## Appendix A

# Bioinformatics workflow

Table A.1: `Velvet` assembly statistics of contigs longer than 1 000 bp for a single lane of paired 76 bp sequences from *Eucalytpus* xylem tissue reads trimmed to different lengths. The assemblies were all performed with a kmer setting of 41. These statistics were used to calculate the assembly score, as discussed in Section 2.3.3 on page 56 and presented in Table 2.6.

| Read length | N | Sum | Min | 1st Quartile | Median | 3rd Quartile | Max | Mean | N50 |
|---|---|---|---|---|---|---|---|---|---|
| 50 | 2 644 | 3 853 938 | 1 000 | 1 118 | 1 300 | 1 611.5 | 6 772 | 1 457.61 | 1 424 |
| 55 | 5 045 | 7 722 735 | 1 000 | 1 138 | 1 342 | 1 709 | 8 078 | 1 530.77 | 1 512 |
| 60 | 6 458 | 10 216 572 | 1 000 | 1 149 | 1 371 | 1 770 | 8 241 | 1 582.00 | 1 574 |
| 65 | 7 165 | 11 547 759 | 1 000 | 1 160.5 | 1 393 | 1 804 | 11 049 | 1 611.69 | 1 609 |
| 70 | 7 548 | 12 288 379 | 1 000 | 1 162 | 1 395 | 1 823 | 11 008 | 1 628.03 | 1 627 |
| 76 | 7 857 | 12 917 451 | 1 000 | 1 164 | 1 415 | 1 848 | 9 925 | 1 644.06 | 1 643 |

## Appendix B

# Extendinator

The `Python` script used for a coverage-assisted re-assembly of contigs, also known as ”extendinator” is provided on the following pages. A graphical representation of the process is provided in Figure 3.1. The program selects an entry from the assembled contigs file, and performs an alignment of the short reads to the selected contig and calculated the true coverage of the contig. After alignment, the program extracts all the short reads together with their respective mate-pairs from a `Berkeley` database, and sends the contig as well as the sampled short reads to `Velvet` with the calculated coverage parameter to perform a directed contig assembly.

```
"""
    Extendinator:
        An interative approach to try and improve contig sizes.

    1) Map all the short reads to a contig, get the reads that mapped.
    2) Extract the pairs
        2a) Connect to a database, get all the reads that match
            2ab) Convert to fasta
    3) Assemble with Velvet
        3a) Parameter range cc_9 ec [9,50,100,200,1000]
        3b)Join the longest assemblies in one file (best_assembly.fa)


    @requires: Biopython
    @requires: bsddb3
    @author: charles.hefer@gmail.com
"""


import sys
import getopt
from datetime import datetime
from Bio import SeqIO
import os
import subprocess
import time
from multiprocessing import Process
import bsddb3

global usage
usage = """
Extendinator: An iterative approach to extext Velvet contigs

Usage: python start_extendinator.py [options] short_reads.fa contigs.fa

++Bowtie options++
\t-f\t--short_reads_type\Either fa for fasta, or fq for fastq, default is fa
\t-b\t--bowtie_mismatch\tNumber of mismatches allowed during the bowtie matching of the short reads to the contig
\t-m\t--max_bowtie_processes\tMax number of bowtie processes
\t-t\t--threads\tNumber of threads for Bowtie, this times the #processes = number of CPUs

++Global options++
\t-h\t\t--help\tThis help message
"""
global cwd
cwd = os.getcwd()
global bowtie_build_cmd
bowtie_build_cmd = "/usr/local/bowtie/bowtie-build"
global bowtie_cmd
bowtie_cmd = "/usr/local/bowtie/bowtie"
global bdb
bdb = "./pairs.db"


class UsageEx(Exception):
    """The standard exception"""
    def __init__(self, msg):
        """
            Sets the exception message
            @var msg: The exception message thrown
        """
        self.msg = msg

def now():
    """
        Converts the current time to a string format

        @requires: datetime.datetime
        @return: A string reprepsentation of datetime.now()
    """
    curr_time = datetime.now()
    return curr_time.strftime("%c")


def get_number_of_processes(process):
    """Returns the number of processes returned by grep
    ps -eaf | grep processname
    Subtract the grep itself, and the extra newline that comes through.
    @var process: The process to grep for
    @type process: String

    @return: The number of process as an int
    """
    num_procs = subprocess.Popen("ps -eaf | grep '%s'" % process, shell=True, stdout=subprocess.PIPE)
    output = num_procs.stdout.readlines()
    i = len(output) - 2
    return i
```

```
def multiprocess_start(cmd):
    """
        Executes the command as a multiprocess
    """
    process = subprocess.call(cmd, shell=True, stdout=subprocess.PIPE)
    return process


def prepare_bowtie_build(dir, filename, max_bowtie_processes):
    """
        Sets the command to run bowtie build on the contig
    """
    #the resulting build has a _ewbt extention
    #and is in the ./bowtie dir
    cmd = "%s %s %s_ewbt" % (bowtie_build_cmd, dir+filename, "./bowtie/"+filename)

    while get_number_of_processes("bowtie_build") >= max_bowtie_processes:
        time.sleep(5)
    process = Process(target=multiprocess_start, args=(cmd,))
    process.start()


def prepare_bowtie_align(short_reads_filename, ewbt_filename, bowtie_mismatch, max_bowtie_processes, threads, short_reads_filetype):
    """
        Aligns the short reads to the file
    """
    cmd = "%s -%s -n %s --alfa=%s.match -p %s %s %s %s.out" % (bowtie_cmd,
                                                                short_reads_filetype,
                                                              bowtie_mismatch,
                                                              "bowtie/"+ewbt_filename,
                                                              threads,
                                                              "bowtie/"+ewbt_filename,
                                                              short_reads_filename,
                                                              "bowtie/"+ewbt_filename)

    while get_number_of_processes("bowtie") >= max_bowtie_processes:
        time.sleep(5)
    process = Process(target=multiprocess_start, args=(cmd,))
    process.start()

def save_biopython_entry(dir, entry, format):
    """
        Saves the biopython object in the correct format
    """
    try:
        handle = open(dir+"/" + entry.name + "/" + entry.name+".fa", "w")
    except IOError, e:
        print(e)
        sys.exit()
    SeqIO.write([entry], handle, format)
    handle.close()
    return dir+"/"+entry.name + "/" + entry.name + ".fa"

def bowtie_watcher(contig, max_bowtie_processes, bowtie_mismatch,short_reads_filename):
    """
        Somehow manages the number of bowtie executables that can be started
    """
    #Get the current number of bowties running
    current = get_number_of_processes("bowtie")
    while current > max_bowtie_processes:
        time.sleep(10)
    else:
        bowtie_dir = prepare_bowtie_dir(contig.name)
        contig_file_name = save_biopython_entry(bowtie_dir, contig, "fasta")
        bowtie_builder(contig_file_name)
        bowtie_aligner(contig_file_name, bowtie_mismatch, short_reads_filename)

def split_fasta_file(handle, dir):
    """
        Takes every entry, create an output file for that entry in the dir
    """
    entries = SeqIO.parse(handle, "fasta")
    for entry in entries:
        out = open(dir+entry.name.replace(" ","").replace("\\","").replace("|","_").replace("/","_").replace("(","_").replace(")","_")
        SeqIO.write([entry], out, "fasta")
        out.close()

def create_mates_file(base_name, database_name):
    """ Iterates over ./bowtie/base_name.match, and returns all the mated
        that is found in the berkeley database
        Creates a file basename.fa in ./mates
    """

    try:
        handle = open("./bowtie/%s.fa_ewbt.match" % (base_name),"r")
        out_handle = open("./mates/%s.fa" % (base_name),"w")
    except IOError, e:
        #No alignments found... can do nothing about that
        #should this be reported?
```

```
        return None
    entries = SeqIO.parse(handle, "fasta")

    mate_pairs = []
    pairs = bsddb3.hashopen(bdb, "r")
    for entry in entries:
        out_handle.write(">%s\n" % entry.name)
        out_handle.write("%s\n" % pairs[entry.name].split(",")[0])
        out_handle.write(">%s\n" % entry.name)
        out_handle.write("%s\n" % pairs[entry.name].split(",")[1])
    out_handle.close()

def faLen_stats(file):
    """
        Returns the result from running faLen on the file
        #TODO: Rewrite use subprocess
    """
    import popen2

    output = []

    cmd = "faLen < %s | stats" % (file)
    process = popen2.Popen3(cmd)
    process.wait()
    result = process.fromchild.readlines()
    for line in result:
        line = line.replace(" ","")
        output.append(line.split("=")[1].rstrip())
    output.append("\n")
    return output


def velveth_runner(filename, kmer):
    """
        Runs velveth on the file, hashing for the kmer
    """
    velvet_exe = "/usr/local/velvet/velveth"
    cmd = "%s ./velvet/%s/assembly %s -fasta -shortPaired ./mates/%s -long ./fasta/%s" % \
    (velvet_exe, filename, kmer, filename, filename)

    while get_number_of_processes("velveth") >= 20:
        time.sleep(5)
    process = Process(target=multiprocess_start, args=(cmd,))
    process.start()
    time.sleep(2)

def get_coverage(filename):
    """
        Returns the coverage value stored in ./mates/cov_stats.csv
    """
    file = open("./mates/cov_stats.csv", "r")
    for line in file:
        if filename in line:
            cols = line.split(",")
            contig_length = int(cols[1])
            bases = int(cols[2].rstrip())
    return bases/float(contig_length)


def velvetg_runner(filename):
    """
        Runs velvetg in the file, hashing for the kmer
    """
    velvet_exe = "/usr/local/velvet/velvetg"
    coverage = get_coverage(filename)

    cmd = "%s ./velvet/%s/assembly -ins_length 200 -ins_length_sd 80 -exp_cov %s -cov_cutoff 8" % \
    (velvet_exe, filename, coverage)

    print cmd

    while get_number_of_processes("velveth") >= 20:
        time.sleep(5)
    process = Process(target=multiprocess_start, args=(cmd,))
    process.start()

def save_longest_entry(entry_name, contigs_file, location):
    """
        Finds the longest entry in the contigs_file, rename it to the
        entry name [minus the extention] , and saves it in the locatoion
    """
    try:
        contigs_handle = open(contigs_file, "r")
        location_handle = open(location+"/%s" % entry_name, "w")
    except IOError, e:
        print(e)

    longest_entry = None
    longest_length = 0
```

```
    entries = SeqIO.parse(contigs_handle, "fasta")
    for entry in entries:
        if len(entry.seq) > longest_entry:
            longest_entry = entry
            longest_length = len(entry.seq)

    #Rename the longest_entry
    longest_entry.id = entry_name.replace(".fa","")
    longest_entry.name = ""
    longest_entry.description = ""
    #write to the location
    SeqIO.write([longest_entry], location_handle, "fasta")
    location_handle.close()
    contigs_handle.close()

    #update the report
    #remove the entries that did not grow for fasta
    #repeat = True

def main(argv = None):
    """
        The main program flow
    """
    print("%s Extendinator started" % now())

    #Get all the arguments
    if argv is None:
        argv = sys.argv
    try:
        try:
            opts, args = getopt.getopt(argv[1:], "b:h:m:t:f:",
                                        ["bowtie_mismatch=",
                                        "max_bowtie_processes=",
                                        "threads=",
                                        "short_reads_type"
                                        "help"])

            bowtie_mismatch = 2
            max_bowtie_processes = 1
            threads = "2"
            short_reads_filetype = "f"


            for opt, value in opts:
                if opt in ("-b", "--bowtie_mismatch"):
                    bowtie_mismatch = value
                if opt in ("-m","--max_bowtie_processes"):
                    max_bowtie_processes = int(value)
                if opt in ("-t", "--threads"):
                    threads = value
                if opt in ("-f", "--short_reads_type"):
                    if value == "fq":
                        short_reads_filetype = "q"
                if opt in ("-h", "--help"):
                    print(usage)
                    raise sys.exit()


        except getopt.error, e:
            print(e)
            raise UsageEx(e)

        #test the presence of the contigs and short read files
        try:
            print("%s Validating the short reads file: %s" % (now(), args[0]))
            short_reads_filename = cwd+"/"+args[0]
            short_reads_handle = open(args[0],"r")
            print("%s Validating the contigs file: %s" % (now(), args[1]))
            contigs_handle = open(args[1],"r")
        except IOError,e:
            print(e)
            raise UsageEx(e)

        #Prepare the directory structure
        #this can be made more intelligent
        try:
            os.system("rm -rf bowtie")
            os.system("rm -rf fasta")
            os.system("rm -rf mates")
            os.system("rm -rf velvet")
        except OSError:
            pass
        try:
            os.mkdir("bowtie")
            os.mkdir("fasta")
            os.mkdir("mates")
            os.mkdir("velvet")
        except OSError, e:
            os.system("rm -rf bowtie/*")
```

```
    os.system("rm -rf fasta/*")
    os.system("rm -rf mates/*")
    os.system("rm - rf velvet/*")

try:
    report_handle = open("report.csv","w")
except IOError, e:
    print(e)
    sys.exit()

#The step is to parse the contigs file
print("%s Parsing the contigs file into ./fasta" % now())
split_fasta_file(contigs_handle, "./fasta/")
contigs_handle.close()

fasta_entries = os.listdir("./fasta")

#generate an file with the initial lengths
print("%s Generate the initial report template" % now())
report_handle.write("Sequence_entry,init_length\n")
for fasta_file in fasta_entries:
    #get the sequence length
    entry_length = int(faLen_stats("./fasta/%s" % fasta_file)[1])
    report_handle.write("%s,%s\n" % (fasta_file, entry_length))
report_handle.close()

while 1:
    fasta_entries = os.listdir("./fasta")

    if len(fasta_entries) == 0:
        break

    print("%s Building the Bowtie indices" % now())
    for fasta_entry in fasta_entries:
        prepare_bowtie_build("./fasta/", fasta_entry, max_bowtie_processes)
    time.sleep(2)
    #Need to wait for all the processes to finish
    while get_number_of_processes("bowtie_build") > 0:
        time.sleep(5)

    print("%s Running Bowtie aligner with %s mismatches" % (now(), bowtie_mismatch))
    print("Stdout from Bowtie to follow...this can be ignored")
    for fasta_entry in fasta_entries:
        prepare_bowtie_align(short_reads_filename, fasta_entry+"_ewbt", bowtie_mismatch, max_bowtie_processes, threads, short_
        #give the os time to register
        time.sleep(2)
    #Need to wait for all the processes to finish
    time.sleep(5)
    while get_number_of_processes("bowtie") > 0:
        time.sleep(5)
    print("%s Done with the Bowtie aligner" % (now()))


    print("%s Preparing to find the mates" % (now()))
    for fasta_entry in fasta_entries:
        #change the name
        fasta_entry = ".".join(fasta_entry.split(".")[:-1])
        create_mates_file(fasta_entry, bdb)
    time.sleep(5)
    print("%s Mates now in ./mates" % now())

    print("%s Calculating the coverage statistics" % now())
    mate_entries = os.listdir("mates")
    try:
        mate_entries.remove("cov_stats.csv")
    except:
        pass
    cov_stats_handle = open("mates/cov_stats.csv", "w")
    cov_stats_handle.write("Contig_name,Lenght,Bases_in_mates")
    cov_stats_handle.write("\n")
    for mate_entry in mate_entries:
        contig_length = int(faLen_stats("./fasta/%s" % mate_entry)[1])
        pairs_bases = int(faLen_stats("./mates/%s" % mate_entry)[1])
        cov_stats_handle.write("%s,%s,%s" % (mate_entry, contig_length, pairs_bases))
        cov_stats_handle.write("\n")
    cov_stats_handle.close()
    time.sleep(5)
    print("%s Finished with the coverage statistics, in ./mates/cov_stats.csv" % now())

    print("%s Preparing for the velvet hashing " % now())
    for entry in mate_entries:
        try:
            os.mkdir("./velvet/%s" % entry)
        except OSError, e:
            pass
        velveth_runner(entry,"31")
    time.sleep(5)
    while get_number_of_processes("velveth") > 0:
        time.sleep(5)
```

```
            print("%s Done with the velvet hashing" % now())

            print("%s Preparing for the velvet assembly " % now())
            for entry in mate_entries:
                velvetg_runner(entry)
            time.sleep(5)
            while get_number_of_processes("velveth") > 0:
                time.sleep(5)
            print("%s Done with the velvet assembly" % now())

            print("%s Getting the longest entry for every assembly" % now())
            for entry in mate_entries:
                #the contigs resides in velvet/entry/assembly/contigs.fa
                save_longest_entry(entry, "velvet/%s/assembly/contigs.fa" % entry, "fasta/")
            print("%s All the longest entries now back in ./fasta" % now())

            print("%s Adding the newest data to the report.csv file" % now())
            #Append to the reports file
            os.system("mv ./report.csv ./report.csv.prev")
            reports_handle = open("report.csv.prev","r")
            report_out_handle = open("report.csv","w")
            report_out_handle.write("Sequence_entry,init_length\n")
            for line in reports_handle:
                if line.startswith("Sequence_entry"):
                    continue
                line = line.rstrip()
                cols = line.split(",")
                #the name of the entry is the first col
                try:
                    entry_length = int(faLen_stats("./fasta/%s" % cols[0])[1])
                    cols.append("%i" % entry_length)
                except IndexError, e:
                    pass
                outline = ",".join(cols)
                report_out_handle.write(outline + "\n")
            report_out_handle.close()
            reports_handle.close()
            print("%s Updated the report.csv file" % now())

            #Now, check the report file, if the last entry is smaller or equal to
            #the second last entry, then call the entry finished
            #remove from ./fasta/
            #and append to finished_contigs.fa
            print("%s remove the contigs that does not want to grow any more" % now())
            report_handle = open("report.csv","r")
            for line in report_handle:
                if line.startswith("Sequence_entry"):
                    continue
                print line
                line = line.rstrip()
                cols = line.split(",")
                if int(cols[-1]) <= int(cols[-2]):
                    print cols[0]
                    os.system("less ./fasta/%s >> finished_contigs.fa" % cols[0])
                    os.system("rm ./fasta/%s" % cols[0])
                    os.system("rm ./mates/%s" % cols[0])
                    print os.listdir("fasta")
                    print os.listdir("mates")

            print("%s And start over again?" % now())
        print("%s Done" % now())


    except UsageEx, err:
        print(usage)

if __name__ == "__main__":
    if len(sys.argv) < 3:
        print(usage)
        sys.exit()
    else:
        sys.exit(main())
```

