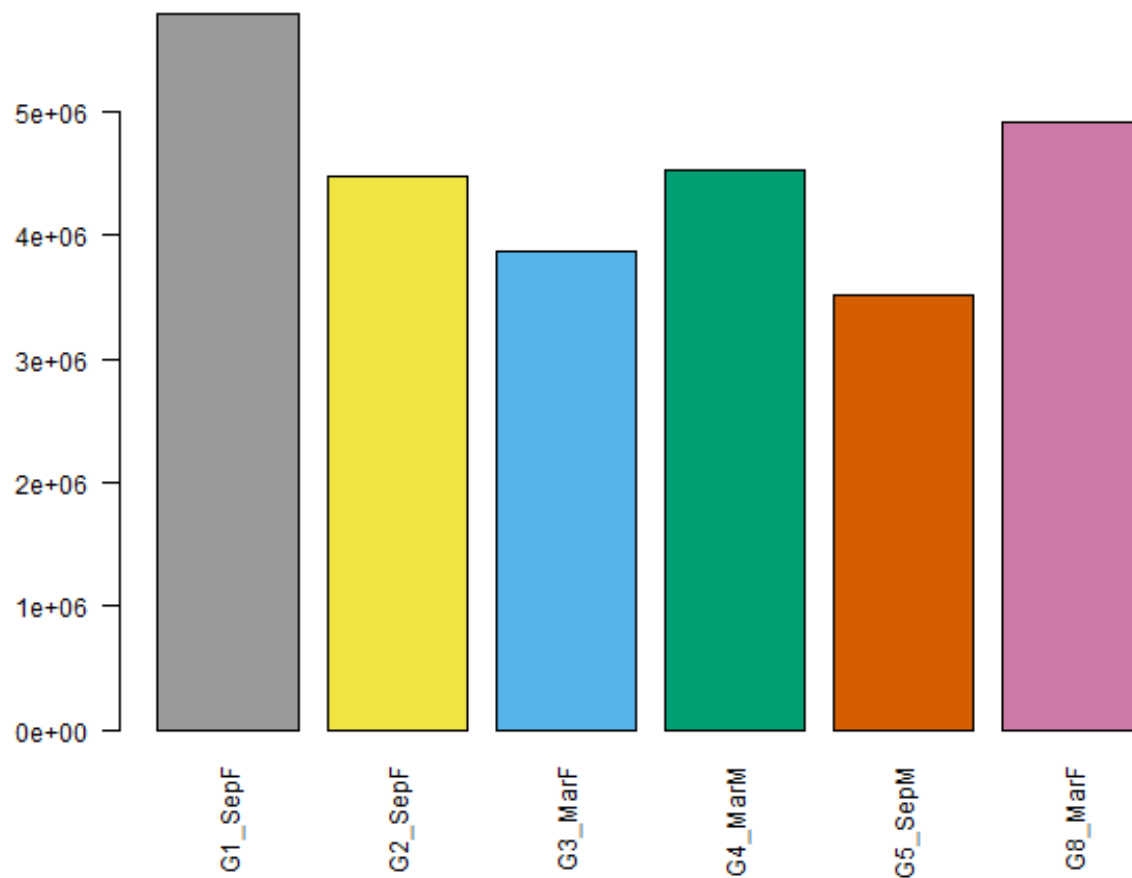


Figure 19: Library size of poly-a selected mRNA sequenced from kidney tissue of six *T. adelaidensis* individuals

Visualise total estimated transcript counts per sample

```
# export the plot as png
png("boxplot_Season.png", width = 600)
boxplot(dge_Season$counts, col = myPalette, las = 2, legend = TRUE)
# title('Boxplot of transcript total estimated counts')
```

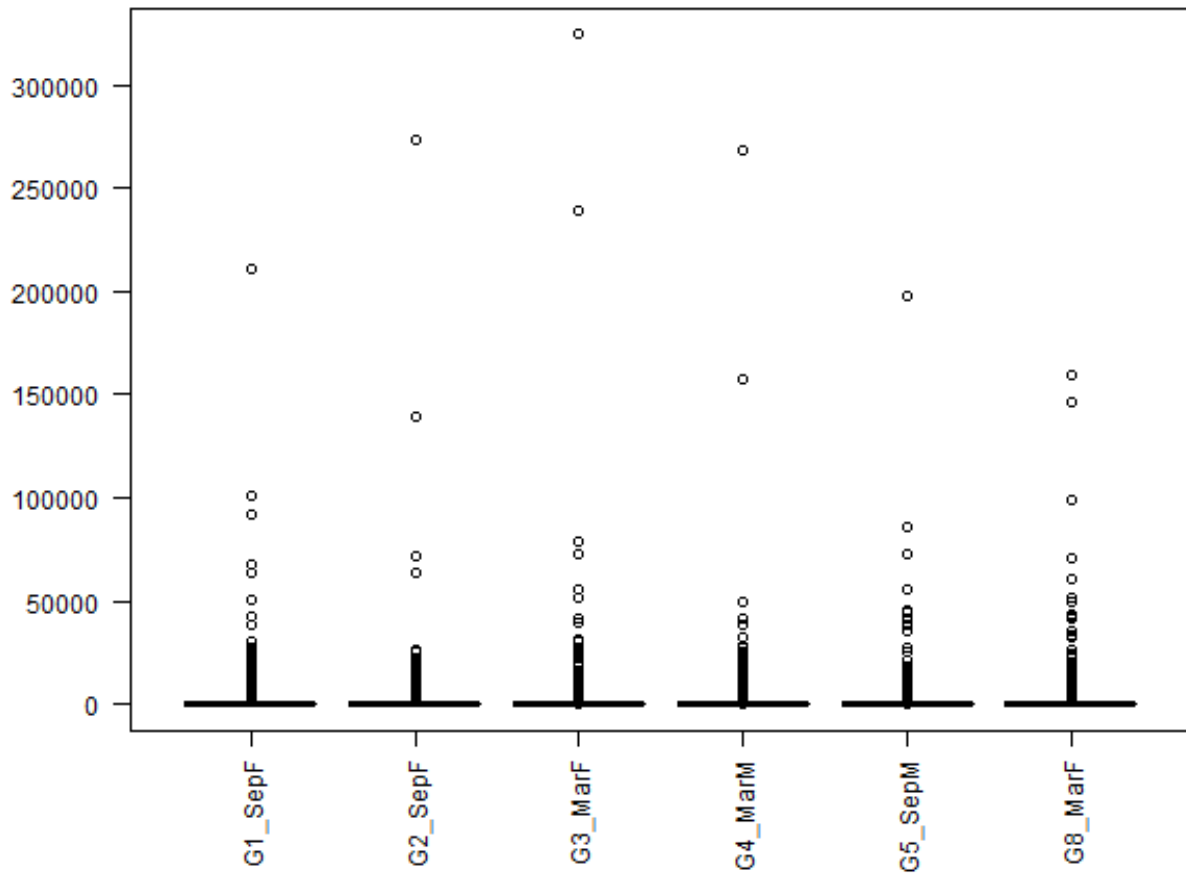


Figure 20: Total estimated counts for poly-a selected mRNA expression data from six *T. adelaidensis* kidney samples

Visualise logged total estimated transcript counts per sample

```
# Get log2 counts per million
logcounts_Season <- cpm(dge_Season, log = TRUE)
# Check distributions of samples using boxplots export the
# plot as png
png("boxplot-logcounts_Season.png", width = 600)
boxplot(logcounts_Season, xlab = "", ylab = "Log2 counts per million",
        las = 2, col = myPalette, legend = TRUE)
# Let's add a blue horizontal line that corresponds to the
# median logCPM
abline(h = median(logcounts_Season), col = myPalette, legend = TRUE)
# title('Boxplots of log Counts Per Million')
```