## Appendix C

# Transcriptome assembly

## C.1. Evaluating contig contiguity of the assembled transcript sequences

### C.1.1. Full length *Eucalyptus* cDNA sequences

The following table contains the 34 full length CDS sequences used to validate the assembly. The functional role of the 33 sequences ranges from transcription factors, transporter genes, structural and developmental proteins, indicating that the assembled transcriptome succesfully assembled near full length genes, including the 5' and 3' UTR regions for a wide variate of mRNA sequences.

| Accession | Contig_id | Description | length | FPKM |
|---|---|---|---|---|
| AB465730.1 | contig_87094 | Eucalyptus grandis AGL mRNA for agamous-like protein, complete cds. | 1184 | 17.98 |
| AB479542.1 | contig_10798 | Eucalyptus grandis mRNA for transcription factor Myb, complete cds. | 666 | 14.02 |
| AB479543.1 | contig_45922 | Eucalyptus grandis mRNA for transcription factor GRAS family protein, complete cds. | 1485 | 13.00 |
| AB479544.1 | contig_94920 | Eucalyptus grandis mRNA for 1-aminoacyclopropane-1-carboxylate oxidase, complete cds. | 1288 | 81.75 |

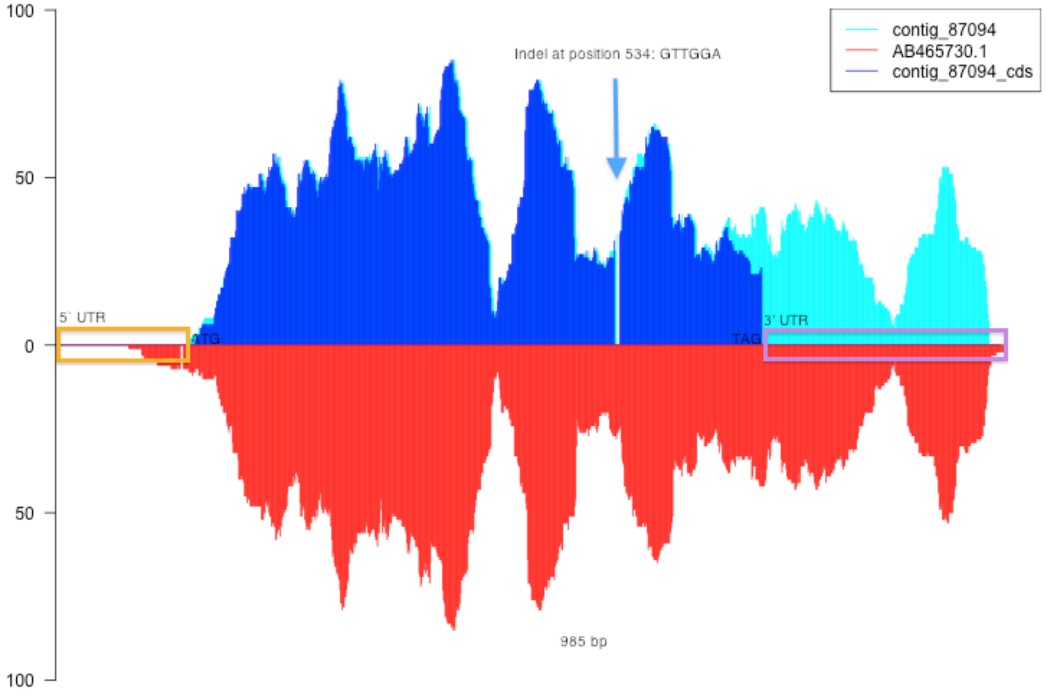
| | | | | |
|---|---|---|---|---|
| AB479545.1 | contig_56935 | Eucalyptus grandis mRNA for transcription factor squamosa promoter binding protein like, complete cds | 1940 | 43.35 |
| AF029976.1 | contig_93436 | Eucalyptus grandis MADS box protein (EGM2) mRNA, complete cds. | 920 | 13.01 |
| AF197329.1 | contig_5550 | Eucalyptus grandis zinc transporter (EgZnT1) mRNA, complete cds. | 1635 | 17.08 |
| AF197330.1 | contig_2649 | Eucalyptus grandis calcineurin-like protein (EgCBL1) mRNA, complete cds. | 951 | 27.21 |
| AY150283.1 | contig_11286 | Eucalyptus grandis fertilization independent endosperm development protein mRNA, complete cds | 1626 | 18.87 |
| AY263807.1 | contig_68957 | Eucalyptus grandis SOC1-like floral activator MADS3 mRNA, complete cds. | 1112 | 21.66 |
| AY263808.1 | contig_52396 | Eucalyptus grandis SOC1-like floral activator MADS4 mRNA, complete cds. | 980 | 8.80 |
| AY263809.1 | contig_6043 | Eucalyptus grandis SVP-like floral repressor mRNA, complete cds. | 855 | 20.09 |
| DQ014506.1 | contig_2805 | Eucalyptus grandis cellulose synthase 2 (CesA2) mRNA, complete cds. | 3471 | 226.37 |
| DQ014507.1 | contig_31 | Eucalyptus grandis cellulose synthase 3 (CesA3) mRNA, complete cds. | 3452 | 220.59 |
| DQ014509.1 | contig_4202 | Eucalyptus grandis cellulose synthase 5 (CesA5) mRNA, complete cds. | 3712 | 137.25 |
| DQ014510.1 | contig_19509 | Eucalyptus grandis cellulose synthase 6 (CesA6) mRNA, complete cds. | 3782 | 97.32 |
| DQ227992.1 | contig_6857 | Eucalyptus grandis thioredoxin h mRNA, complete cds. | 354 | 133.93 |

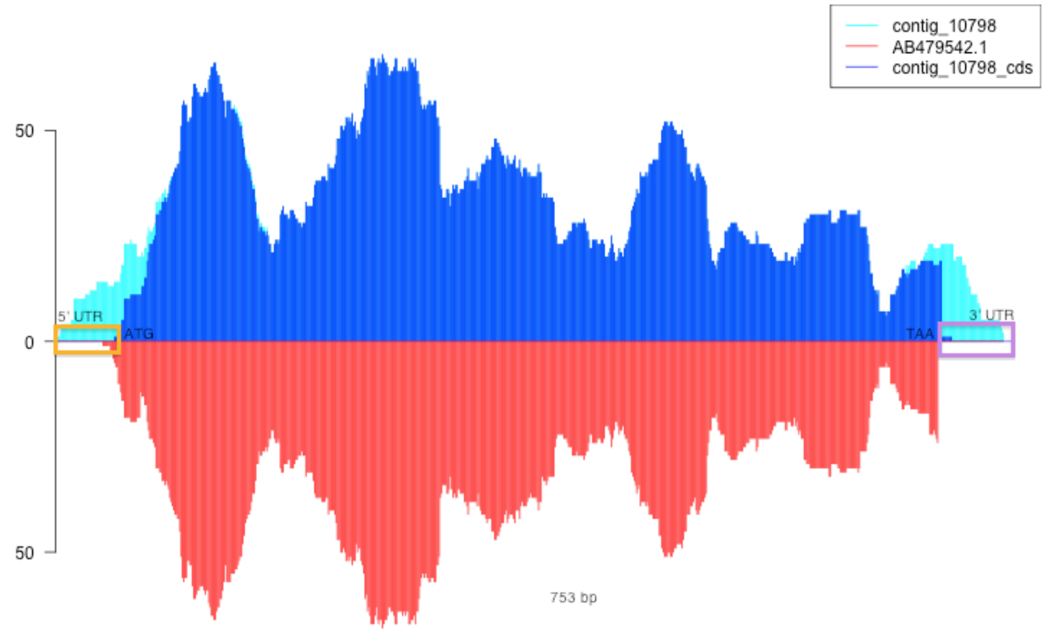
| | | | | |
|---|---|---|---|---|
| DQ227993.1 | contig_69050 | Eucalyptus grandis sucrose synthase (SuSy1) mRNA, complete cds. | 2498 | 250.38 |
| DQ227994.1 | contig_40644 | Eucalyptus grandis sucrose synthase (SuSy3) mRNA, complete cds. | 2508 | 220.28 |
| EF179384.1 | contig_24067 | Eucalyptus grandis UDP-glucose dehydrogenase (UGDH) mRNA, complete cds. | 1443 | 812.03 |
| EF534216.1 | contig_319 | Eucalyptus grandis fasciclin-like arabinogalactan protein (FLA1) mRNA, complete cds. | 1179 | 666.30 |
| EF534217.1 | contig_4434 | Eucalyptus grandis fasciclin-like arabinogalactan protein (FLA2) mRNA, complete cds. | 1125 | 180.66 |
| EF534218.1 | contig_2707 | Eucalyptus grandis fasciclin-like arabinogalactan protein (FLA3) mRNA, complete cds. | 1033 | 224.10 |
| EF534219.1 | contig_2477 | Eucalyptus grandis beta-tubulin (TUB1) mRNA, complete cds. | 1583 | 285.33 |
| EF534220.1 | contig_64905 | Eucalyptus grandis beta-tubulin (TUB2) mRNA, complete cds. | 1654 | 55.93 |
| EF534223.1 | contig_4441 | Eucalyptus grandis beta-tubulin (TUB5) mRNA, complete cds. | 1607 | 307.08 |
| EF534224.1 | contig_100 | Eucalyptus grandis alpha-tubulin (TUA1) mRNA, complete cds. | 1657 | 674.32 |
| EU737107.1 | contig_2692 | Eucalyptus grandis UTP-glucose 1 phosphate uridylyltransferase (UGP) mRNA, complete cds. | 1431 | 153.30 |
| EU737108.1 | contig_33128 | Eucalyptus grandis UDP-D-glucuronate carboxy-lyase (UXS1) mRNA, complete cds. | 1041 | 158.60 |
| EU770570.1 | contig_2246 | Eucalyptus grandis iron-sulfer cluster scaffold protein ISU1 (ISU1) mRNA, complete cds. | 756 | 78.07 |

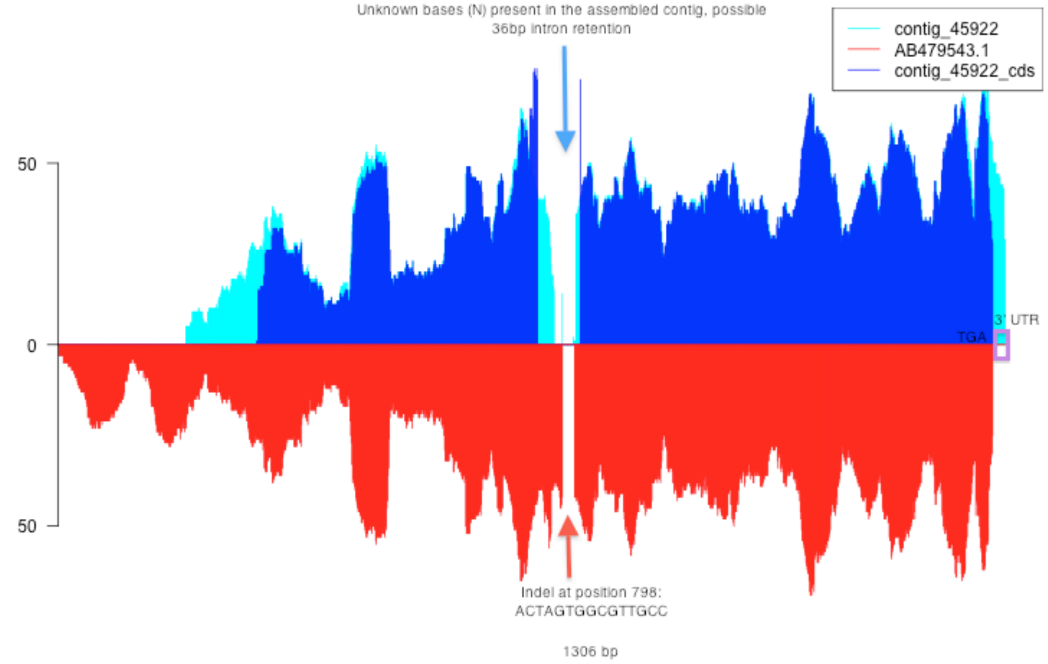
| | | | | |
|---|---|---|---|---|
| EU770571.1 | contig_31483 | Eucalyptus grandis iron-sulfer cluster scaffold protein NFU4 (NFU4) mRNA, partial cds. | 869 | 13.30 |
| EU770572.1 | contig_15010 | Eucalyptus grandis iron-sulfer cluster scaffold protein ISA1 (ISA1) mRNA, partial cds. | 822 | 25.81 |
| EU770573.1 | contig_25291 | Eucalyptus grandis iron-sulfer cluster scaffold protein NFS1 (NFS1) mRNA, partial cds. | 871 | 16.29 |

### C.1.2. Alignment coverage graphs of the 33 full length cDNA sequences and assembled contigs

Comparison of 33 *de novo*-assembled contigs of the *Eucalyptus grandis* x *Eucalyptus urophylla* clone compared to the reference contigs obtained from Sanger sequencing. Peak heights indicates the actual coverage per base (CPB) across the contig. The CBP of the assembled contig is shown in cyan, the CBP of the predicted CDS in dark blue, and the CPB of the reference sequence in red. Where present, the 5' UTR (orange box) and the 3' UTR (purple box) is indicated. Large gaps in the global alignment between the sequences are indicated by gaps in the graph, and possible reasons for the gap annotated on each graph. The graphs are also available as supplementary material for the article by Mizrachi *et al.* (2010).

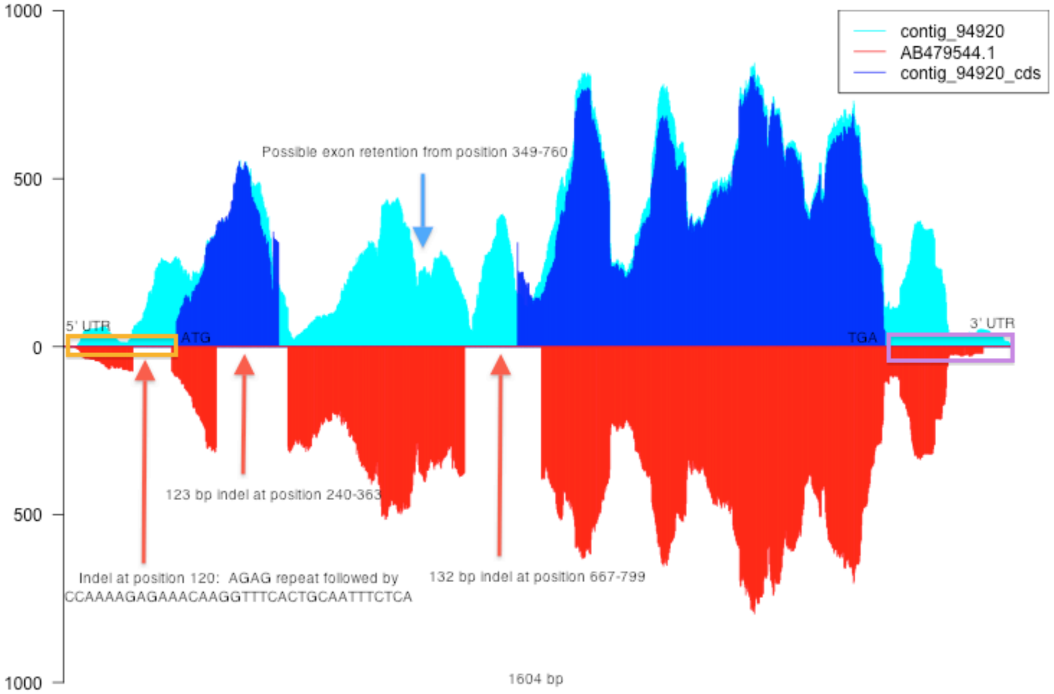

100
50
0
50
100
contig_87094
AB465730.1
contig_87094_cds
Indel at position 534: GTTGGA
5` UTR
ATG
TAG
3' UTR
985 bp


50
0
50
contig_10798
AB479542.1
contig_10798_cds
5' UTR
ATG
TAA
3' UTR
753 bp



Unknown bases (N) present in the assembled contig, possible 36bp intron retention

Indel at position 798: ACTAGTGGCGTTGCC

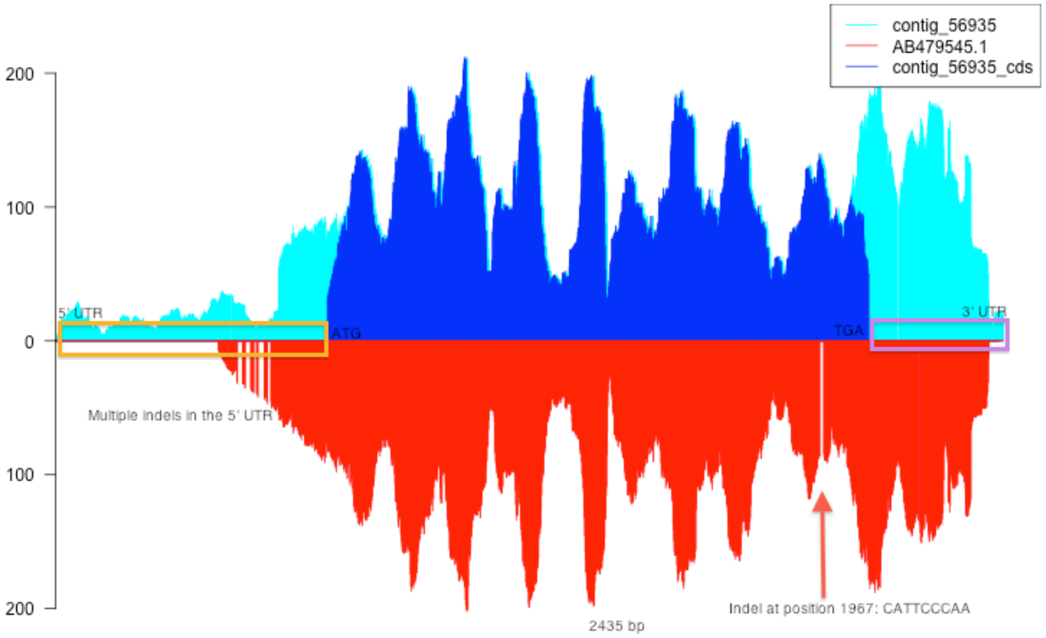


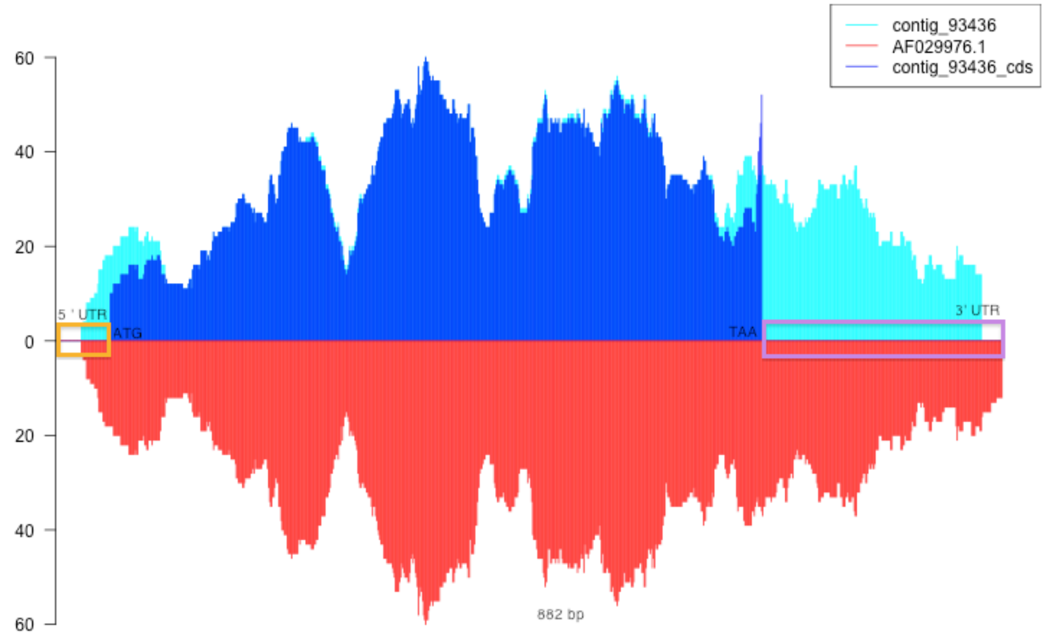
Possible exon retention from position 349-760

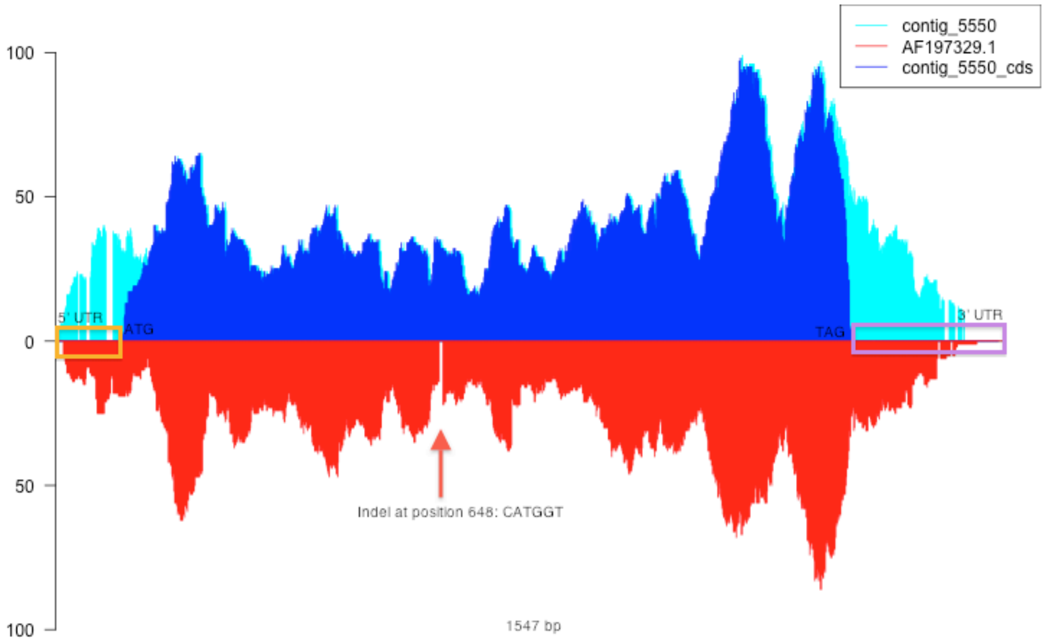
123 bp indel at position 240-363

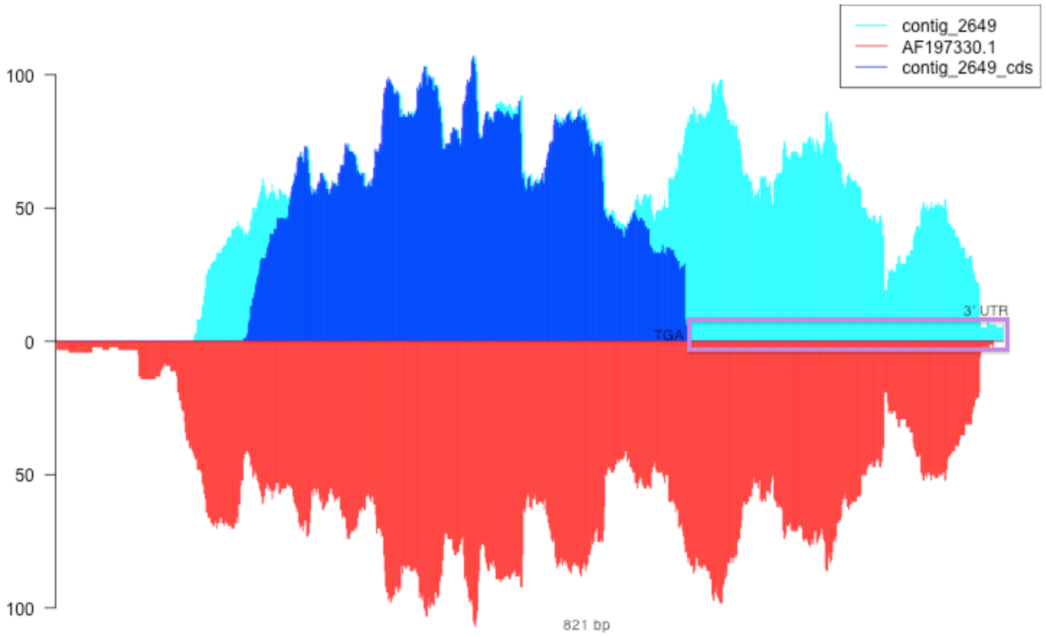
Indel at position 120: AGAG repeat followed by CCAAAAGAGAAACAAGGTTTCACTGCAATTTCTCA

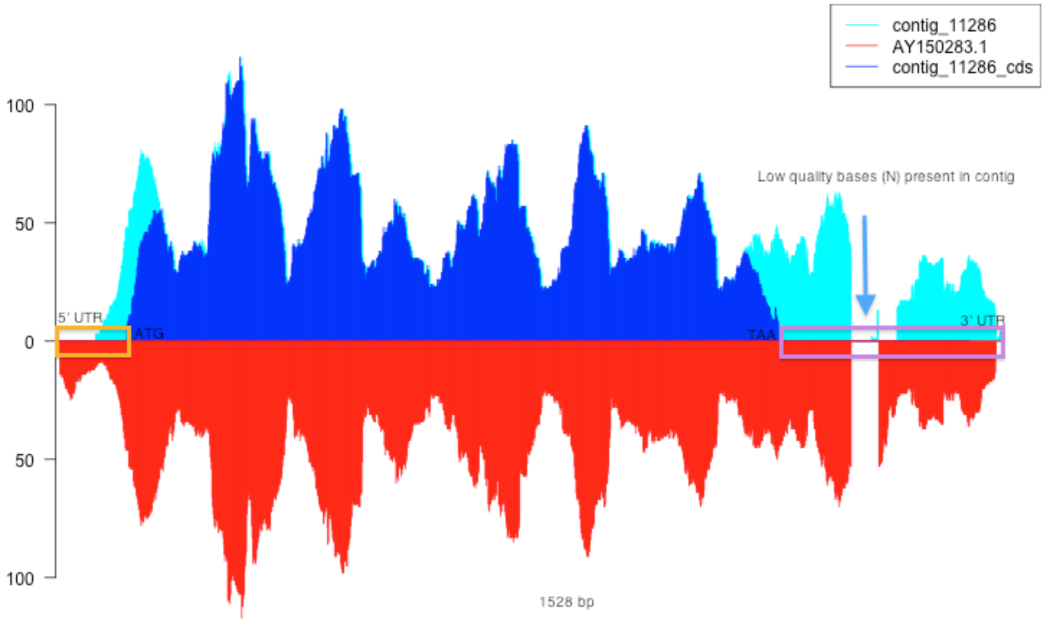
132 bp indel at position 667-799

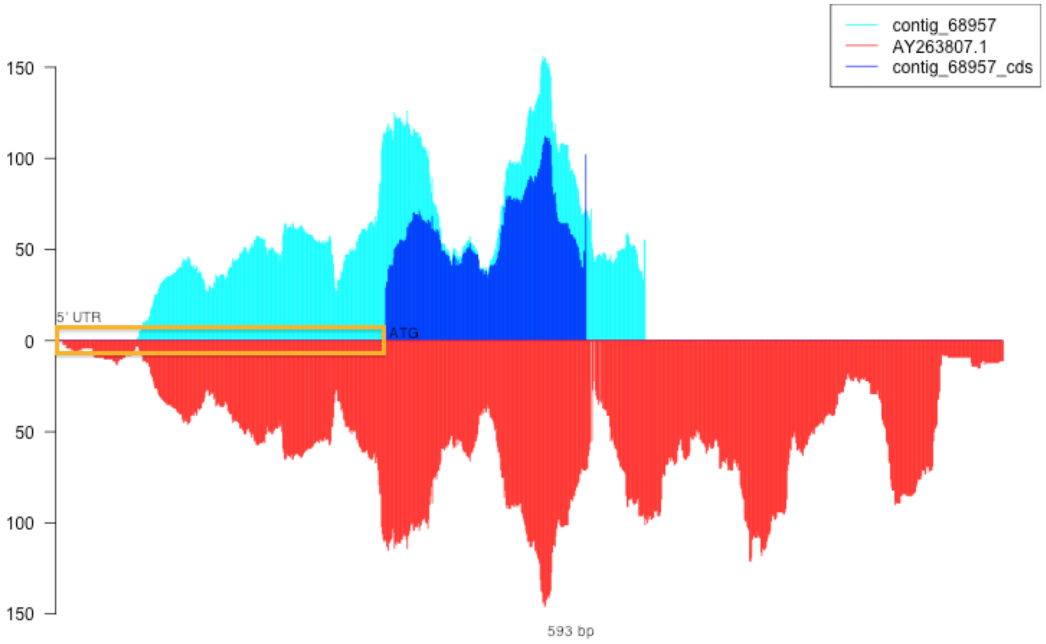

contig_56935
AB479545.1
contig_56935_cds
200
100
0
100
200
5' UTR
ATG
TGA
3' UTR
Multiple indels in the 5' UTR
Indel at position 1967: CATTCCCAA
2435 bp

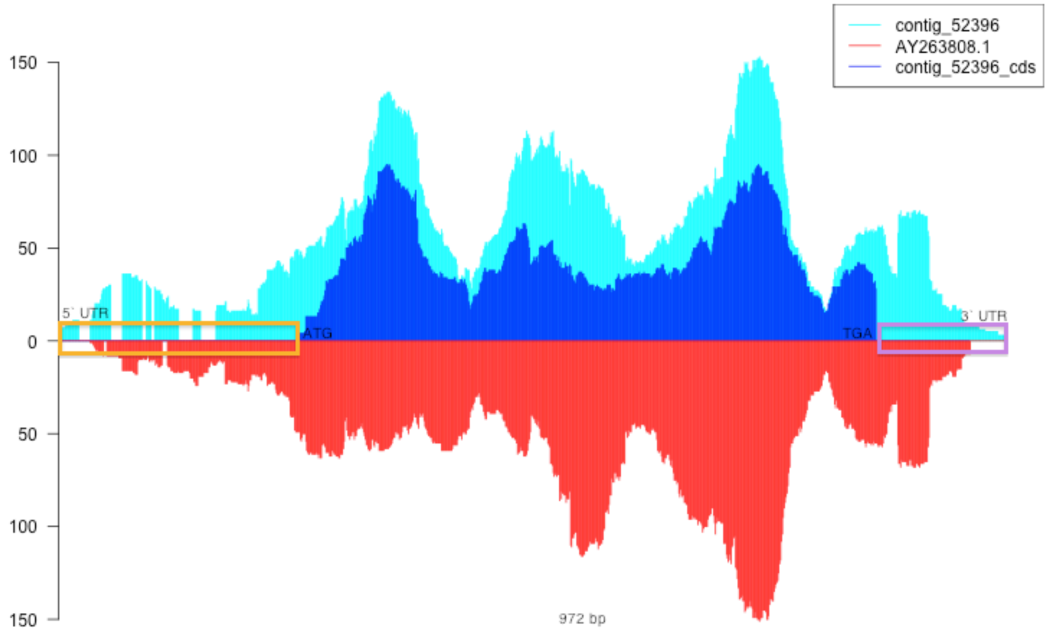

contig_93436
AF029976.1
contig_93436_cds
60
40
20
0
20
40
60
5 ' UTR
ATG
TAA
3' UTR
882 bp

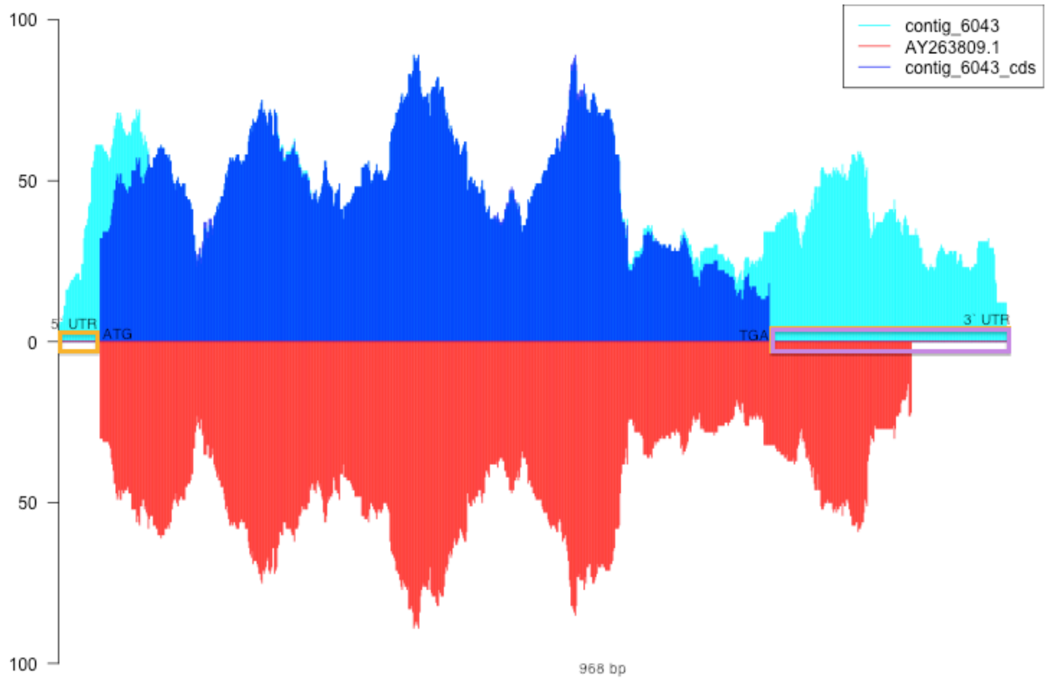

contig_5550
AF197329.1
contig_5550_cds
100
50
0
50
100
5' UTR
ATG
TAG
3' UTR
Indel at position 648: CATGGT
1547 bp