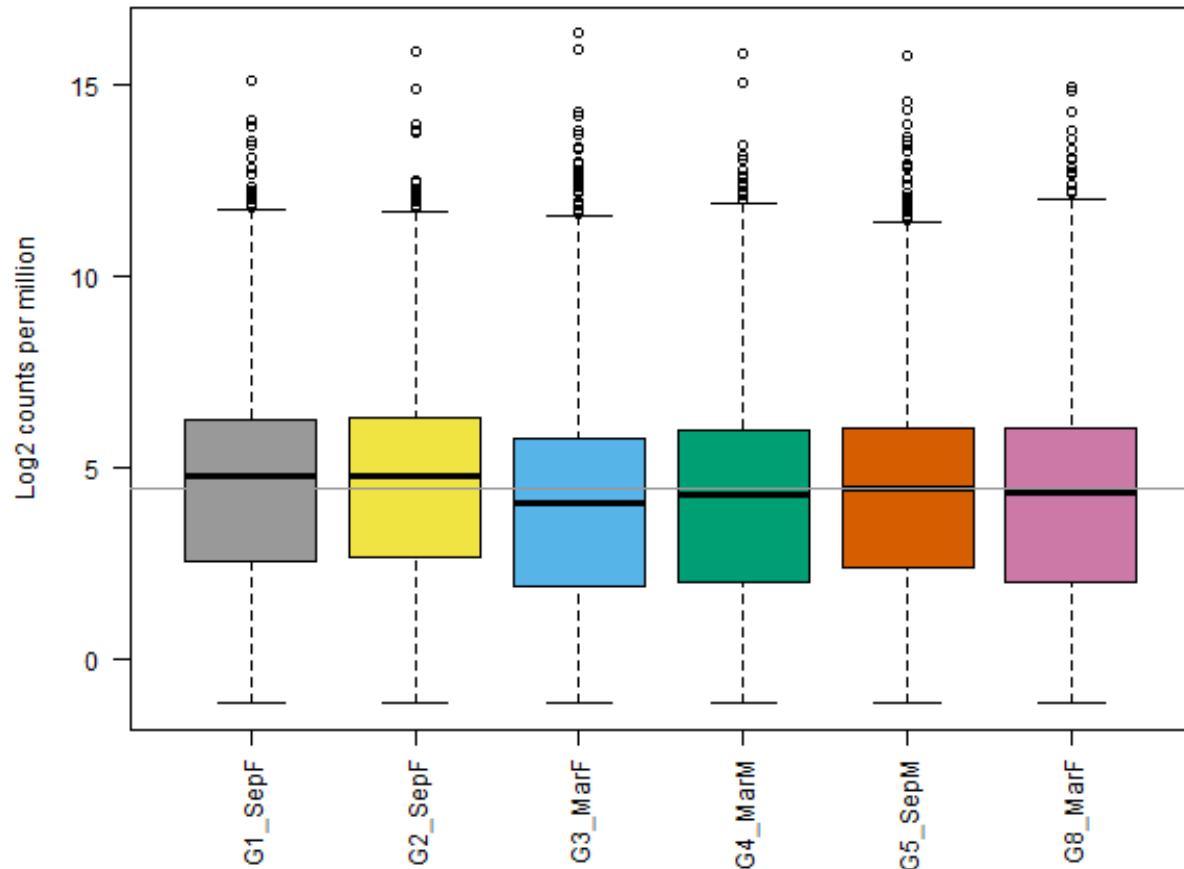


Figure 21: Log2 of estimated counts per million, for poly-a selected mRNA expression data from six *T. adelaidensis* kidney samples

Visualise sample variation using an MDS plot

```
# export the plot as png
png("MDS_Season.png", width = 600)
plotMDS(dge_Season, col = myPalette)
```

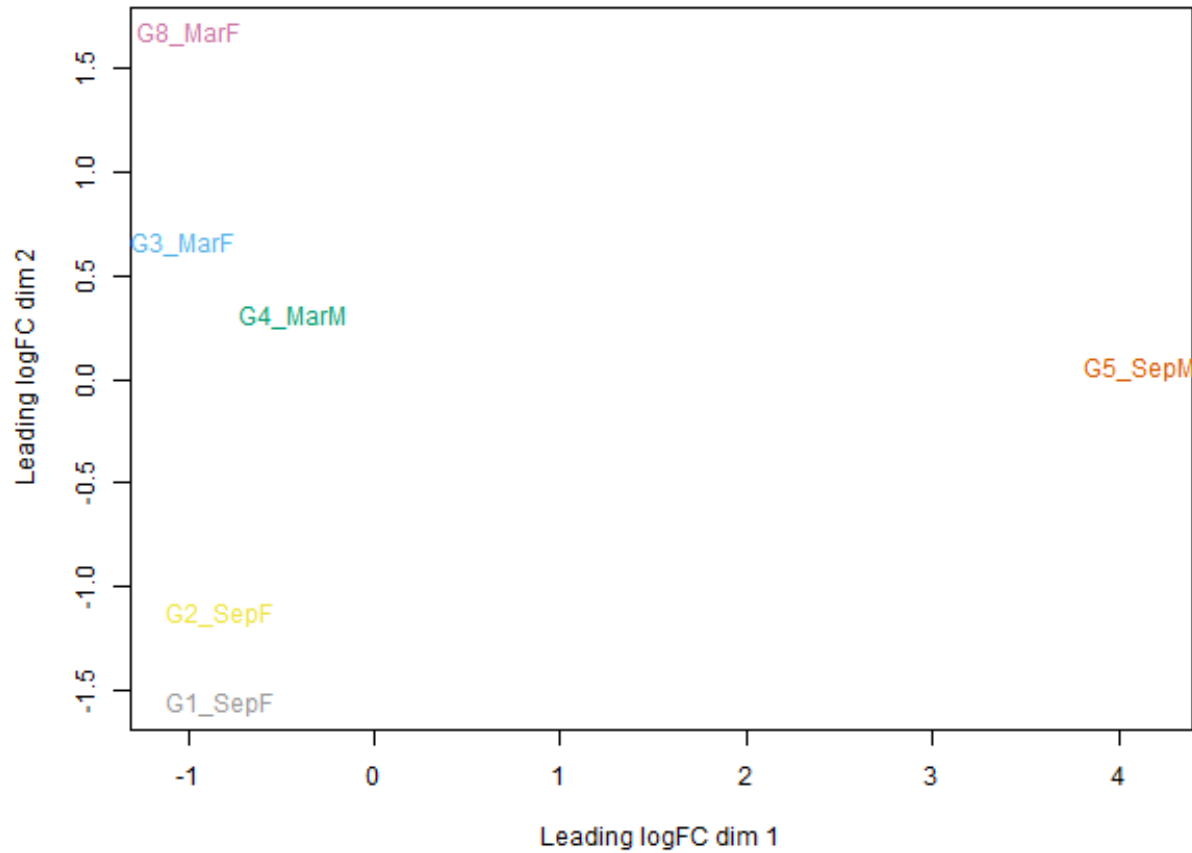


Figure 22: MDS plot of variation among six *T. adelaidensis* individuals

Prepare data for a HeatMap (based off of [this document](#))

Estimate the variance for each row in the logcounts matrix

```
var_genes_season <- apply(logcounts_Season, 1, var)
head(var_genes_season)
```

```
## PB.2.1|002537|path0:1-1624(+)|transcript/18304
## 2.129658e-01
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564
## 2.533823e-01
## PB.10.1|004815|path1:5-1713(+)|transcript/17534
## 1.112634e-01
## PB.11.1|004815|path2:1-3039(+)|transcript/3426
## 8.949062e-01
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401
## 2.129924e-30
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993
## 6.973540e-01
```

Transcript IDs for the top 250 most variable transcripts

```
select_var_decT_season <- names(sort(var_genes_season, decreasing = TRUE))[1:250]
head(select_var_decT_season)
```

```
## [1] "PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227"
## [2] "PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961"
## [3] "PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195"
## [4] "PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702"
## [5] "PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823"
## [6] "PB.966.1|257403|path2:1-1611(+)|transcript/18188"
```

Subset logcounts matrix

```
highly_variable_lcpm_season <- logcounts_Season[select_var_decT_season,
]
dim(highly_variable_lcpm_season)
```

```
## [1] 250 6
```

```
head(highly_variable_lcpm_season)
```

```
##
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227  G1_SepF  G2_SepF
## PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961  8.4777062 -1.17531272
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195  2.6269095  2.65902383
## PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702  7.5221514 -1.17531272
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823  0.1566818 -0.65153689
## PB.966.1|257403|path2:1-1611(+)|transcript/18188  -0.1472913  0.03140609
##
##                                     G3_MarF  G4_MarM
```

```
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227 -0.04654642 -0.5650195
## PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961 0.96325415 0.2590954
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 2.63110803 10.8339962
## PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702 -0.52485253 -0.5919392
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823 -1.17531272 4.0999879
## PB.966.1|257403|path2:1-1611(+)|transcript/18188 -0.07249537 -0.1405300
##
## G5_SepM G8_MarF
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227 11.56898532 -0.63723533
## PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961 7.49680086 7.98334543
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 10.93486157 2.02845409
## PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702 -0.01304149 8.04184470
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823 9.85798545 -0.62606240
## PB.966.1|257403|path2:1-1611(+)|transcript/18188 10.40291931 -0.01877115
```