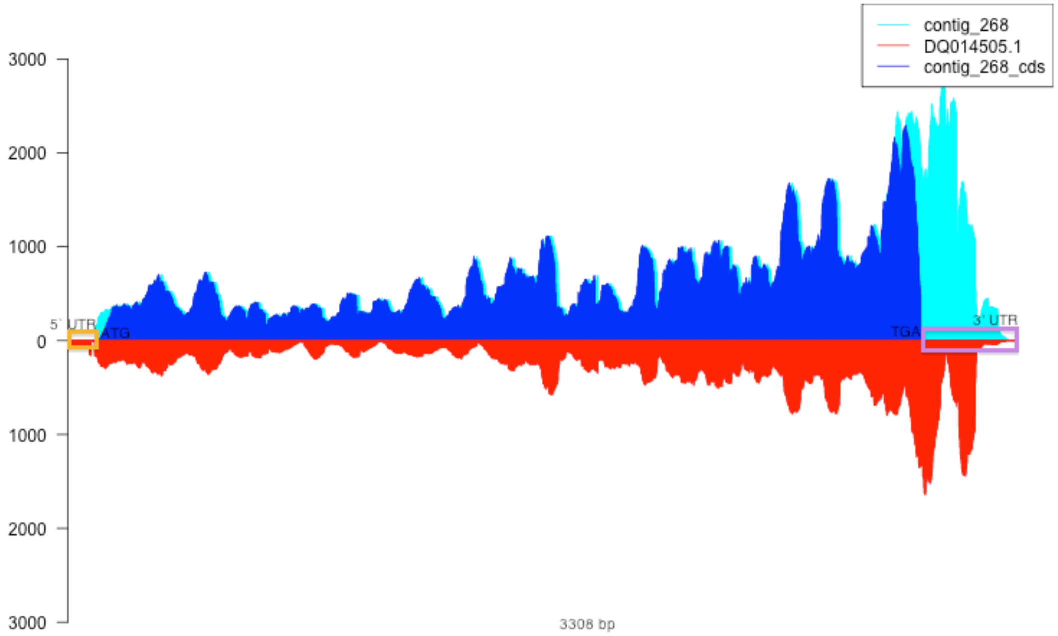

contig_2649
AF197330.1
contig_2649_cds
100
50
0
50
100
TGA
3' UTR
821 bp

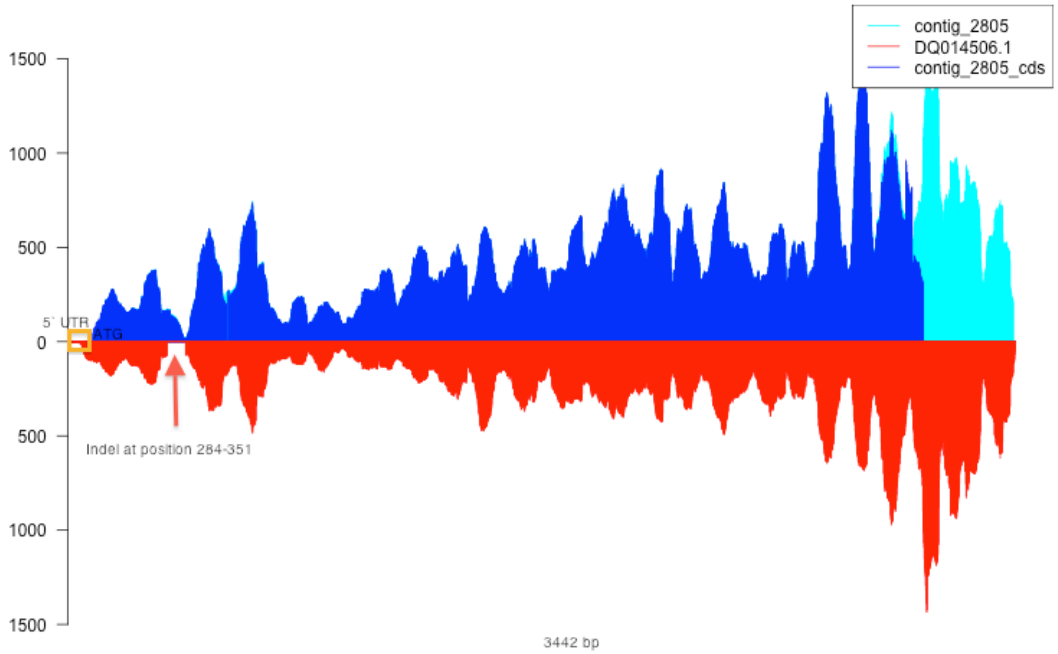

contig_11286
AY150283.1
contig_11286_cds
100
50
0
50
100
5' UTR
ATG
TAA
3' UTR
Low quality bases (N) present in contig
1528 bp

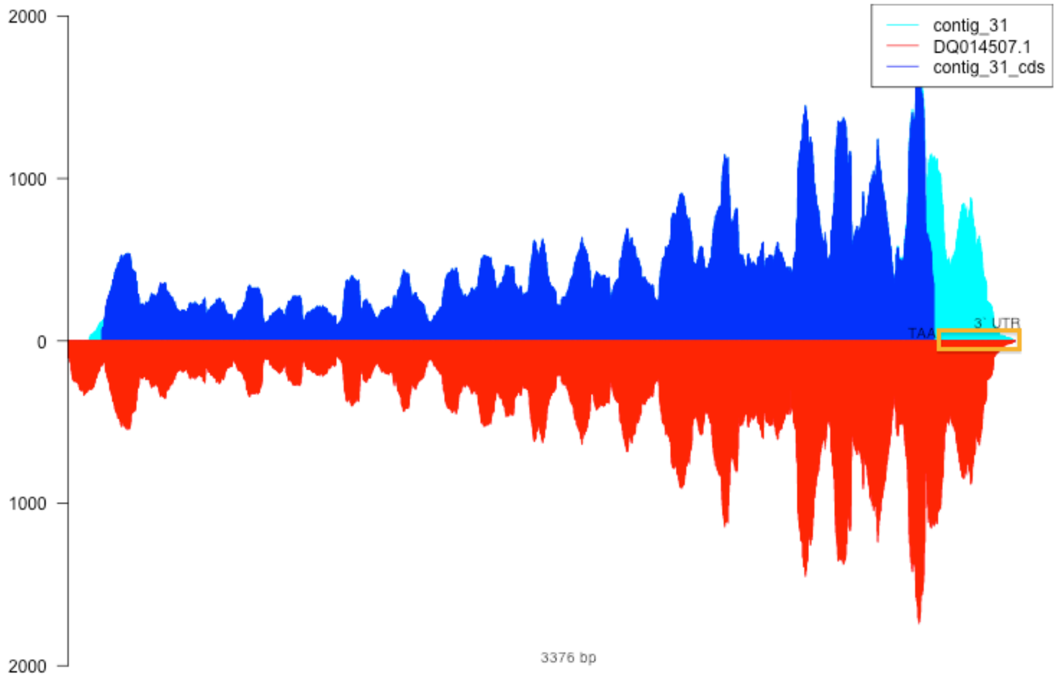

contig_68957
AY263807.1
contig_68957_cds
150
100
50
0
50
100
150
5' UTR
ATG
593 bp

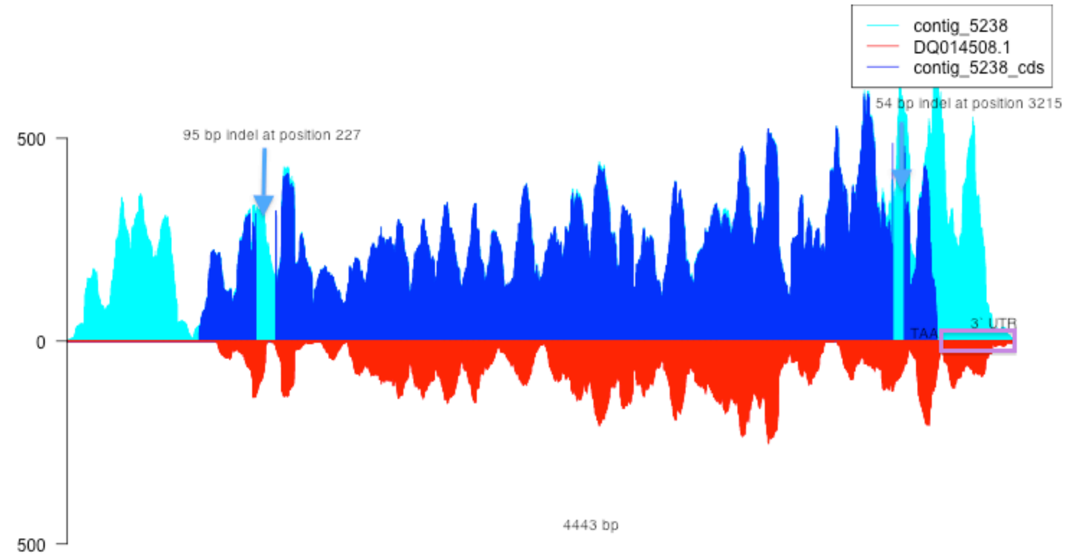

contig_52396
AY263808.1
contig_52396_cds
150
100
50
0
50
100
150
5` UTR
ATG
TGA
3` UTR
972 bp

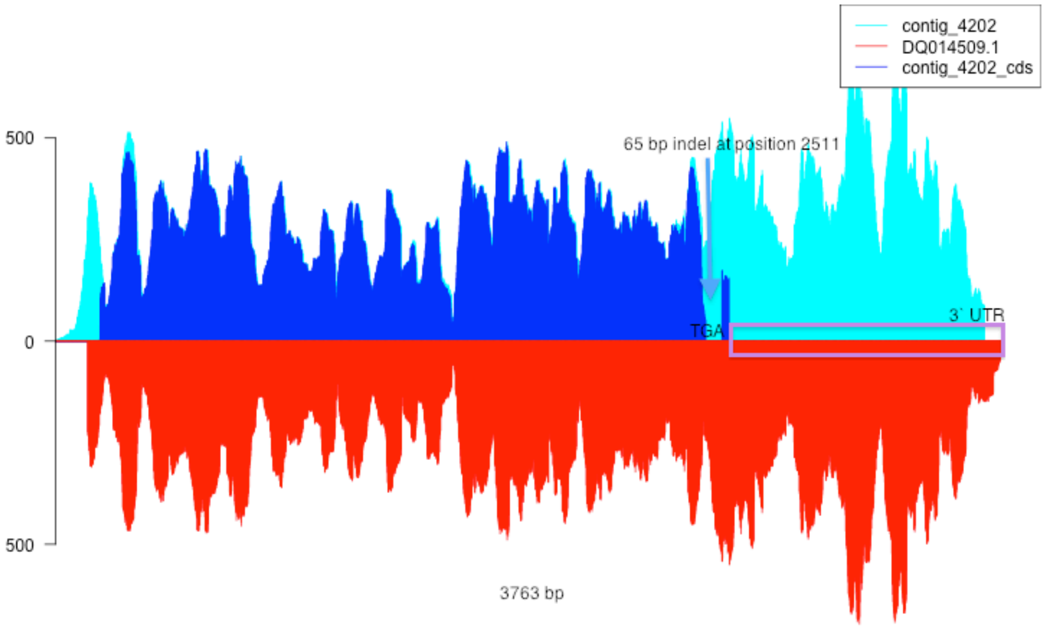

contig_6043
AY263809.1
contig_6043_cds
100
50
0
50
100
5` UTR
ATG
TGA
3` UTR
968 bp

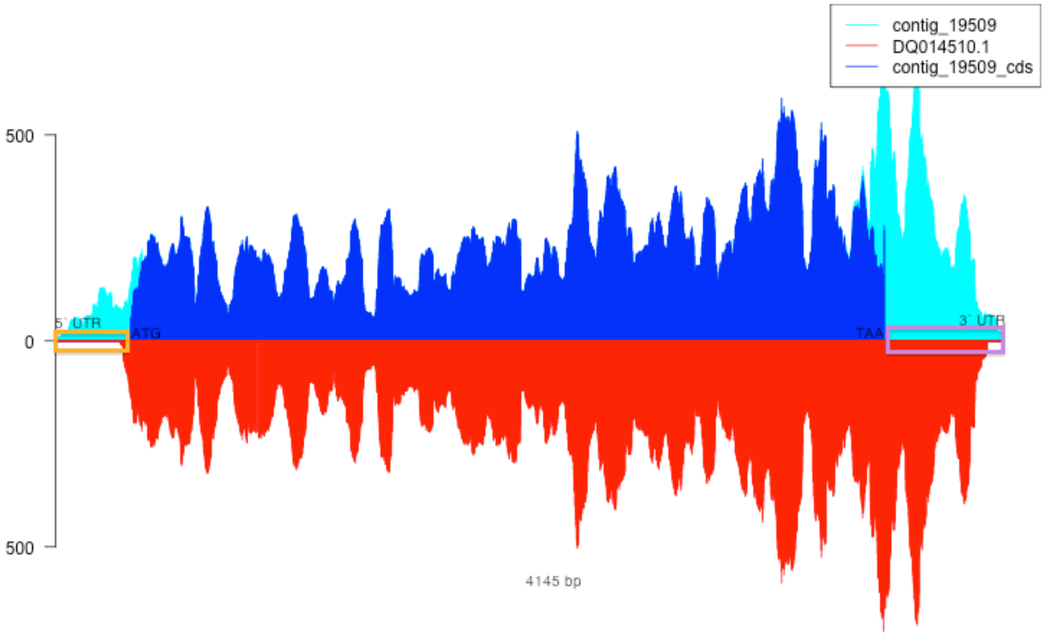

contig_268
DQ014505.1
contig_268_cds
3000
2000
1000
0
1000
2000
3000
5` UTR
ATG
TGA
3' UTR
3308 bp


contig_2805
DQ014506.1
contig_2805_cds
1500
1000
500
0
500
1000
1500
5` UTR
ATG
Indel at position 284-351
3442 bp


2000
1000
0
1000
2000
contig_31
DQ014507.1
contig_31_cds
TAA
3' UTR
3376 bp


500
0
500
contig_5238
DQ014508.1
contig_5238_cds
95 bp indel at position 227
54 bp indel at position 3215
TAA
3' UTR
4443 bp


contig_4202
DQ014509.1
contig_4202_cds
500
0
500
65 bp indel at position 2511
TGA
3` UTR
3763 bp


contig_19509
DQ014510.1
contig_19509_cds
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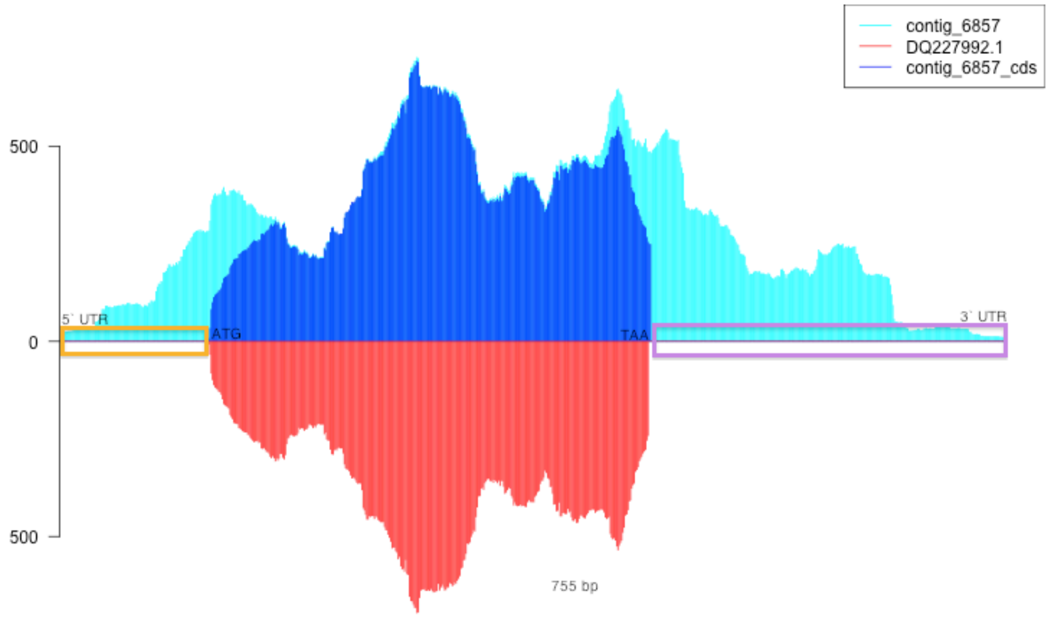
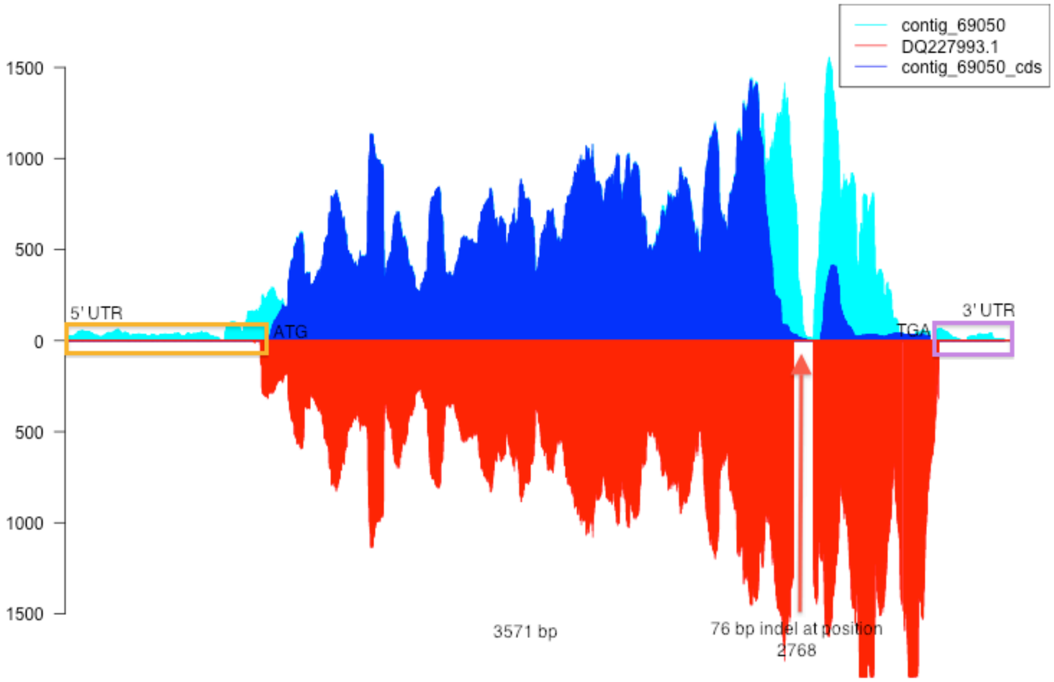
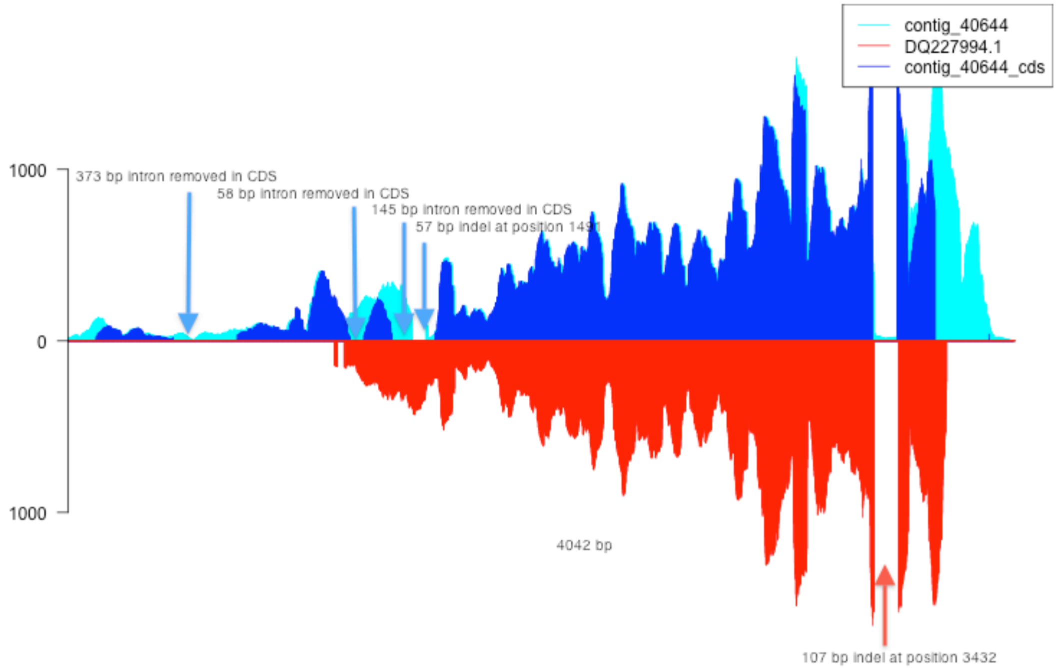
0
500
5` UTR
ATG
TAA
3` UTR
4145 bp


contig_6857
DQ227992.1
contig_6857_cds
500
0
500
5` UTR
ATG
TAA
3` UTR
755 bp


contig_69050
DQ227993.1
contig_69050_cds
1500
1000
500
0
500
1000
1500
5' UTR
ATG
TGA
3' UTR
3571 bp
76 bp indel at position 2768


contig_40644
DQ227994.1
contig_40644_cds
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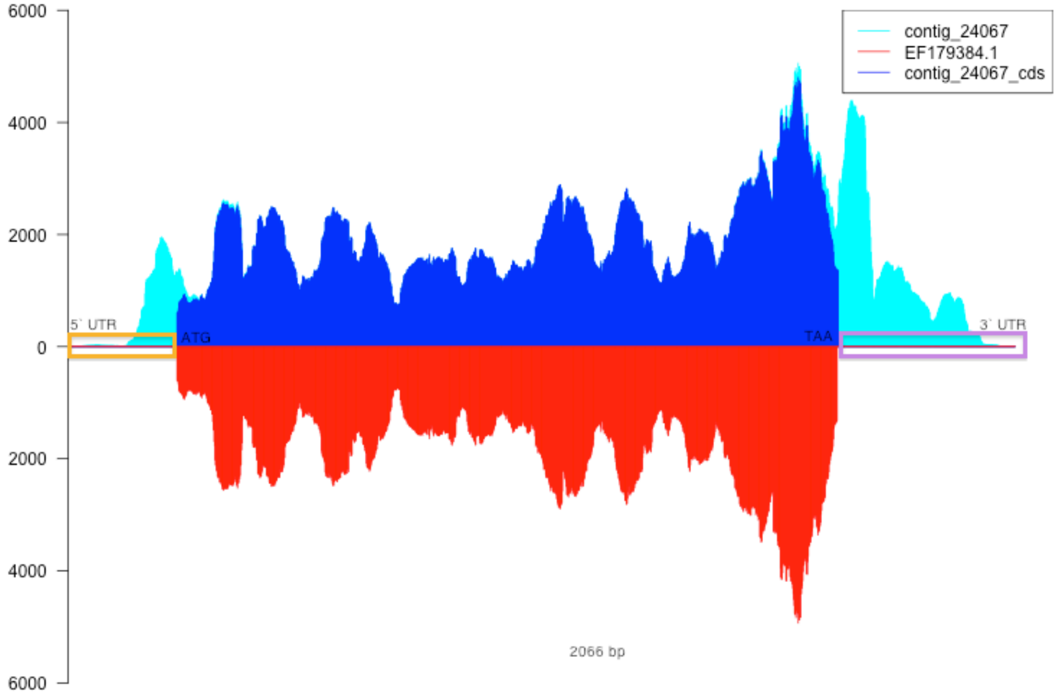
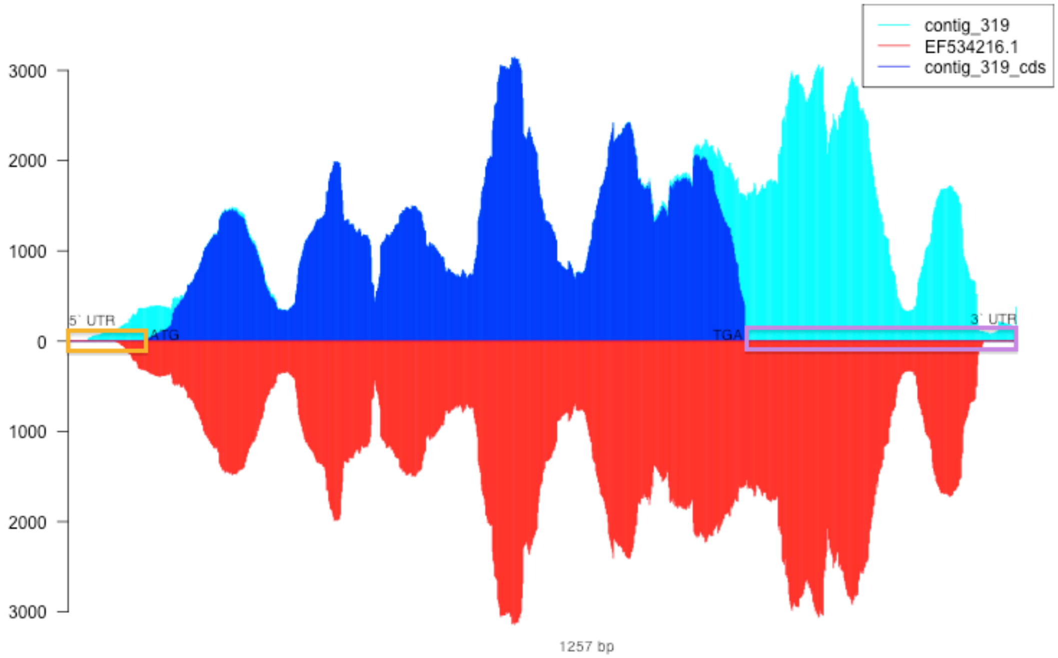
0
1000
373 bp intron removed in CDS
58 bp intron removed in CDS
145 bp intron removed in CDS
57 bp indel at position 1491
4042 bp
107 bp indel at position 3432


contig_24067
EF179384.1
contig_24067_cds
6000
4000
2000
0
2000
4000
6000
5' UTR
ATG
TAA
3' UTR
2066 bp


contig_319
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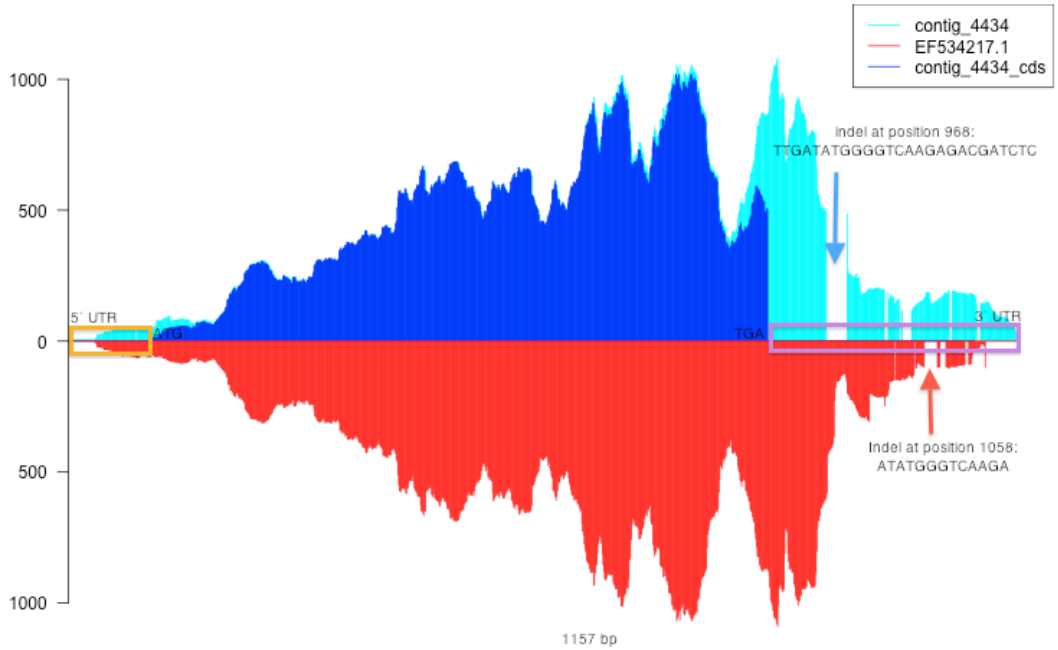
3000
2000
1000
0
1000
2000
3000
5` UTR
ATG
TGA
3` UTR
1257 bp


contig_4434
EF534217.1
contig_4434_cds
1000
500
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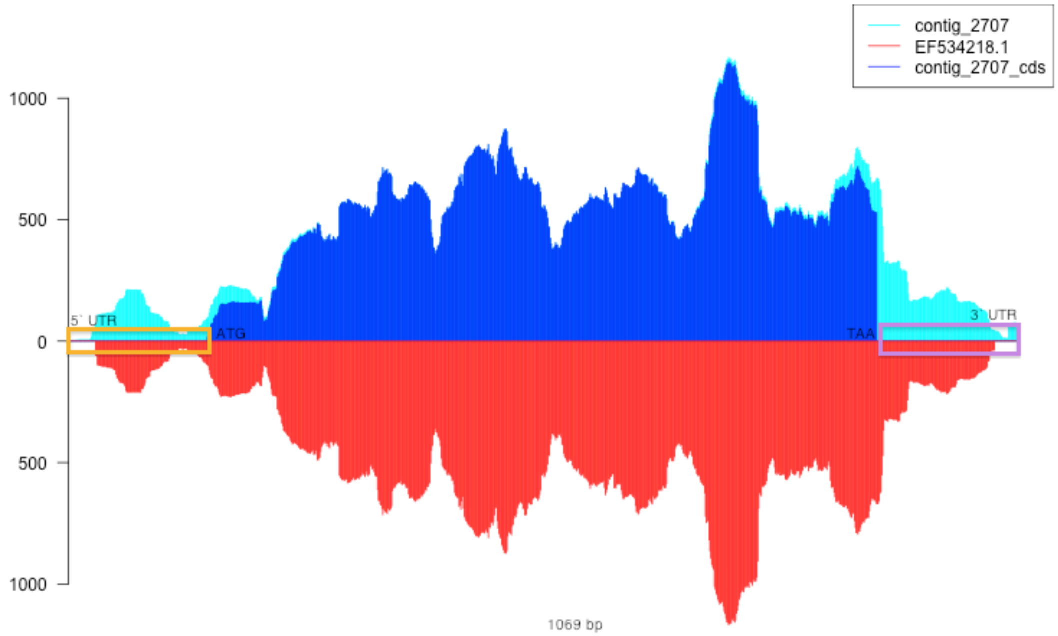
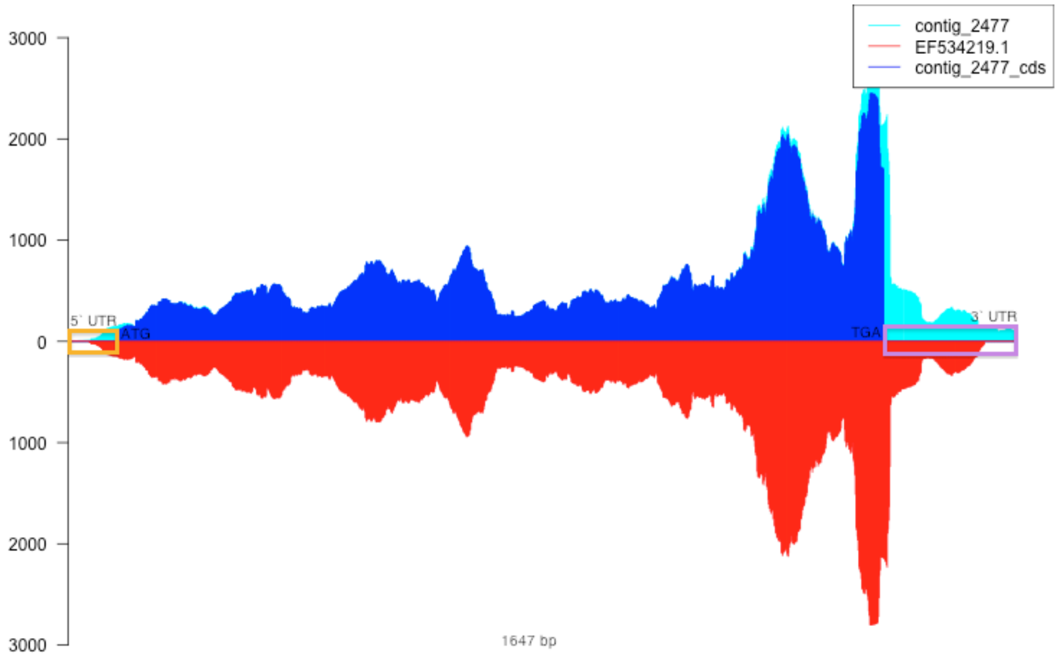
500
1000
5` UTR
ATG
TGA
3` UTR
Indel at position 968:
TTGATATGGGGTCAAGAGACGATCTC
Indel at position 1058:
ATATGGGTCAAGA
1157 bp


contig_2707
EF534218.1
contig_2707_cds
1000
500
0
500
1000
5` UTR
ATG
TAA
3` UTR
1069 bp


contig_2477
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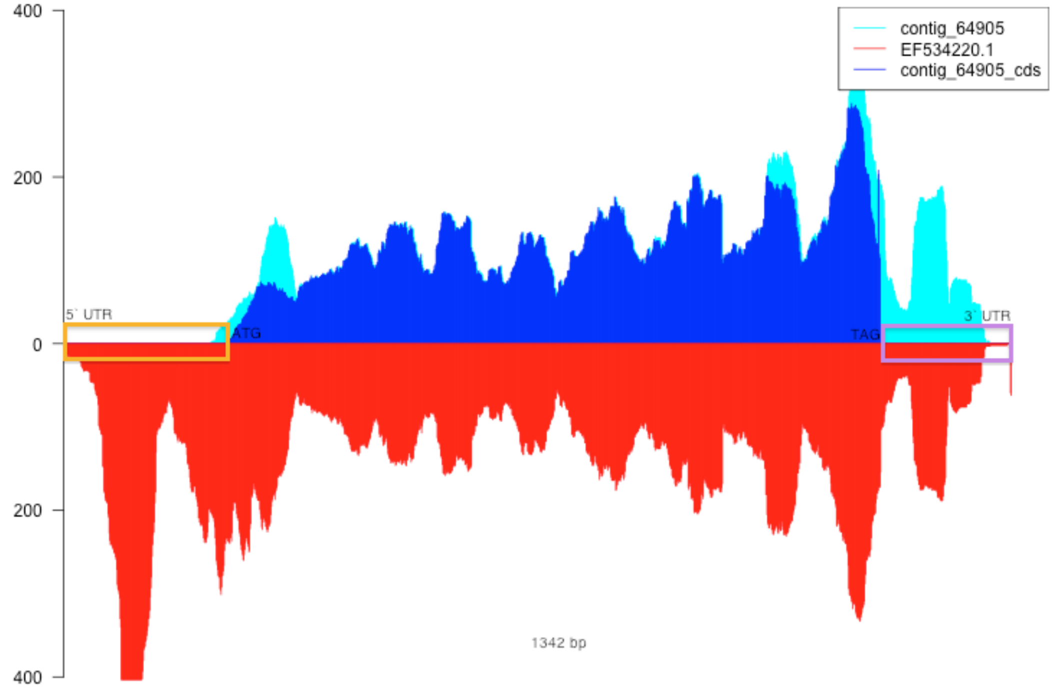
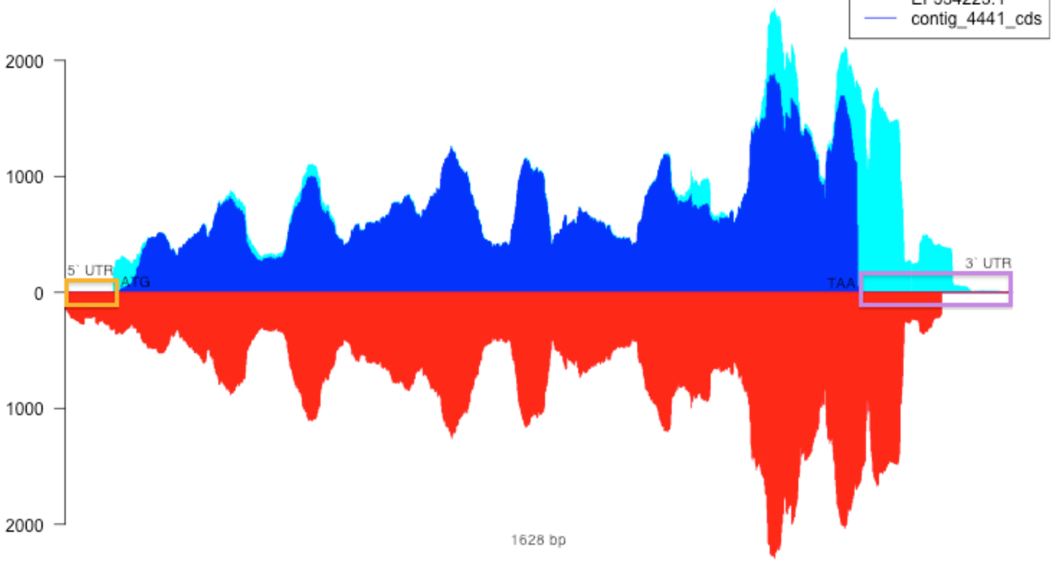
contig_2477_cds
3000
2000
1000
0
1000
2000
3000
5` UTR
ATG
TGA
3` UTR
1647 bp