Reorder columns to cluster Season factor groups, and sexes

```
col.order_season <- c("G1_SepF", "G2_SepF", "G5_SepM", "G3_MarF",
  "G8_MarF", "G4_MarM")
highly_variable_lcpm_season <- highly_variable_lcpm_season[,
  col.order_season]
```

```
head(highly_variable_lcpm_season)
```

```
##
## G1_SepF G2_SepF
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227 0.7541505 0.82792179
## PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961 8.4777062 -1.17531272
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 2.6269095 2.65902383
## PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702 7.5221514 -1.17531272
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823 0.1566818 -0.65153689
## PB.966.1|257403|path2:1-1611(+)|transcript/18188 -0.1472913 0.03140609
##
## G5_SepM G3_MarF
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227 11.56898532 -0.04654642
## PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961 7.49680086 0.96325415
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 10.93486157 2.63110803
## PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702 -0.01304149 -0.52485253
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823 9.85798545 -1.17531272
## PB.966.1|257403|path2:1-1611(+)|transcript/18188 10.40291931 -0.07249537
##
## G8_MarF G4_MarM
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227 -0.63723533 -0.5650195
## PB.5298.2|c8653e|path1:2-1500(+)|transcript/19961 7.98334543 0.2590954
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 2.02845409 10.8339962
## PB.5264.3|c67b04|path4:35-1046(+)|transcript/22702 8.04184470 -0.5919392
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823 -0.62606240 4.0999879
## PB.966.1|257403|path2:1-1611(+)|transcript/18188 -0.01877115 -0.1405300
```

Colour palette

```
mypalette <- brewer.pal(11, "RdYlBu")
morecols <- colorRampPalette(mypalette)
```

Colour vector for season_group factor variable: note - factor groups changed to match column reordering in highly_variable_lcpm March=1 September=2

```
Reordered_season_group <- factor(c(2, 2, 2, 1, 1, 1))  
col.cell_season <- c("chartreuse3", "orange")[Reordered_season_group]
```

Create the HeatMap

```
png(file = "High_var_genes.heatmap-season.png", width = 7.5 *
    300, height = 5 * 300, res = 300, pointsize = 8)
# 5 x 300 pixels height = 5*300, res = 300, # 300 pixels per
# inch
heatmap.2(highly_variable_lcpm_season, col = rev(morecols(50)),
    Colv = FALSE, trace = "none", main = "Top 250 most variable genes across samples",
    ColSideColors = col.cell_season, scale = "row", margins = c(8,
        4), srtCol = 45, labRow = "", )
```

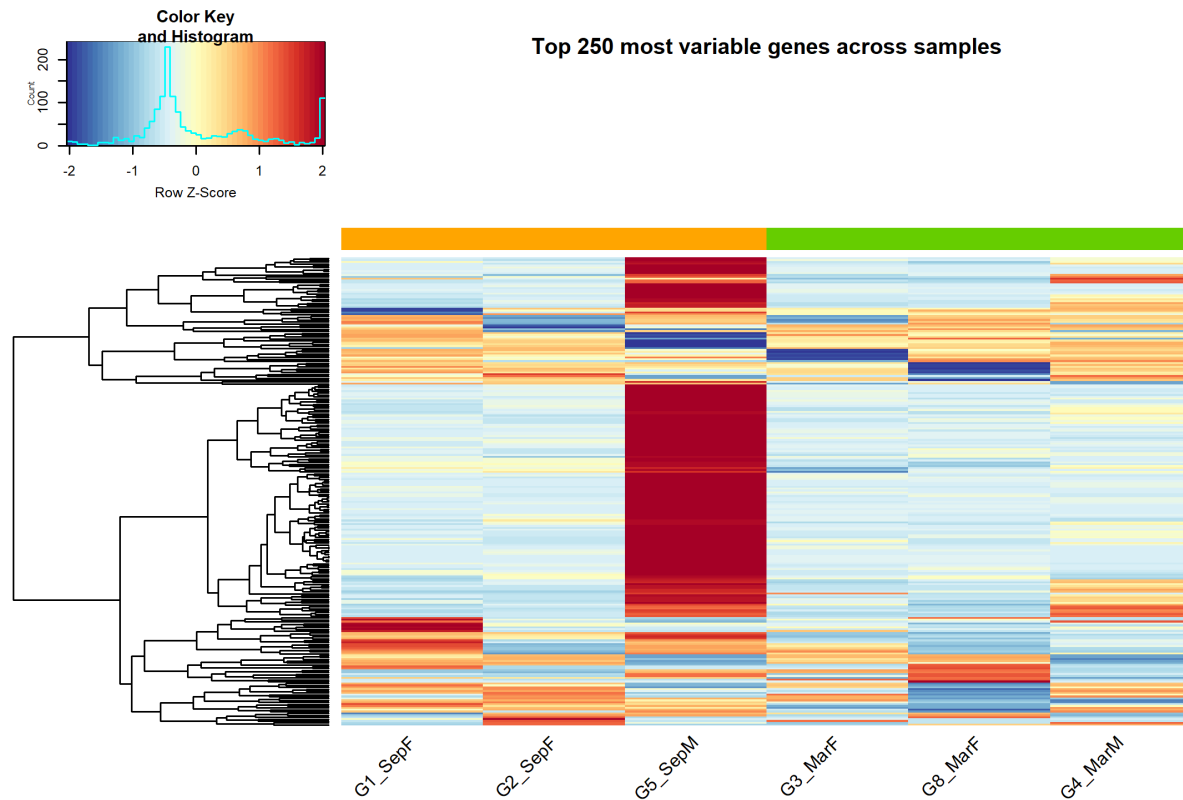


Figure 23: Top 250 most variable transcripts among six *T. adelaidensis* individuals, based on total counts per million values

Transcript IDs for the bottom 250 most variable transcripts = i.e the least variable transcripts

```
select_var_decF_season <- names(sort(var_genes_season, decreasing = FALSE))[1:250]
head(select_var_decF_season)
```

```
## [1] "PB.11.2|004815|path2:1047-3035(+)|transcript/13401"
## [2] "PB.28.1|016cdc|path0:1-842(+)|transcript/23336"
## [3] "PB.32.1|017c97|path0:1-1679(+)|transcript/17216"
## [4] "PB.40.1|019024|path0:1-4793(+)|transcript/8273"
## [5] "PB.130.4|056771|path1:13-5910(+)|transcript/2903"
## [6] "PB.159.1|068d5c|path0:1-2199(+)|transcript/10689"
```

Subset logcounts matrix

```
low_variable_lcpm_season <- logcounts_Season[select_var_decF_season,
]
dim(low_variable_lcpm_season)
```

```
## [1] 250 6
```

```
head(low_variable_lcpm_season)
```

```
##                               G1_SepF  G2_SepF
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 -1.175313 -1.175313
## PB.28.1|016cdc|path0:1-842(+)|transcript/23336      -1.175313 -1.175313
## PB.32.1|017c97|path0:1-1679(+)|transcript/17216     -1.175313 -1.175313
## PB.40.1|019024|path0:1-4793(+)|transcript/8273      -1.175313 -1.175313
## PB.130.4|056771|path1:13-5910(+)|transcript/2903    -1.175313 -1.175313
## PB.159.1|068d5c|path0:1-2199(+)|transcript/10689    -1.175313 -1.175313
##                               G3_MarF  G4_MarM
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 -1.175313 -1.175313
## PB.28.1|016cdc|path0:1-842(+)|transcript/23336      -1.175313 -1.175313
## PB.32.1|017c97|path0:1-1679(+)|transcript/17216     -1.175313 -1.175313
## PB.40.1|019024|path0:1-4793(+)|transcript/8273      -1.175313 -1.175313
## PB.130.4|056771|path1:13-5910(+)|transcript/2903    -1.175313 -1.175313
## PB.159.1|068d5c|path0:1-2199(+)|transcript/10689    -1.175313 -1.175313
##                               G5_SepM  G8_MarF
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 -1.175313 -1.175313
## PB.28.1|016cdc|path0:1-842(+)|transcript/23336      -1.175313 -1.175313
## PB.32.1|017c97|path0:1-1679(+)|transcript/17216     -1.175313 -1.175313
## PB.40.1|019024|path0:1-4793(+)|transcript/8273      -1.175313 -1.175313
## PB.130.4|056771|path1:13-5910(+)|transcript/2903    -1.175313 -1.175313
## PB.159.1|068d5c|path0:1-2199(+)|transcript/10689    -1.175313 -1.175313
```