400
200
0
200
400
contig_64905
EF534220.1
contig_64905_cds
5` UTR
ATG
TAG
3` UTR
1342 bp


2000
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0
1000
2000
contig_4441
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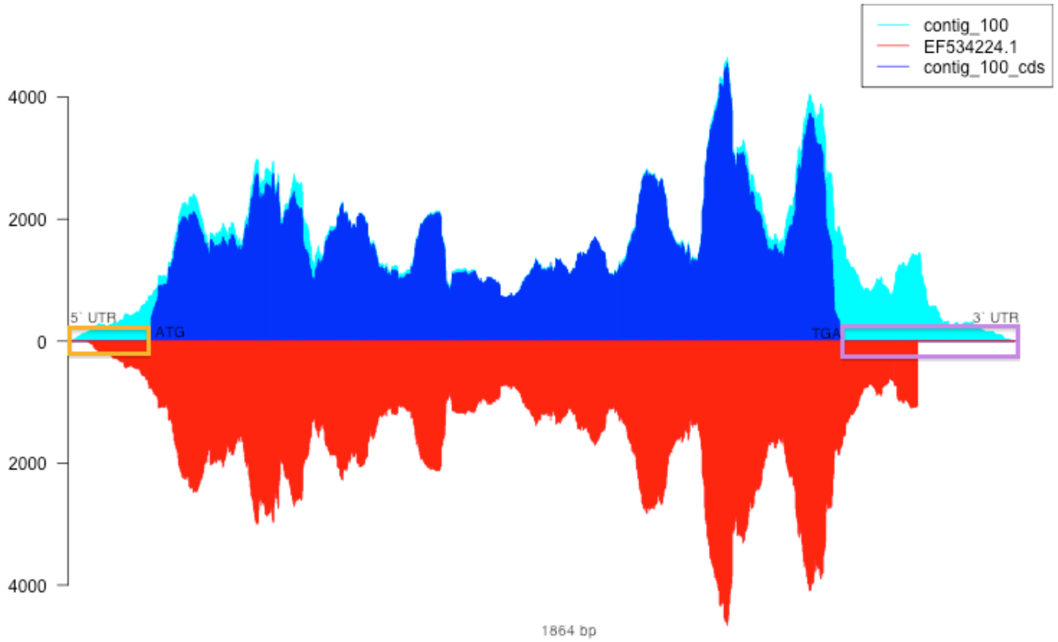
contig_4441_cds
5` UTR
ATG
TAA
3` UTR
1628 bp


contig_100
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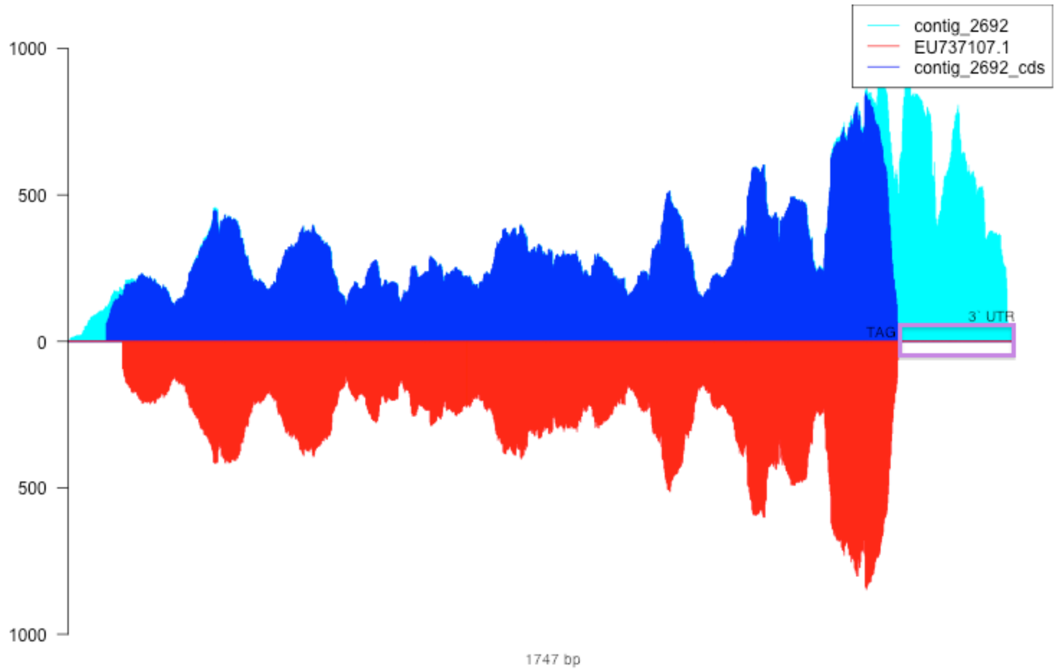
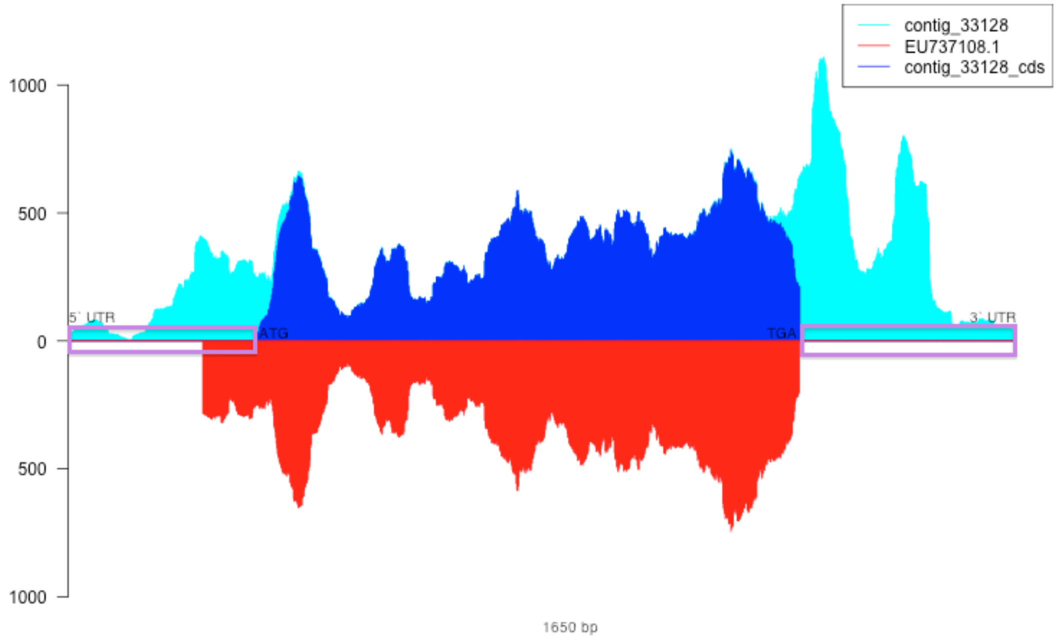
contig_100_cds
4000
2000
0
2000
4000
5` UTR
ATG
TGA
3` UTR
1864 bp


contig_2692
EU737107.1
contig_2692_cds
1000
500
0
500
1000
TAG
3` UTR
1747 bp


contig_33128
EU737108.1
contig_33128_cds
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500
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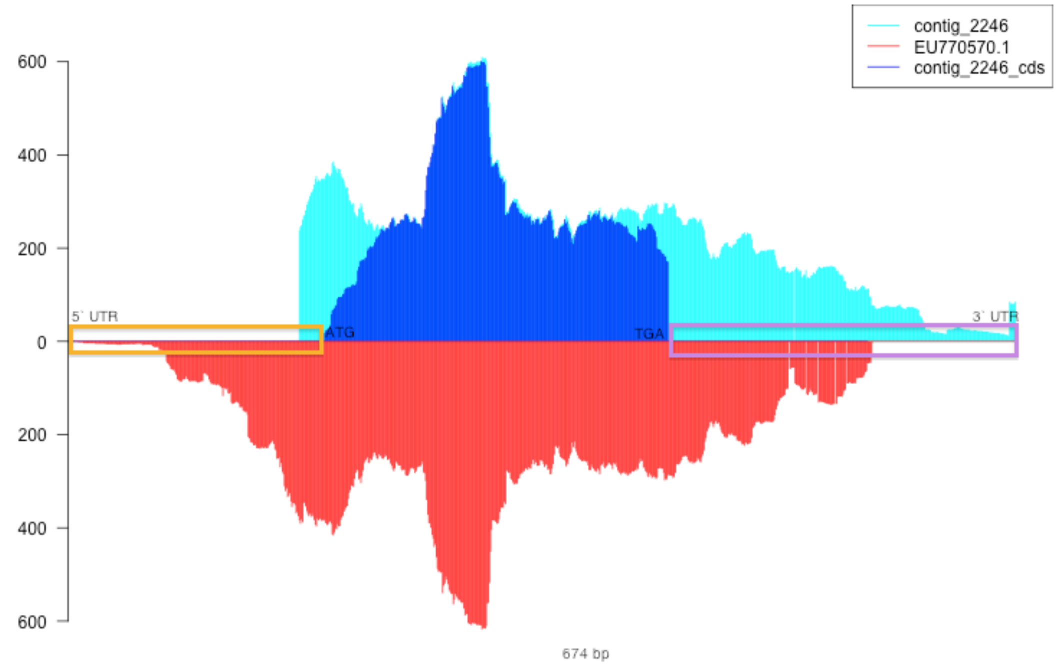
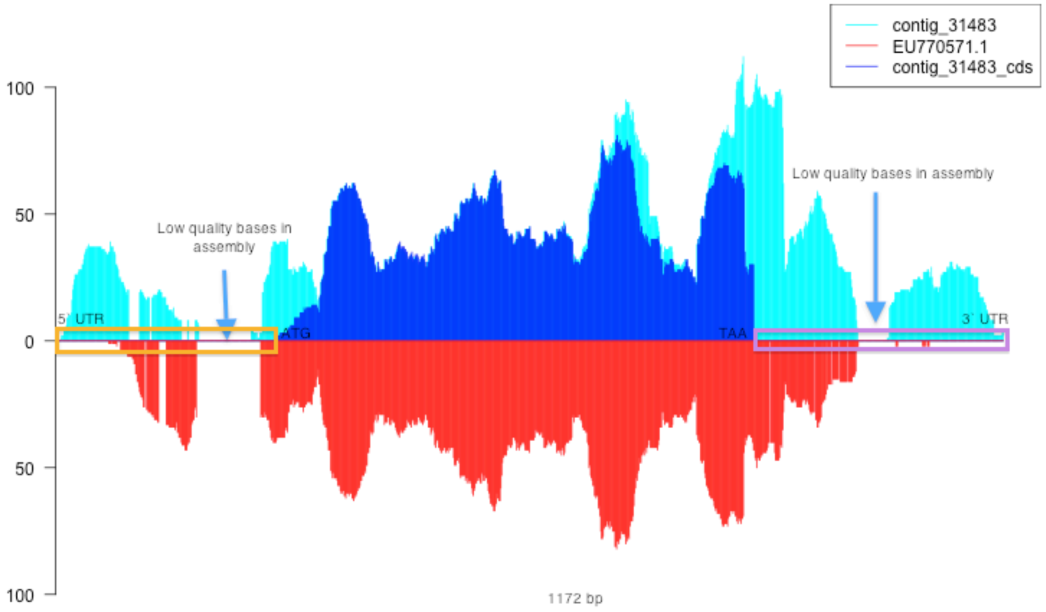
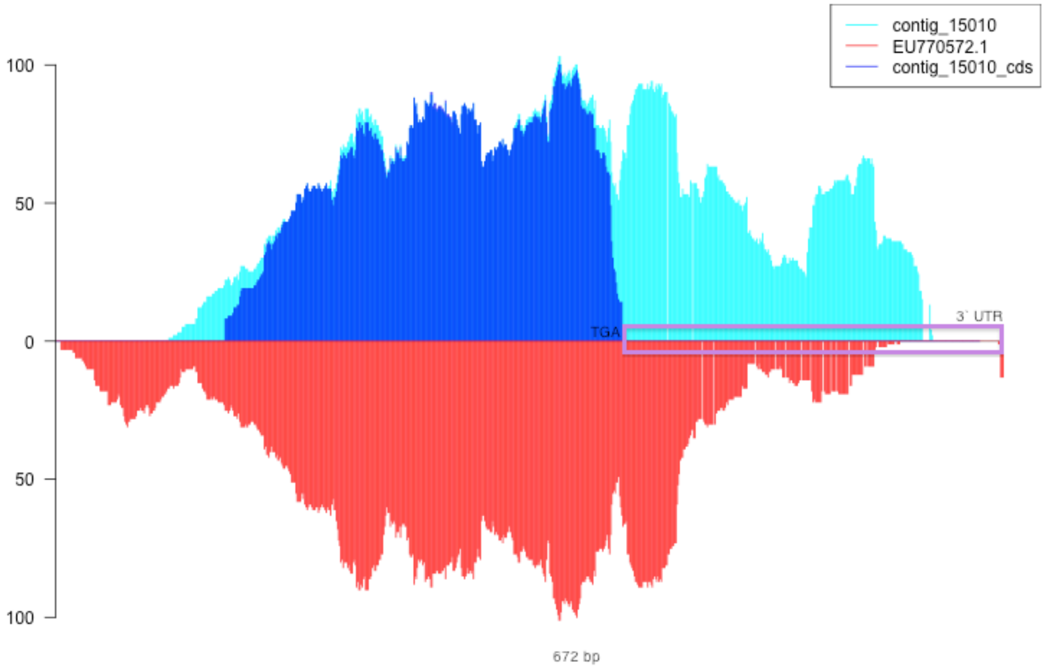
500
1000
5` UTR
ATG
TGA
3` UTR
1650 bp


contig_2246
EU770570.1
contig_2246_cds
600
400
200
0
200
400
600
5` UTR
ATG
TGA
3` UTR
674 bp


contig_31483
EU770571.1
contig_31483_cds
100
50
0
50
100
Low quality bases in assembly
Low quality bases in assembly
5` UTR
ATG
TAA
3` UTR
1172 bp


contig_15010
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contig_15010_cds
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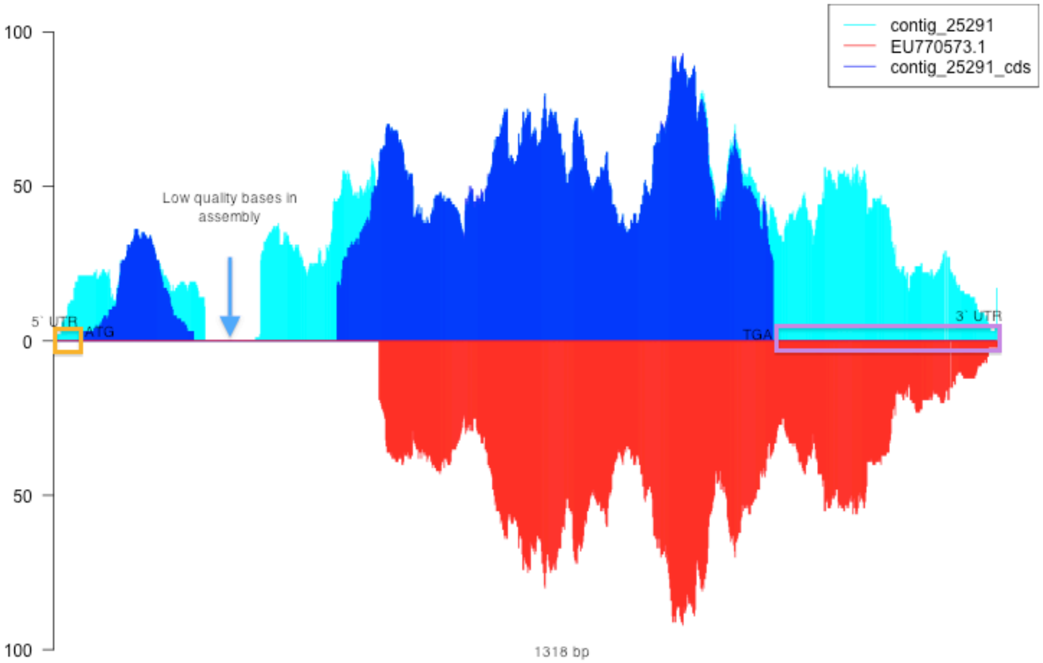
50
0
50
100
TGA
3` UTR
672 bp


contig_25291
EU770573.1
contig_25291_cds
100
50
0
50
100
Low quality bases in assembly
5` UTR
ATG
TGA
3` UTR
1318 bp

### C.1.3. Alignment of contig 68291 before and after extension

The complete alignment of contig or node 68291 before and after the coverage-assisted re-assembly of the dataset. The aligment was performed with the the `ClustalW` program, and no editing of the alignment was performed. The alignment shows that although 1 485 bases was reportedly added to the contig during extension, these bases mostly consisted of the extension of a low quality region containing Ns. The extension did however resolve a 88 bp region of these low quality bases. The contig after extension also showed removed regions at the start and end of the original contig, due to the presence of a polyA region at the beginning of the sequence. An extract from the alignment is presented in Figure 3.6.

```
NODE_68291_before TTTTTTTTTTTTTTTTTTTTTTA[illegible]AGAGAGGCTTGGAGTGGTCAGGACTCTTA    80
NODE_68291_after  --------------------------------------------------------------------------------

                                                                              **    * * ***  * *
NODE_68291_before TTCCAGAGAGAAAGCATCAGCGCGGGCTGTCAAAGCTTCAATGAACGTATAGTTTGTTACTGAAGCGGAGATAAAGGAGA   160
NODE_68291_after  -----------------------------------------------------------AAAAAAAAAAAAAAAAAAAAA    21

                    **     ***********************************************************************
NODE_68291_before GTAAGNNNNCAAGCAAAGCTGGTGCTTTTTGTGCATCTCCATTGACTTGGCCATTTGGTCCATTGATAAGTTCGGCAACT   240
NODE_68291_after  AAAAAAAAACAAGCAAAGCTGGTGCTTTTTGTGCATCTCCATTGACTTGGCCATTTGGTCCATTGATAAGTTCGGCAACT   101

                  ********************************************************************************
NODE_68291_before CAGGCGATTCCTGGCTTTGCGTCGGTGAGTTCTTCGTGATCTGGCAATGGCGTCGGCTCTAGCCGGCGATGATTTGGCTA   320
NODE_68291_after  CAGGCGATTCCTGGCTTTGCGTCGGTGAGTTCTTCGTGATCTGGCAATGGCGTCGGCTCTAGCCGGCGATGATTTGGCTA   181

                  ********************************************************************************
NODE_68291_before GATCCACGAGCAGCCGCCGGAGCTGGGCCTCCGGGAGCCACCGGAGCTGGGCCTCCACGAGCTTCCGGGAGGCGTGGAAC   400
NODE_68291_after  GATCCACGAGCAGCCGCCGGAGCTGGGCCTCCGGGAGCCACCGGAGCTGGGCCTCCACGAGCTTCCGGGAGGCGTGGAAC   261

                  ********************************************************************************
NODE_68291_before GGCCCGCCAGATGTGTTCGCGCGGAGCGGGAGGCAGGACGACGAGGAGGAGCTCCGGTGGGCCGCCATCGAACGGCTGCC   480
NODE_68291_after  GGCCCGCCAGATGTGTTCGCGCGGAGCGGGAGGCAGGACGACGAGGAGGAGCTCCGGTGGGCCGCCATCGAACGGCTGCC   341

                  ********************************************************************************
NODE_68291_before AACGTATGACCGCCTCCGAAAAGGCATGCTGAAGCAAGTACTTGATACTGGGAGGGTGGTCCAGCAAGAAGTGGACGTGA   560
NODE_68291_after  AACGTATGACCGCCTCCGAAAAGGCATGCTGAAGCAAGTACTTGATACTGGGAGGGTGGTCCAGCAAGAAGTGGACGTGA   421

                  ********************************************************************************
NODE_68291_before CCAACCTCGGAATGCAGGACAAGAAGCAGTTGATGGAGAGCATCCTTAAGGTTGCGGAAGAAGACAATGAGAGGTTCTTG   640
NODE_68291_after  CCAACCTCGGAATGCAGGACAAGAAGCAGTTGATGGAGAGCATCCTTAAGGTTGCGGAAGAAGACAATGAGAGGTTCTTG   501

                  ********************************************************************************
NODE_68291_before AGGAGATTGAGAGACAGGACTGATAGGGTCGGGATCGAAATTCCGAAGATCGAAGTCCGGTGTGAGCATTTATCTGTAGA   720
NODE_68291_after  AGGAGATTGAGAGACAGGACTGATAGGGTCGGGATCGAAATTCCGAAGATCGAAGTCCGGTGTGAGCATTTATCTGTAGA   581

                  ********************************************************************************
NODE_68291_before AGGAGACGTGTACGTTGGAAGCAGAGCTCTCCCTACCCTTCTCAATGCCACTATGAACGCGATAGAGAGTGTTCTTGGAC   800
NODE_68291_after  AGGAGACGTGTACGTTGGAAGCAGAGCTCTCCCTACCCTTCTCAATGCCACTATGAACGCGATAGAGAGTGTTCTTGGAC   661

                  ********************************************************************************
NODE_68291_before TTATTCGGCTAGCCCCATCGAAGAAGAGAAAAATTCAGATACTTAAGGACGTGAACGGATTAGTCAGGCCTTCGAGGATG   880
NODE_68291_after  TTATTCGGCTAGCCCCATCGAAGAAGAGAAAAATTCAGATACTTAAGGACGTGAACGGATTAGTCAGGCCTTCGAGGATG   741

                  ********************************************************************************
NODE_68291_before ACCCTACTTTTGGGTCCACCGGGAGCTGGGAAGACAACATTGTTGCTGGCACTTGCTGGGAAACTAGACAGCGATCTGAG   960
NODE_68291_after  ACCCTACTTTTGGGTCCACCGGGAGCTGGGAAGACAACATTGTTGCTGGCACTTGCTGGGAAACTAGACAGCGATCTGAG   821

                  ********************************************************************************
NODE_68291_before GGTAACGGGAAAAGTCACCTACTGTGGTCACGAGCTAAACGAATTTGTTCCTCAAAGGACTTGCGCTTATATCAGCCAAC  1040
NODE_68291_after  GGTAACGGGAAAAGTCACCTACTGTGGTCACGAGCTAAACGAATTTGTTCCTCAAAGGACTTGCGCTTATATCAGCCAAC   901

                  ********************************************************************************
NODE_68291_before ATGATCTTCACTATGGGGAAATGACAGTTAGAGAGACATTGGACTTCTCGGGTCGCTGTTTGGGTGTAGGGACAAGGTAT  1120
NODE_68291_after  ATGATCTTCACTATGGGGAAATGACAGTTAGAGAGACATTGGACTTCTCGGGTCGCTGTTTGGGTGTAGGGACAAGGTAT   981

                  ********************************************************************************
NODE_68291_before GAGATGCTTGCAGAACTCTCCAGGCGAGAGAGGGAAGCCGGAATCAAACCTGATCCCGAAATTGACGCTTTTATGAAGGC  1200
NODE_68291_after  GAGATGCTTGCAGAACTCTCCAGGCGAGAGAGGGAAGCCGGAATCAAACCTGATCCCGAAATTGACGCTTTTATGAAGGC  1061

                  ********************************************************************************
NODE_68291_before CACAGCTCTGTCGGGTCAAGAGACAAGCTTGGTCACTGATTATATACTCAAGATTCTTGGATTGGATATCTGTGCAGACA  1280
NODE_68291_after  CACAGCTCTGTCGGGTCAAGAGACAAGCTTGGTCACTGATTATATACTCAAGATTCTTGGATTGGATATCTGTGCAGACA  1141

                  ********************************************************************************
NODE_68291_before TTATGGTCGGAGATGAGATGCGAAGGGGCATTTCAGGTGGACAAAAAAAGCGTCTTACAACCGGAGAGATGTTAGTAGGA  1360
NODE_68291_after  TTATGGTCGGAGATGAGATGCGAAGGGGCATTTCAGGTGGACAAAAAAAGCGTCTTACAACCGGAGAGATGTTAGTAGGA  1221

                  ********************************************************************************
NODE_68291_before CCAGCAAAGGCTCTTTTTATGGATGAAATATCCACAGGGTTGGACAGTTCCACTACTTTTCAAATTTGCAAATTCATGAG  1440
NODE_68291_after  CCAGCAAAGGCTCTTTTTATGGATGAAATATCCACAGGGTTGGACAGTTCCACTACTTTTCAAATTTGCAAATTCATGAG  1301

                  ********************************************************************************
NODE_68291_before GCAGATGGTTCATATTATGGATGTCACCATGATCATCTCATTGCTTCAGCCGGCTCCTGAGACTTATGATCTCTTCGATG  1520
NODE_68291_after  GCAGATGGTTCATATTATGGATGTCACCATGATCATCTCATTGCTTCAGCCGGCTCCTGAGACTTATGATCTCTTCGATG  1381
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                  ***********************                            *****************************
NODE_68291_before ACATTATCCTTCTCTCGGAGGGT                            ACGTCCTCGAGTTTTTCGAGCACATGGGA  1600
NODE_68291_after  ACATTATCCTTCTCTCGGAGGGTCAAGTCGTCTACCAAGGTCCACGAGAGAACGTCCTCGAGTTTTTCGAGCACATGGGA  1461

                  ********************************************************************************
NODE_68291_before TTCAAGTGCCCTGAAAGGAAAGGAGTTGCCGACTTCTTGCAAGAAGTGACATCTAAGAAAGATCAAGAACAGTATTGGTT  1680
NODE_68291_after  TTCAAGTGCCCTGAAAGGAAAGGAGTTGCCGACTTCTTGCAAGAAGTGACATCTAAGAAAGATCAAGAACAGTATTGGTT  1541

                  ********************************************************************************
NODE_68291_before CAAGAAGAACCAACCTTTCCAATACGTTTCTGTAGATGATTTCGTGCATGGATTCAAATCTTTTCACATTGGCCAACATC  1760
NODE_68291_after  CAAGAAGAACCAACCTTTCCAATACGTTTCTGTAGATGATTTCGTGCATGGATTCAAATCTTTTCACATTGGCCAACATC  1621

                  ******************************************************************************
NODE_68291_before TGTCATCCGATCTTAGGATTCCTTATGACAAATCAAAAACTCACCCAGCTGCACTAGTCAAAGAGAAATACGGGNNNN--  1838
NODE_68291_after  TGTCATCCGATCTTAGGATTCCTTATGACAAATCAAAAACTCACCCAGCTGCACTAGTCAAAGAGAAATACGGGNNNNGC  1701


NODE_68291_before --------------------------------------------------------------------------------  1838
NODE_68291_after  ACTAGTCAAAGAGAAATACGGGATTTCAAATATGGAGCTGTTCAAGGCATGCTTTGCCAGAGAATGGCTACTAATGAAGC  1781

                       ***************************************************************************
NODE_68291_before -----TCCTTTGTTTACATATTCAAGACCACCCAGATCACTATCATGTCGCTTATTGCTCTGACGGTGTTCCTTAGGACT  1913
NODE_68291_after  GAAACTCCTTTGTTTACATATTCAAGACCACCCAGATCACTATCATGTCGCTTATTGCTCTGACGGTGTTCCTTAGGACT  1861

                  ********************************************************************************
NODE_68291_before GAAATGCCAGTAGGGTCAGTGCAAGATGGAGGGAAGTTTTTTGGAGCACTTTTCTTCAGCTTGATCAATGTCATGTTCAA  1993
NODE_68291_after  GAAATGCCAGTAGGGTCAGTGCAAGATGGAGGGAAGTTTTTTGGAGCACTTTTCTTCAGCTTGATCAATGTCATGTTCAA  1941

                  ********************************************************************************
NODE_68291_before TGGAATGGCGGAACTTGCAATGACCGTTTTCAGGCTTCCTGTGTTCTATAAGCAGAGAGATTTCTTGTTTTACCCCGCTT  2073
NODE_68291_after  TGGAATGGCGGAACTTGCAATGACCGTTTTCAGGCTTCCTGTGTTCTATAAGCAGAGAGATTTCTTGTTTTACCCCGCTT  2021

                  ********************************************************************************
NODE_68291_before GGGCTTTCGGCTTGCCTATTTGGGTCCTCCGAATTCCGTTGTCATTCATGGAATCAGGGATATGGATCATCTTAACATAC  2153
NODE_68291_after  GGGCTTTCGGCTTGCCTATTTGGGTCCTCCGAATTCCGTTGTCATTCATGGAATCAGGGATATGGATCATCTTAACATAC  2101

                  ********************************************************************************
NODE_68291_before TACACCATTGGCTTCGCTCCAGCGGCCAGCAGGTTCTTCAAGCAATTCTTGGCATTCTTTGGCATCCATCAGATGGCACT  2233
NODE_68291_after  TACACCATTGGCTTCGCTCCAGCGGCCAGCAGGTTCTTCAAGCAATTCTTGGCATTCTTTGGCATCCATCAGATGGCACT  2181

                  ********************************************************************************
NODE_68291_before GTCCCTCTTTCGGTTCATTGCTGCAGTTGGGAGAACTCAGGTTGTCGCAAACACCCTGGGAACCTTCACTTTGCTAATGG  2313
NODE_68291_after  GTCCCTCTTTCGGTTCATTGCTGCAGTTGGGAGAACTCAGGTTGTCGCAAACACCCTGGGAACCTTCACTTTGCTAATGG  2261

                  ********************************************************************************
NODE_68291_before TTTTCGTTCTTGGAGGATTTATTGTTTCCAAAAACGACATCGAGCCATGGATGATATGGGGATATTACGTATCTCCTATG  2393
NODE_68291_after  TTTTCGTTCTTGGAGGATTTATTGTTTCCAAAAACGACATCGAGCCATGGATGATATGGGGATATTACGTATCTCCTATG  2341

                  ********************************************************************************
NODE_68291_before ATGTATGGGCAAAATGCTATAGTGATGAATGAATTCCTCGACAAAAGATGGAGCACGCGTAACGAGGATACTAGAATTAA  2473
NODE_68291_after  ATGTATGGGCAAAATGCTATAGTGATGAATGAATTCCTCGACAAAAGATGGAGCACGCGTAACGAGGATACTAGAATTAA  2421

                  ********************************************************************************
NODE_68291_before TGAGCCCACAGTTGGAAAAGTGCTTTTGAAGTCTCGAGGTTTCTTCGTACAAGAATATTGGTATTGGATCTGCATTGGAG  2553
NODE_68291_after  TGAGCCCACAGTTGGAAAAGTGCTTTTGAAGTCTCGAGGTTTCTTCGTACAAGAATATTGGTATTGGATCTGCATTGGAG  2501

                  ********************************************************************************
NODE_68291_before CACTGTTTGGGTTTTCACTCCTCTTCAACATCTTGTTTGTTGCAGCATTGACTTGGTTAAATCCTTTGGGAGATGCAAAA  2633
NODE_68291_after  CACTGTTTGGGTTTTCACTCCTCTTCAACATCTTGTTTGTTGCAGCATTGACTTGGTTAAATCCTTTGGGAGATGCAAAA  2581

                  ********************************************************************************
NODE_68291_before GCAGTTGTCTCGGATGAAGAGGCGGATAAGAAGAAAAACAAATCATTGTCTTCGCAACTTGCGAAAGAAGGAATCGACAT  2713
NODE_68291_after  GCAGTTGTCTCGGATGAAGAGGCGGATAAGAAGAAAAACAAATCATTGTCTTCGCAACTTGCGAAAGAAGGAATCGACAT  2661

                  ********************************************************************************
NODE_68291_before GCAAGTGAGAAGTTCTTCTGAAATCGTTAGCACTTCAGAGAATATACAGAGAAGAGGGATGGTTCTGCCATTCCAACCCC  2793
NODE_68291_after  GCAAGTGAGAAGTTCTTCTGAAATCGTTAGCACTTCAGAGAATATACAGAGAAGAGGGATGGTTCTGCCATTCCAACCCC  2741

                  ********************************************************************************
NODE_68291_before TTTCTCTTGCGTTCAACCATGTGAACTACTACGTGGATATGCCTGCAGAAATGAAGAGTCAAGGAGTTGAGGAAGACCGT  2873
NODE_68291_after  TTTCTCTTGCGTTCAACCATGTGAACTACTACGTGGATATGCCTGCAGAAATGAAGAGTCAAGGAGTTGAGGAAGACCGT  2821

                  ********************************************************************************
NODE_68291_before CTCCAACTGTTGAGAGATGTCAGTGGCGCTTTCAGACCAGGGGTACTCACAGCATTGGTCGGGGTTAGTGGTGCTGGAAA  2953
NODE_68291_after  CTCCAACTGTTGAGAGATGTCAGTGGCGCTTTCAGACCAGGGGTACTCACAGCATTGGTCGGGGTTAGTGGTGCTGGAAA  2901
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                  *********************** [illegible] *******************************
NODE_68291_before GACAACCCTCATGGATGTGCTAG [illegible] AGGAAGTATTAGCATCTCCGGATACCCTA   3033
NODE_68291_after  GACAACCCTCATGGATGTGCTAGCAGGAAGGAAGACAGGTGGTTACATAGAAGGAAGTATTAGCATCTCCGGATACCCTA   2981

                  ********************************************************************************
NODE_68291_before AAAACCAATCAACGTTTGCTCGGGTCAGTGGTTACTGTGAACAGAACGACATTCACTCGCCTAACGTCACTGTCTACGAA   3113
NODE_68291_after  AAAACCAATCAACGTTTGCTCGGGTCAGTGGTTACTGTGAACAGAACGACATTCACTCGCCTAACGTCACTGTCTACGAA   3061

                  ********************************************************************************
NODE_68291_before TCCCTCCTATACTCAGCCTGGCTTCGTCTTTCTTCCGACATTAAGACTCAAACTCGCAAGATGTTTGTGGAAGAAGTTAT   3193
NODE_68291_after  TCCCTCCTATACTCAGCCTGGCTTCGTCTTTCTTCCGACATTAAGACTCAAACTCGCAAGATGTTTGTGGAAGAAGTTAT   3141

                  ********************************************************************************
NODE_68291_before GGAGTTGGTTGAGCTCAACCCTATCAGAAACGCGCTTGTCGGGCTTCCTGGTGTCGATGGCCTTTCGACTGAGCAAAGAA   3273
NODE_68291_after  GGAGTTGGTTGAGCTCAACCCTATCAGAAACGCGCTTGTCGGGCTTCCTGGTGTCGATGGCCTTTCGACTGAGCAAAGAA   3221

                  ********************************************************************************
NODE_68291_before AGCGGCTGACAATAGCTGTAGAGTTGGTGGCTAATCCATCTATTATCTTTATGGACGAACCAACCTCCGGCCTTGATGCT   3353
NODE_68291_after  AGCGGCTGACAATAGCTGTAGAGTTGGTGGCTAATCCATCTATTATCTTTATGGACGAACCAACCTCCGGCCTTGATGCT   3301

                  ********************************************************************************
NODE_68291_before AGAGCAGCCGCCATCGTGATGCGTACGGTGAGGAACACGGTGGATACAGGGAGGACTGTTGTTTGCACGATTCACCAGCC   3433
NODE_68291_after  AGAGCAGCCGCCATCGTGATGCGTACGGTGAGGAACACGGTGGATACAGGGAGGACTGTTGTTTGCACGATTCACCAGCC   3381

                  ********************************************************************************
NODE_68291_before GAGCATTGACATTTTTGAAGCTTTTGATGAGTTGCTATTAATGAAAAGAGGCGGGCGGGTCATTTATGCTGGCCCTCTTG   3513
NODE_68291_after  GAGCATTGACATTTTTGAAGCTTTTGATGAGTTGCTATTAATGAAAAGAGGCGGGCGGGTCATTTATGCTGGCCCTCTTG   3461

                  ********************************************************************************
NODE_68291_before GTCGCCATTCCCACAAGCTCGTAGAATATTTTGAGGCTGTCCCAGGGGTTCCGAAGATCAGGGATGGTCACAATCCAGCC   3593
NODE_68291_after  GTCGCCATTCCCACAAGCTCGTAGAATATTTTGAGGCTGTCCCAGGGGTTCCGAAGATCAGGGATGGTCACAATCCAGCC   3541

                  ********************************************************************************
NODE_68291_before ACATGGATGCTTGAAGTGAGTGCTCCGGCAGTTGAGGCTCAGCTCGAGGTCGACTTCGCAGATATTTACCCAAACTCTGA   3673
NODE_68291_after  ACATGGATGCTTGAAGTGAGTGCTCCGGCAGTTGAGGCTCAGCTCGAGGTCGACTTCGCAGATATTTACCCAAACTCTGA   3621

                  ********************************************************************************
NODE_68291_before CCTTTATAAGCGGAACCAAGACCTGATCAAAGAGCTTAGTACCCCAGCCCCAGGCTGCAAAGATCTCCACTTCCCTACCG   3753
NODE_68291_after  CCTTTATAAGCGGAACCAAGACCTGATCAAAGAGCTTAGTACCCCAGCCCCAGGCTGCAAAGATCTCCACTTCCCTACCG   3701

                  ********************************************************************************
NODE_68291_before AGTACTCACAACCTTTCCTCACTCAGTGCAAGGCTTGTTTCTGGAAACAGCACTGGTCTTACTGGAGAAATCCTCAGTAC   3833
NODE_68291_after  AGTACTCACAACCTTTCCTCACTCAGTGCAAGGCTTGTTTCTGGAAACAGCACTGGTCTTACTGGAGAAATCCTCAGTAC   3781

                  ********************************************************************************
NODE_68291_before AACGCCATCCGGTTCTTTATGACCATAGTCATCGCCATTTTGTTTGGTTTAATATTCTGGGATAAAGGACAGCAGACGAC   3913
NODE_68291_after  AACGCCATCCGGTTCTTTATGACCATAGTCATCGCCATTTTGTTTGGTTTAATATTCTGGGATAAAGGACAGCAGACGAC   3861

                  ********************************************************************************
NODE_68291_before CAAGCAACAAGACCTGATGAATCTTTTGGGAGCCATGTACGCAGCTGTGCTTTTCCTTGGGGCCACAAATGCTTCTGCTG   3993
NODE_68291_after  CAAGCAACAAGACCTGATGAATCTTTTGGGAGCCATGTACGCAGCTGTGCTTTTCCTTGGGGCCACAAATGCTTCTGCTG   3941

                  ********************************************************************************
NODE_68291_before TGCAGTCTATAGTCGCCATTGAGAGGACAGTCTTCTACCGTGAACGAGCAGCTGGAATGTACTCTCCGCTGCCATACGCA   4073
NODE_68291_after  TGCAGTCTATAGTCGCCATTGAGAGGACAGTCTTCTACCGTGAACGAGCAGCTGGAATGTACTCTCCGCTGCCATACGCA   4021

                  ********************************************************************************
NODE_68291_before TTTGCTCAGGTGGCTATTGAGACAATTTATGTAGCGATTCAGACATTGGTCTACAGTCTTCTCCTTTACTCGATGATTGG   4153
NODE_68291_after  TTTGCTCAGGTGGCTATTGAGACAATTTATGTAGCGATTCAGACATTGGTCTACAGTCTTCTCCTTTACTCGATGATTGG   4101

                  ********************************************************************************
NODE_68291_before GTTCGAGTGGAAGGCGGGGAAGTTCTTGTGGTTCTACTACTACATACTGATGTGCTTCATCTACTTCACGATGTATGGAA   4233
NODE_68291_after  GTTCGAGTGGAAGGCGGGGAAGTTCTTGTGGTTCTACTACTACATACTGATGTGCTTCATCTACTTCACGATGTATGGAA   4181

                  ********************************************************************************
NODE_68291_before TGATGGTTGTAGCATTGACACCAGGCCACCAGATAGCTGCCATTGTGATGTCCTTCTTCCTGAGCTTCTGGAACTTGTTC   4313
NODE_68291_after  TGATGGTTGTAGCATTGACACCAGGCCACCAGATAGCTGCCATTGTGATGTCCTTCTTCCTGAGCTTCTGGAACTTGTTC   4261

                  ********************************************************************************
NODE_68291_before TCTGGCTTCCTTATCCCTAGGCCGCAAATTCCTGTATGGTGGAGGTGGTATTACTGGGCTTCACCAGTGGCATGGACGCT   4393
NODE_68291_after  TCTGGCTTCCTTATCCCTAGGCCGCAAATTCCTGTATGGTGGAGGTGGTATTACTGGGCTTCACCAGTGGCATGGACGCT   4341

                  ********************************************************************************
NODE_68291_before GTACGGTCTTGTCACCTCTCAAGTGGGCGACAAGAATGGCAATCTCGAAATACCAGGAGCCGGCAACATGCCGTTGAAGC   4473
NODE_68291_after  GTACGGTCTTGTCACCTCTCAAGTGGGCGACAAGAATGGCAATCTCGAAATACCAGGAGCCGGCAACATGCCGTTGAAGC   4421
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                  ********************************************************************************
NODE_68291_before AGTTCCTGAAGGTAGAACTGGGTTTTGACTACAGCTTCCTCCCCGCTGTCGCGGTTGCTCACATCGGCTGGGTCCTTCTC  4553
NODE_68291_after  AGTTCCTGAAGGTAGAACTGGGTTTTGACTACAGCTTCCTCCCCGCTGTCGCGGTTGCTCACATCGGCTGGGTCCTTCTC  4501

                  ********************************************************************************
NODE_68291_before TTTTTCTTTGTCTTCGCTTACGGCATCAAGTTCCTCAATTTCCAGAGGAGATAAAACCGATGGCAAACAGTTCTCACTTT  4633
NODE_68291_after  TTTTTCTTTGTCTTCGCTTACGGCATCAAGTTCCTCAATTTCCAGAGGAGATAAAACCGATGGCAAACAGTTCTCACTTT  4581

                  ********************************************************************************
NODE_68291_before CTGGCTAGATTTTGAAACGTTAAACGTAGGCCCATCATGTAAATTAAGGATGATAGGCGACTAAAGAGTCTCCCTCCTCC  4713
NODE_68291_after  CTGGCTAGATTTTGAAACGTTAAACGTAGGCCCATCATGTAAATTAAGGATGATAGGCGACTAAAGAGTCTCCCTCCTCC  4661

                  ************************************************************************* ******
NODE_68291_before TGTTTTCTTCACTTTTCAGTAAGTCTTGCTTTTGTAACACTAGCATTCTTTGTCACCGCTGCTTCATTGGACTGAGAGCG  4793
NODE_68291_after  TGTTTTCTTCACTTTTCAGTAAGTCTTGCTTTTGTAACACTAGCATTCTTTGTCACCGCTGCTTCATTGGACTTAGAGCG  4741

                  ** *******
NODE_68291_before TCAGTTAATTGTAAGAGACAAATAATTAATTTGAAATGCAAACGAGTGGTGTG  4846
NODE_68291_after  TCGGTTAATT-------------------------------------------  4751
```