Reorder columns to cluster Season factor groups, and sexes

```
col.order_season <- c("G1_SepF", "G2_SepF", "G5_SepM", "G3_MarF",
  "G8_MarF", "G4_MarM")
low_variable_lcpm_season <- low_variable_lcpm_season[, col.order_season]
```

```
head(low_variable_lcpm_season)
```

```
##                               G1_SepF   G2_SepF
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 -1.175313 -1.175313
## PB.28.1|016cdc|path0:1-842(+)|transcript/23336      -1.175313 -1.175313
## PB.32.1|017c97|path0:1-1679(+)|transcript/17216     -1.175313 -1.175313
## PB.40.1|019024|path0:1-4793(+)|transcript/8273      -1.175313 -1.175313
## PB.130.4|056771|path1:13-5910(+)|transcript/2903    -1.175313 -1.175313
## PB.159.1|068d5c|path0:1-2199(+)|transcript/10689    -1.175313 -1.175313
##                               G5_SepM    G3_MarF
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 -1.175313 -1.175313
## PB.28.1|016cdc|path0:1-842(+)|transcript/23336     -1.175313 -1.175313
## PB.32.1|017c97|path0:1-1679(+)|transcript/17216    -1.175313 -1.175313
## PB.40.1|019024|path0:1-4793(+)|transcript/8273     -1.175313 -1.175313
## PB.130.4|056771|path1:13-5910(+)|transcript/2903   -1.175313 -1.175313
## PB.159.1|068d5c|path0:1-2199(+)|transcript/10689   -1.175313 -1.175313
##                               G8_MarF    G4_MarM
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 -1.175313 -1.175313
## PB.28.1|016cdc|path0:1-842(+)|transcript/23336     -1.175313 -1.175313
## PB.32.1|017c97|path0:1-1679(+)|transcript/17216    -1.175313 -1.175313
## PB.40.1|019024|path0:1-4793(+)|transcript/8273     -1.175313 -1.175313
## PB.130.4|056771|path1:13-5910(+)|transcript/2903   -1.175313 -1.175313
## PB.159.1|068d5c|path0:1-2199(+)|transcript/10689   -1.175313 -1.175313
```

Colour palette

```
mypalette <- brewer.pal(11, "RdYlBu")
morecols <- colorRampPalette(mypalette)
```

Colour vector for season_group factor variable: note - factor groups changed to match column reordering in low_variable_lcpm March=1 September=2

```
Reordered_season_group <- factor(c(2, 2, 2, 1, 1, 1))
col.cell_season <- c("chartreuse3", "orange")[Reordered_season_group]
```


Create the HeatMap

```
png(file = "Low_var_genes.heatmap-season.png", width = 7.5 *
    300, height = 5 * 300, res = 300, pointsize = 8)
# 5 x 300 pixels height = 5*300, res = 300, # 300 pixels per
# inch
heatmap.2(low_variable_lcpm_season, col = rev(morecols(50)),
    Colv = FALSE, trace = "none", main = "Bottom 250 most variable genes across samples",
    ColSideColors = col.cell_season, scale = "row", margins = c(8,
    4), srtCol = 45, labRow = "", )
```

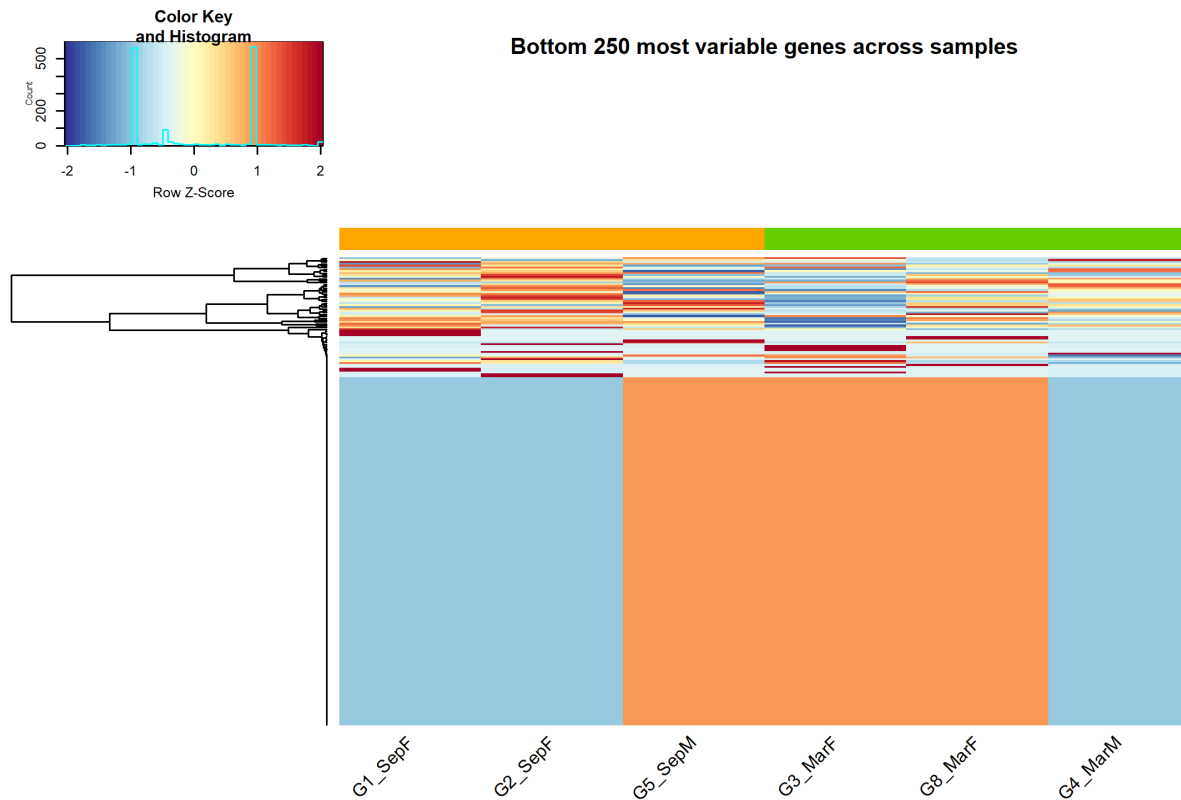


Figure 24: Bottom 250 most variable transcripts among six *T. adelaidensis* individuals, based on total counts per million values

4.4.3 Calculations

```
# Data with actual counts, not TPM = 'dge'
head(dge_Season)
```

```
## An object of class "DGEList"
## $counts
##
##          G1_SepF    G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.682740e-07 1.363036
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.639676e+01 28.388835
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 7.179928e+01 33.065458
##
##          G3_MarF    G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.0000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.4894863 0.9870162
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 10.4093586 4.4945206
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.0000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 10.3920012 12.2814560
##
##          G5_SepM    G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 29.50226 12.42200
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 10.39200 25.50764
##
## $samples
##      group lib.size norm.factors
## G1_SepF    2  5796184           1
## G2_SepF    2  4480604           1
## G3_MarF    1  3867272           1
## G4_MarM    1  4517543           1
## G5_SepM    2  3518940           1
## G8_MarF    1  4920453           1
##
## $genes
##
##          Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1530 1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 2065 1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 1612 1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2999 2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1965 1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 2714 2381.281
##
##          Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.253130
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 1.288037
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 1.058506
```

```
head(myTPM_Season)
```

```
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      G1_SepF      G2_SepF
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      8.410000e+02  746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      3.682740e-07   1.363036
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      2.639676e+01  28.388835
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  0.000000e+00   0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      7.179928e+01  33.065458
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      G3_MarF      G4_MarM
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      49.0000000    113.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      645.0000000   997.0000000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      0.4894863     0.9870162
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  10.4093586    4.4945206
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      0.0000000     0.0000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      10.3920012    12.2814560
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      G5_SepM      G8_MarF
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      77.00000      147.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      415.00000    1577.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      0.00000      0.00000
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  29.50226     12.42200
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      0.00000      0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      10.39200     25.50764
```

Filter out genes with less than 5 reads in 2 samples for each transcript

```
filter_Season <- apply(dge_Season, 1, function(x) length(x[x >
5]) >= 2)
filtered_Season <- dge_Season[filter_Season, ]
head(filtered_Season)
```

```
## An object of class "DGEList"
## $counts
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      G1_SepF      G2_SepF      G3_MarF
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      179.00000    118.00000    49.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      841.00000    746.00000    645.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      26.39676     28.38883    10.40936
## PB.14.1|006283|path0:1-1955(+)|transcript/13821     71.79928     33.06546    10.39200
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774      708.75635    442.39668    326.26755
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774      624.00000    527.00000    351.00000
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      G4_MarM      G5_SepM      G8_MarF
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      113.0000000   77.00000     147.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426      997.0000000   415.00000    1577.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      4.494521     29.50226     12.42200
## PB.14.1|006283|path0:1-1955(+)|transcript/13821     12.281456    10.39200     25.50764
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774      508.756184    306.91270    552.99585
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774      336.000000    281.00000    719.00000
##
## $samples
##      group lib.size norm.factors
## G1_SepF      2  5796184          1
## G2_SepF      2  4480604          1
```

```
## G3_MarF      1  3867272      1
## G4_MarM      1  4517543      1
## G5_SepM      2  3518940      1
## G8_MarF      1  4920453      1
##
## $genes
##                                     Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      2065      1732.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       2999      2666.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       2714      2381.281
## PB.14.1|006283|path0:1-1955(+)|transcript/13821      1920      1587.281
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774       3583      3250.281
##
##                                     Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       1.288037
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       1.058506
## PB.14.1|006283|path0:1-1955(+)|transcript/13821      1.084999
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774       1.000000
```

Run calculations

```
dge_Season <- calcNormFactors(dge_Season)
dge_Season <- estimateCommonDisp(dge_Season)
dge_Season <- estimateTagwiseDisp(dge_Season)
```

4.4.4 Differentially expressed genes:

View the top ten ‘differentially expressed genes’

```
results_Season <- exactTest(dge_Season)
topTags(results_Season)
```

```
## Comparison of groups: 2-1
##                                     Length EffectiveLength
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      1940      1607.2807
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      2000      1667.2807
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825     1608      1275.2807
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462       3086      2753.2807
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387        1086       753.2807
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548     1750      1417.2807
## PB.2003.2|4ab8e8|path0:1458-4018(+)|transcript/6743       2535      2202.2807
## PB.499.1|124cf9|path4:1-2080(+)|transcript/11611         2071      1738.2807
## PB.3885.3|918572|path1:1406-3716(+)|transcript/9242      2278      1945.2807
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227       1771      1438.2807
##
##                                     Overdispersion      logFC
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      3.166184  4.937224
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      1.621394  2.891897
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825     1.000000  5.024827
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462       1.044347  2.898051
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387        1.242289  3.088832
```

```

## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548      1.000000  4.336311
## PB.2003.2|4ab8e8|path0:1458-4018(+)|transcript/6743      1.179403  2.473788
## PB.499.1|124cf9|path4:1-2080(+)|transcript/11611        1.358840  2.448778
## PB.3885.3|918572|path1:1406-3716(+)|transcript/9242      5.786371 -5.384475
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227      1.141272 11.520001
##
##                                     logCPM      PValue
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      5.413096 3.967872e-09
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      5.501158 5.425738e-08
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825    3.551103 2.893073e-07
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462      5.042973 8.245973e-07
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387      4.962496 8.724447e-07
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548    3.466335 2.370305e-06
## PB.2003.2|4ab8e8|path0:1458-4018(+)|transcript/6743      5.788082 3.024335e-06
## PB.499.1|124cf9|path4:1-2080(+)|transcript/11611        5.654586 3.673456e-06
## PB.3885.3|918572|path1:1406-3716(+)|transcript/9242      1.510773 3.716870e-06
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227      9.130702 3.880790e-06
##
##                                     FDR
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      3.893673e-05
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      2.662138e-04
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825    9.463241e-04
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462      1.712260e-03
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387      1.712260e-03
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548    3.488516e-03
## PB.2003.2|4ab8e8|path0:1458-4018(+)|transcript/6743      3.488516e-03
## PB.499.1|124cf9|path4:1-2080(+)|transcript/11611        3.488516e-03
## PB.3885.3|918572|path1:1406-3716(+)|transcript/9242      3.488516e-03
## PB.4788.1|b34d56|path10:2-1859(+)|transcript/15227      3.488516e-03

```

Output the top 25 table of values based on calculated PValue

```

tab_Season <- topTags(results_Season, n = 25, sort.by = "PValue")
head(tab_Season)

```

```

## Comparison of groups: 2-1
##
##                                     Length EffectiveLength
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      1940      1607.2807
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      2000      1667.2807
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825    1608      1275.2807
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462      3086      2753.2807
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387      1086       753.2807
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548    1750      1417.2807
##
##                                     Overdispersion      logFC
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      3.166184 4.937224
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      1.621394 2.891897
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825    1.000000 5.024827
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462      1.044347 2.898051
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387      1.242289 3.088832
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548    1.000000 4.336311
##
##                                     logCPM      PValue
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836      5.413096 3.967872e-09
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418      5.501158 5.425738e-08
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825    3.551103 2.893073e-07
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462      5.042973 8.245973e-07

```

```
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387      4.962496 8.724447e-07
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548 3.466335 2.370305e-06
##                                                    FDR
## PB.3849.1|906dad|path19:1-2010(+)|transcript/12836    3.893673e-05
## PB.499.11|124cf9|path4:2108-4109(+)|transcript/7418    2.662138e-04
## PB.7745.1|transcript/17825:1-1637(+)|transcript/17825 9.463241e-04
## PB.8897.1|transcript/3462:1-3118(+)|transcript/3462    1.712260e-03
## PB.2869.1|6b0af0|path2:1-1117(+)|transcript/22387      1.712260e-03
## PB.7443.1|transcript/15548:1-1797(+)|transcript/15548 3.488516e-03
```

```
write.table(tab_Season, file = "Kgenelist-Top25_Season.txt")
```

Output the entire table of values

```
tab_Season_all <- topTags(results_Season, n = Inf, sort.by = "PValue")
write.table(tab_Season_all, file = "Kgenelist_Season.txt")
```

Output summary expression data

```
dim(results_Season)
```

```
## [1] 9813    3
```

```
summary(de_Season <- decideTests(results_Season))
```

```
##          2-1
## Down      25
## NotSig 9631
## Up        157
```

```
write.table(summary, file = "summary_Season.txt")
```

```
## Error in as.data.frame.default(x[[i]], optional = TRUE): cannot coerce class '"function"' to a data.frame
```

```
detags_Season <- rownames(dge_Season)[as.logical(de_Season)]
head(detags_Season)
```

```
## [1] "PB.147.1|0620ba|path0:1-1756(+)|transcript/23387"
## [2] "PB.193.1|07b09f|path0:1-2724(+)|transcript/21409"
## [3] "PB.193.7|07b09f|path0:5-2523(+)|transcript/22910"
## [4] "PB.222.2|0889ce|path13:1-894(+)|transcript/23126"
## [5] "PB.225.1|089810|path3:1-2255(+)|transcript/24270"
## [6] "PB.329.1|0bd3d9|path3:1-656(+)|transcript/24223"
```

```
head(results_Season)
```

```
## An object of class "DGEEexact"
## $table
##
##          logFC      logCPM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 -0.01384282  4.6123326
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 -0.97428387  7.5877885
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 -0.37656880 -0.9010547
## PB.11.1|004815|path2:1-3039(+)|transcript/3426  1.38669548  2.1987804
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000000 -1.2129277
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993  0.79643615  2.5138513
##
##          PValue
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 0.98619206
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 0.05750860
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 1.00000000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 0.06492398
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1.00000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 0.26397635
##
## $comparison
## [1] "1" "2"
##
## $genes
##
##          Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1530 1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 2065 1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 1612 1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2999 2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1965 1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 2714 2381.281
##
##          Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.253130
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 1.288037
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 1.058506
```

Visualise the logged Fold Change against Average logged counts per million, in expression of transcripts between season 1 (March/April) and 2 (September) using plotsmear: red indicates PValue < 0.05

```
png("plotsmear_Season.png", width = 600)
plotSmear(results_Season, de.tags = detags_Season)
```

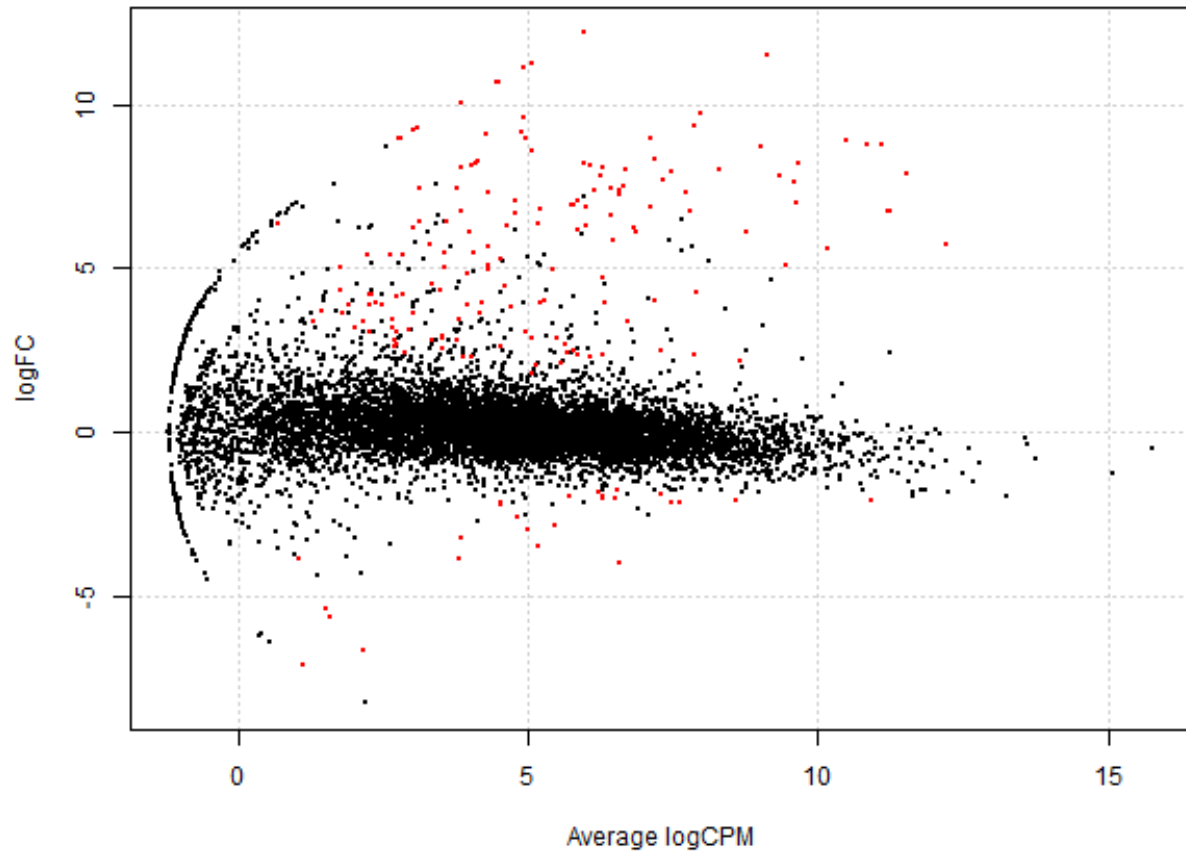


Figure 25: Log Fold Change vs average Log counts per million for expression level comparing September to March season variables among six *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values <0.05.

Visualise differential expression of transcripts between season 1 (March/April) and 2 (September) with negative Logged PValue against Logged Fold Change in expression: Red indicates a transcript with PValue < 0.05, and Log Fold change > 2

```
volcanoData_Season <- cbind(results_Season$table$logFC,
  ↪ -log10(results_Season$table$PValue))
colnames(volcanoData_Season) <- c("logFC", "negLogPval")
DEGs_Season <- results_Season$table$PValue < 0.05 & abs(results_Season$table$logFC) >
  2

point.col_Season <- ifelse(DEGs_Season, "red", "black")
png("volcanoData_Season.png", width = 600)
plot(volcanoData_Season, pch = 16, col = point.col_Season, cex = 0.5)
# pch is shape 1-26 are vectors, cex is size
```

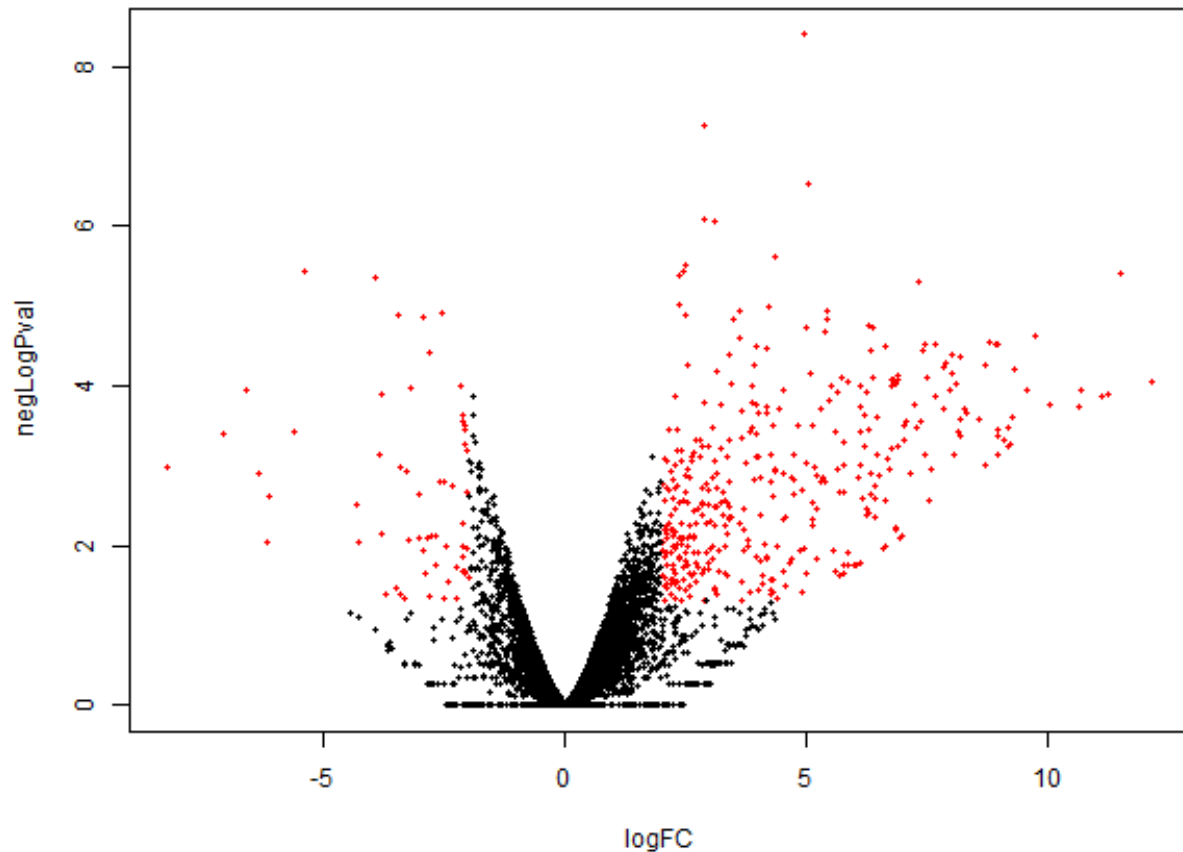


Figure 26: Negative Log of the p-value against Log Fold Change in expression level comparing September to March season variables among six *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values < 0.05 and also a log fold change > 2.

4.4.5 Group Factor: Sex

Create the EdgeR DGE list for use in subsequent analyses

```
dge_Sex <- DGEList(counts = Kcaught$counts/Kcaught$annotation$Overdispersion,
  genes = Kcaught$annotation, group = sex_group)
# View(dge_Sex) names(dge_Sex)
dge_Sex
```

```
## An object of class "DGEList"
## $counts
##
##          G1_SepF    G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.682740e-07 1.363036
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.639676e+01 28.388835
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
##
##          G3_MarF    G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.0000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.4894863 0.9870162
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 10.4093586 4.4945206
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.0000000
##
##          G5_SepM    G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 147.000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 1577.000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 0.000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 29.50226 12.422
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 0.000
## 9808 more rows ...
##
## $samples
##      group lib.size norm.factors
## G1_SepF    1  5796184           1
## G2_SepF    1  4480604           1
## G3_MarF    1  3867272           1
## G4_MarM    2  4517543           1
## G5_SepM    2  3518940           1
## G8_MarF    1  4920453           1
##
## $genes
##
##          Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1530 1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 2065 1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 1612 1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2999 2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1965 1632.281
##
##          Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.253130
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 1.288037
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 1.000000
## 9808 more rows ...
```

Obtain Counts Per Million to standardise count data comparison

```
myTPM_Sex <- dge_Sex$counts
# head(myTPM_Sex)
```

4.4.6 Initial Data Exploration

Which values in myCPM are greater than 0.5? This produces a logical matrix with TRUEs and FALSEs. TRUE values are samples with > 0.5 counts in that sample per million

```
thresh_Sex <- myTPM_Sex > 0.5
# This produces a logical matrix with TRUEs and FALSEs
head(thresh_Sex)
```

```
##                                G1_SepF G2_SepF G3_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534     FALSE      TRUE     FALSE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  FALSE     FALSE     FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      TRUE      TRUE      TRUE
##                                G4_MarM G5_SepM G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      TRUE      TRUE      TRUE
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      TRUE      TRUE      TRUE
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      TRUE     FALSE     FALSE
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       TRUE      TRUE      TRUE
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401  FALSE     FALSE     FALSE
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      TRUE      TRUE      TRUE
```

Raw Counts per million and library sizes will be consistent with the Season comparison above. There is no need to plot them again however scripts are included to create separate R objects for downstream application.

CPM distribution with logged CPM

```
# export the plot as png
png("plotDensities_Sex.png", width = 600)
unfilteredExpr_Sex <- cpm(dge_Sex, log = T)
plotDensities(unfilteredExpr_Sex, col = myPalette, legend = TRUE)
```

Library sizes per sample

```
# export the plot as png
png("LibSize_Sex.png", width = 600)
# The names argument tells the barplot to use the sample
# names on the x-axis The last argument rotates the axis
# names
barplot(dge_Sex$samples$lib.size, names = colnames(dge_Sex),
        las = 2, col = myPalette, legend = TRUE)
```

Total estimated transcript counts per sample

```
# export the plot as png
png("boxplot_Sex.png", width = 600)
boxplot(dge_Sex$counts, col = myPalette, las = 2, legend = TRUE)
# title('Boxplot of transcript total estimated counts')
```

Logged total estimated transcript counts per sample

```
# Get log2 counts per million
logcounts_Sex <- cpm(dge_Sex, log = TRUE)
# Check distributions of samples using boxplots export the
# plot as png
png("boxplot-logcounts_Sex.png", width = 600)
boxplot(logcounts_Sex, xlab = "", ylab = "Log2 counts per million",
        las = 2, col = myPalette, legend = TRUE)
# Let's add a blue horizontal line that corresponds to the
# median logCPM
abline(h = median(logcounts_Sex), col = myPalette, legend = TRUE)
# title('Boxplots of log Counts Per Million')
```

4.4.7 Calculations

```
# Data with actual counts, not TPM = 'dge'
head(dge_Sex)
```

```
## An object of class "DGEList"
## $counts
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.790000e+02 118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 8.410000e+02 746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 3.682740e-07 1.363036
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2.639676e+01 28.388835
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00 0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 7.179928e+01 33.065458
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 49.0000000 113.0000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 645.0000000 997.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.4894863 0.9870162
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 10.4093586 4.4945206
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.0000000 0.0000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 10.3920012 12.2814560
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 77.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 415.00000 1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534 0.00000 0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 29.50226 12.42200
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000 0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 10.39200 25.50764
##
## $samples
## group lib.size norm.factors
```

```
## G1_SepF      1  5796184      1
## G2_SepF      1  4480604      1
## G3_MarF      1  3867272      1
## G4_MarM      2  4517543      1
## G5_SepM      2  3518940      1
## G8_MarF      1  4920453      1
##
## $genes
##                                     Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1530      1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      2065      1732.281
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      1612      1279.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       2999      2666.281
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1965      1632.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       2714      2381.281
##
##                                     Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      3.253130
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       1.288037
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993       1.058506
```

```
head(myTPM_Sex)
```

```
##                                     G1_SepF      G2_SepF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304   1.790000e+02  118.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564   8.410000e+02  746.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534   3.682740e-07   1.363036
## PB.11.1|004815|path2:1-3039(+)|transcript/3426    2.639676e+01  28.388835
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.000000e+00   0.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993    7.179928e+01  33.065458
##
##                                     G3_MarF      G4_MarM
## PB.2.1|002537|path0:1-1624(+)|transcript/18304   49.00000000  113.0000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564  645.00000000 997.0000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534    0.4894863    0.9870162
## PB.11.1|004815|path2:1-3039(+)|transcript/3426   10.4093586    4.4945206
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000000    0.0000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993   10.3920012   12.2814560
##
##                                     G5_SepM      G8_MarF
## PB.2.1|002537|path0:1-1624(+)|transcript/18304    77.00000    147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564  415.00000  1577.00000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534    0.00000    0.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426    29.50226    12.42200
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401 0.00000    0.00000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993    10.39200    25.50764
```

Filter out genes with less than 5 reads in 2 samples for each transcript

```
filter_Sex <- apply(dge_Sex, 1, function(x) length(x[x > 5]) >=
2)
filtered_Sex <- dge_Sex[filter_Sex, ]
head(filtered_Sex)
```

```
## An object of class "DGEList"
## $counts
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 179.00000 118.00000 49.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 841.00000 746.00000 645.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 26.39676 28.38883 10.40936
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 71.79928 33.06546 10.39200
## PB.14.1|006283|path0:1-1955(+)|transcript/13821 708.75635 442.39668 326.26755
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774 624.00000 527.00000 351.00000
##
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 113.000000 77.00000 147.00000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 997.000000 415.00000 1577.00000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 4.494521 29.50226 12.42200
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 12.281456 10.39200 25.50764
## PB.14.1|006283|path0:1-1955(+)|transcript/13821 508.756184 306.91270 552.99585
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774 336.000000 281.00000 719.00000
##
## $samples
## group lib.size norm.factors
## G1_SepF 1 5796184 1
## G2_SepF 1 4480604 1
## G3_MarF 1 3867272 1
## G4_MarM 2 4517543 1
## G5_SepM 2 3518940 1
## G8_MarF 1 4920453 1
##
## $genes
## Length EffectiveLength
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1530 1197.281
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 2065 1732.281
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 2999 2666.281
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 2714 2381.281
## PB.14.1|006283|path0:1-1955(+)|transcript/13821 1920 1587.281
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774 3583 3250.281
##
## Overdispersion
## PB.2.1|002537|path0:1-1624(+)|transcript/18304 1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564 1.000000
## PB.11.1|004815|path2:1-3039(+)|transcript/3426 1.288037
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993 1.058506
## PB.14.1|006283|path0:1-1955(+)|transcript/13821 1.084999
## PB.15.1|006e23|path0:1-3614(+)|transcript/1774 1.000000
```

Run calculations

```
dge_Sex <- calcNormFactors(dge_Sex)
dge_Sex <- estimateCommonDisp(dge_Sex)
dge_Sex <- estimateTagwiseDisp(dge_Sex)
```

4.4.8 Differentially expressed genes:

View the top ten 'differentially expressed genes'

```
results_Sex <- exactTest(dge_Sex)
topTags(results_Sex)
```

```
## Comparison of groups: 2-1
```

```
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195      Length EffectiveLength
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021      1103      770.2807
## PB.602.1|167374|path2:1-1363(+)|transcript/21155      1304      971.2807
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546      1705     1372.2807
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575       3066     2733.2807
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146      2280     1947.2807
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860      905      572.3308
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901      896      563.3308
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823    1337     1004.2807
## PB.2180.1|51f17e|path0:1-673(+)|transcript/24169      469      146.6201
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195      Overdispersion      logFC
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021      1.229384      8.651305
## PB.602.1|167374|path2:1-1363(+)|transcript/21155      2.615079      9.331293
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546      1.000000     10.079098
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575       1.000000      9.038182
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146      1.000000      9.027936
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860      1.039333      8.125067
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901      1.539932      8.299550
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823    6.372376     10.820135
## PB.2180.1|51f17e|path0:1-673(+)|transcript/24169      1.076723      6.617776
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195      logCPM      PValue
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021      9.417486     8.355261e-45
## PB.602.1|167374|path2:1-1363(+)|transcript/21155      5.478547     1.527083e-39
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546      4.803360     1.529268e-34
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575       6.697083     6.072447e-32
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146      5.015148     1.451210e-26
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860      3.754487     3.194292e-22
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901      7.398882     4.491435e-22
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823    7.068196     2.267222e-19
## PB.2180.1|51f17e|path0:1-673(+)|transcript/24169      7.443993     3.231998e-17
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195      FDR
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021      8.199017e-41
## PB.602.1|167374|path2:1-1363(+)|transcript/21155      7.492632e-36
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546      5.002236e-31
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575       1.489723e-28
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146      2.848144e-23
## PB.637.1|1826ff|path3:1-1155(+)|transcript/22860      5.224265e-19
## PB.4152.1|9a8a98|path2:1-992(+)|transcript/22901      6.296350e-19
## PB.2493.6|5d0eeb|path5:27-5305(+)|transcript/20823    2.781031e-16
## PB.2180.1|51f17e|path0:1-673(+)|transcript/24169      2.523955e-14
## PB.2180.1|51f17e|path0:1-673(+)|transcript/24169      7.924228e-14
```

Output the top 25 table of values based on calculated PValue


```
tab_Sex <- topTags(results_Sex, n = 25, sort.by = "PValue")
head(tab_Sex)
```

```
## Comparison of groups: 2-1
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 1136 803.2807
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021 1103 770.2807
## PB.602.1|167374|path2:1-1363(+)|transcript/21155 1304 971.2807
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546 1705 1372.2807
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575 3066 2733.2807
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146 2280 1947.2807
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 1.229384 8.651305
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021 2.615079 9.331293
## PB.602.1|167374|path2:1-1363(+)|transcript/21155 1.000000 10.079098
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546 1.000000 6.074782
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575 1.000000 9.038182
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146 1.000000 9.027936
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 9.417486 8.355261e-45
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021 5.478547 1.527083e-39
## PB.602.1|167374|path2:1-1363(+)|transcript/21155 4.803360 1.529268e-34
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546 6.697083 6.072447e-32
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575 5.015148 1.451210e-26
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146 3.754487 3.194292e-22
##
## PB.2769.1|6751e7|path0:4-1327(+)|transcript/21195 8.199017e-41
## PB.2770.1|6751e7|path1:1-1210(+)|transcript/22021 7.492632e-36
## PB.602.1|167374|path2:1-1363(+)|transcript/21155 5.002236e-31
## PB.725.1|1b70a4|path2:1-1745(+)|transcript/16546 1.489723e-28
## PB.979.1|25edbb|path0:1-3100(+)|transcript/3575 2.848144e-23
## PB.5826.1|dd0edc|path1:1-2280(+)|transcript/9146 5.224265e-19
```

```
write.table(tab_Sex, file = "Kgenelist-Top25_Sex.txt")
```

Output the entire table of values

```
tab_Sex_all <- topTags(results_Sex, n = Inf, sort.by = "PValue")
write.table(tab_Sex_all, file = "Kgenelist_Sex.txt")
```

Output summary expression data

```
dim(results_Sex)
```

```
## [1] 9813 3
```

```
summary(de_Sex <- decideTests(results_Sex))
```

```
## 2-1
## Down 25
## NotSig 9467
## Up 321
```



```
## PB.2.1|002537|path0:1-1624(+)|transcript/18304      1.000000
## PB.3.2|00361c|path0:43-2240(+)|transcript/10564      1.000000
## PB.10.1|004815|path1:5-1713(+)|transcript/17534      3.253130
## PB.11.1|004815|path2:1-3039(+)|transcript/3426       1.288037
## PB.11.2|004815|path2:1047-3035(+)|transcript/13401   1.000000
## PB.13.1|004c10|path2:1-2811(+)|transcript/4993      1.058506
```

Visualise the logged Fold Change against Average logged counts per million, in expression of transcripts between sex 1 (Female) and 2 (Male) using plotsmear: red indicates PValue < 0.05

```
png("plotsmear_Sex.png", width = 600)
plotSmear(results_Sex, de.tags = detags_Sex)
```

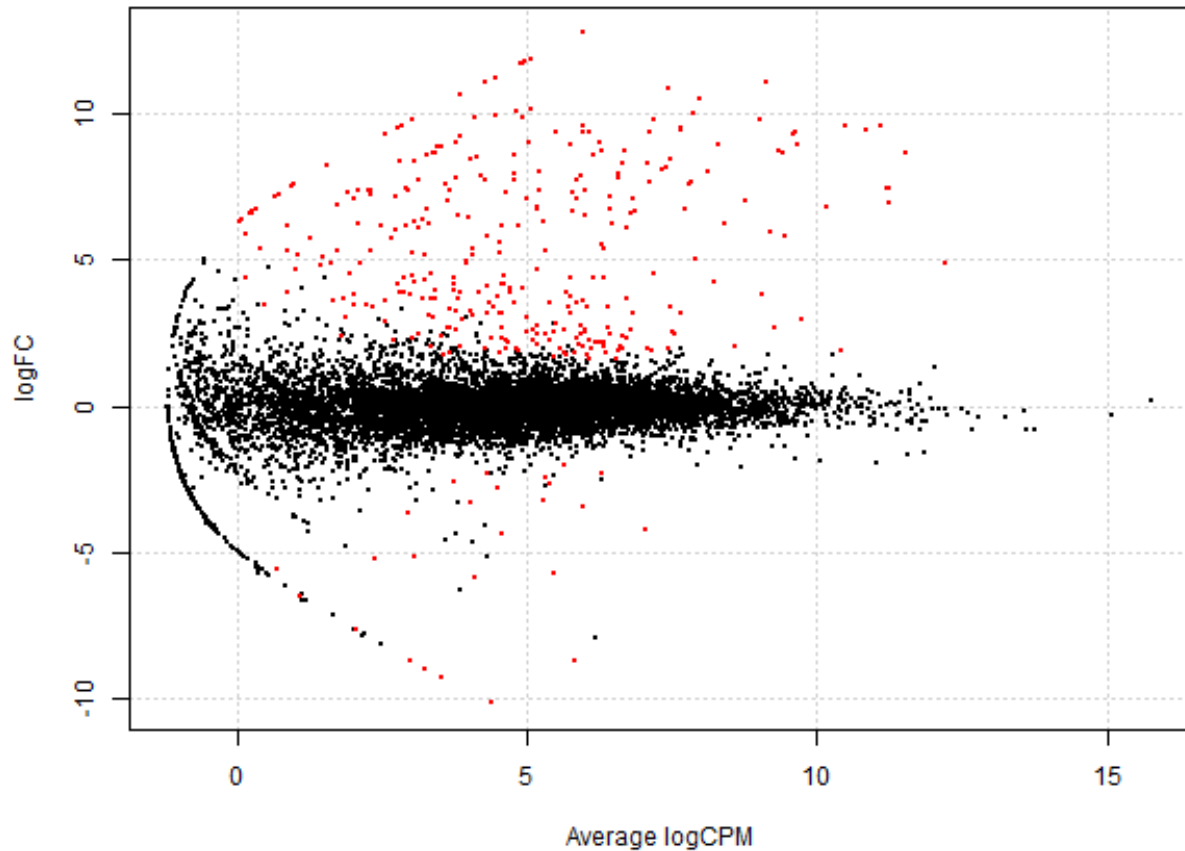


Figure 27: Log Fold Change vs average Log counts per million for expression level comparing Male to Female sex variables among six *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values < 0.05.

Visualise differential expression of transcripts between sex 1 (Female) and 2 (Male) with negative Logged PValue against Logged Fold Change in expression: Red indicates a transcript with PValue < 0.05, and Log Fold change > 2

```
volcanoData_Sex <- cbind(results_Sex$table$logFC, -log10(results_Sex$table$PValue))
colnames(volcanoData_Sex) <- c("logFC", "negLogPval")
DEGs_Sex <- results_Sex$table$PValue < 0.05 & abs(results_Sex$table$logFC) >
  2

point.col_Sex <- ifelse(DEGs_Sex, "red", "black")
png("volcanoData_Sex.png", width = 600)
plot(volcanoData_Sex, pch = 16, col = point.col_Sex, cex = 0.5)
# pch is shape 1-26 are vectors, cex is size
```

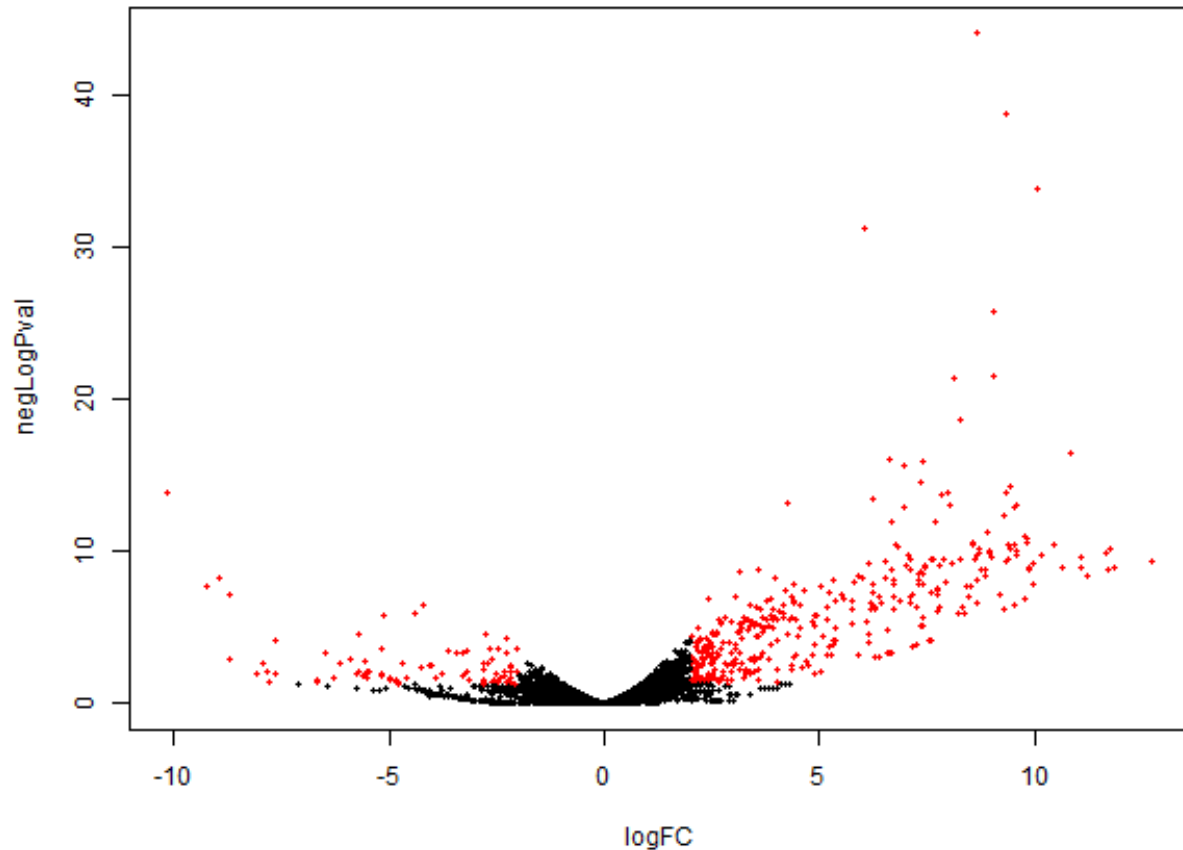


Figure 28: Negative Log of the p-value against Log Fold Change in expression level comparing Male to Female sex variables among six *T. adelaidensis* individuals. Red dots indicate transcripts with a significant change over season, with p-values <0.05 and also a log fold change >2.

4.5 Sequencing batch effects

Note G1, G2, G3, G4, G5 and G8 were all sequenced together on an Illumina Hiseq and library prep was done with the same kit. G6 and G7 were prepared in a separate batch using a different kit and sequenced on an Illumina Hiseq with fewer samples, and have a much higher read depth. These 2 represent a September Male, and March Female, respectively.

The removal of G6 and G7 from the dataset results in two groups of 3 per season, each with 2 females and 1 male.