**Appendix D**

# *De novo* assembled expressed gene catalog of a fast-growing *Eucalyptus* tree produced by Illumina mRNA-Seq

Mizrachi et al. BMC Genomics 2010, **11**:681
http://www.biomedcentral.com/1471-2164/11/681

BMC Genomics

**RESEARCH ARTICLE** **Open Access**

# *De novo* assembled expressed gene catalog of a fast-growing *Eucalyptus* tree produced by Illumina mRNA-Seq

Eshchar Mizrachi[1†], Charles A Hefer[2†], Martin Ranik[1], Fourie Joubert[2], Alexander A Myburg[1*]

## Abstract

**Background:** *De novo* assembly of transcript sequences produced by short-read DNA sequencing technologies offers a rapid approach to obtain expressed gene catalogs for non-model organisms. A draft genome sequence will be produced in 2010 for a *Eucalyptus* tree species (*E. grandis*) representing the most important hardwood fibre crop in the world. Genome annotation of this valuable woody plant and genetic dissection of its superior growth and productivity will be greatly facilitated by the availability of a comprehensive collection of expressed gene sequences from multiple tissues and organs.

**Results:** We present an extensive expressed gene catalog for a commercially grown *E. grandis* × *E. urophylla* hybrid clone constructed using only Illumina mRNA-Seq technology and *de novo* assembly. A total of 18,894 transcript-derived contigs, a large proportion of which represent full-length protein coding genes were assembled and annotated. Analysis of assembly quality, length and diversity show that this dataset represent the most comprehensive expressed gene catalog for any *Eucalyptus* tree. mRNA-Seq analysis furthermore allowed digital expression profiling of all of the assembled transcripts across diverse xylogenic and non-xylogenic tissues, which is invaluable for ascribing putative gene functions.

**Conclusions:** *De novo* assembly of Illumina mRNA-Seq reads is an efficient approach for transcriptome sequencing and profiling in *Eucalyptus* and other non-model organisms. The transcriptome resource (Eucspresso, http://eucspresso.bi.up.ac.za/) generated by this study will be of value for genomic analysis of woody biomass production in *Eucalyptus* and for comparative genomic analysis of growth and development in woody and herbaceous plants.

## Background

Ultra-high-throughput second-generation DNA sequencing technologies from companies such as Roche (454 pyrosequencing), Illumina (sequencing by synthesis, Solexa GA) and Applied Biosystems (sequencing by ligation, SOLiD), are increasingly being used for novel exploratory genomics in small to medium-sized laboratories. "Short-read" (36 - 72 nt) technologies such as those of Illumina and Applied Biosystems have proven to be exceptionally successful in a wide variety of whole-transcriptome investigations [1-5], but most of these studies have relied on prior sequence knowledge such as an annotated genome for qualitative and quantitative transcriptome analyses.

Genome assembly of short sequences without any auxiliary knowledge has primarily utilized 454 sequencing data, due to the longer individual read lengths of 150-400 base pairs (bp). However, short-read sequencing (Illumina GA and SOLiD) has been successfully used for *de novo* assembly of small bacterial genomes (2-5 Mbp), where 36 bp reads have been assembled [6-8] and hybrid approaches, where genomes are *de novo* assembled using a combination of reads from multiple sequencing platforms to overcome the inherent limitations of each technology, have been used to successfully assemble genomes of up to 40 Mbp [9,10]. More recently, the sequencing of the giant panda genome was demonstrated [11] using *de novo* assembly of sequence derived from a single platform (Illumina), but utilizing a

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combination of different insert sizes, allowing assembly of an estimated 94% of the genome (2.25 Gbp). *De novo* assembly of large, highly repetitive and highly heterozygous eukaryotic genomes from short-read data remains a challenge.

In transcriptome studies, 454 pyrosequencing has proven very useful for generating ESTs representing the majority of expressed genes. This has enabled gene discovery in a variety of previously uncharacterized eukaryotic organisms with no or little *a priori* DNA sequence information [12-16]. However, relatively few published studies have attempted *de novo* assembly of whole-transcriptome sequences from short-read data such as that generated by Illumina GA or SOLiD technologies. Assembly of short (36-72 bp) read data into accurate, contiguous transcript sequences has only recently been reported [17-19] demonstrating that assembly of long, potentially full-length, transcript assemblies is indeed possible.

*Eucalyptus* tree species and hybrids presently constitute the most widely planted (≈ 20 Mha) and commercially important hardwood fibre crop in the world. They are mainly utilized for timber, pulp and paper production [20]. Their fast growth rates and wide adaptability may in future allow sustainable and cost efficient production of woody biomass for bioenergy generation [21,22]. *Eucalyptus* will soon be only the second forest plantation genus (after *Populus*) for which a reference genome sequence will be completed by end 2010 [23]. To support the genome annotation effort, there is much value in having a dataset of genes with strong transcriptional evidence across a range of tissues and developmental stages. Until recently, limited amounts of *Eucalyptus* EST/unigene data were available in public databases, mainly due to the fact that commercial interests have necessitated private EST collections [24]. As of March 2010, aside from a mixed-species collection of ≈56,000 nucleotide sequences on NCBI (≈ 37,000 of which are Sanger EST sequences) and which contain extensive redundancy, the largest effort to date to generate a comprehensive catalogue of expressed genes in a single *Eucalyptus* species was based on 454 sequencing of cDNA fragments from *E. grandis* trees [15]. While this study provided an excellent representation of expressed genes and gene ontology classes in *E. grandis*, the relatively short lengths of the assembled contigs (mean length of 389 bp for all contigs longer than 200 bp) meant that very few complete gene models were represented. There remains therefore a fundamental need for a high-quality expressed gene catalog for *Eucalyptus*, to support genome annotation efforts and discern authentically expressed genes from predicted gene models, as well as for future genomics research, which will include transcriptome, proteome and metabolome profiling.

In the process of producing such a high-quality expressed gene catalog for *Eucalyptus*, we addressed three main questions: First, is it feasible to *de novo* assemble Illumina mRNA-Seq data into contiguous, near full-length gene model sequences for *Eucalyptus*? Second, what genes make up the expressed gene catalog for a fast-growing *Eucalyptus* plantation tree? Finally, can we re-use the mRNA-Seq data to create a tissue and organ-specific digital expression profile for each assembled contig? We addressed these questions by generating a comprehensive set of expressed gene sequences from a commercially grown *Eucalyptus* hybrid (*E. grandis* × *E. urophylla*) clone using Illumina mRNA-Seq technology and *de novo* short-read assembly. We report herein the complete annotation of the expressed gene catalog based on comparative analysis with the published *Arabidopsis thaliana* [25], *Populus trichocarpa* [26] and *Vitis vinifera* [27] protein-coding datasets. We describe an interactive database of annotated transcript sequences, coding sequences (CDSs) and derived protein sequences (Eucspresso, http://eucspresso.bi.up.ac.za/, CA Hefer, E Mizrachi, AA Myburg, F Joubert, unpublished), which will be continuously updated and curated in association with the *Eucalyptus* Genome Network (EUCAGEN, http://www.eucagen.org) as part of an effort to initiate a publicly accessible database for *Eucalyptus* transcriptomics research similar to that produced for *Populus* [28].

## Results

### *De novo* assembly, validation and annotation of contigs

In total, 62 million paired-end reads of raw mRNA-Seq data (6.90 Gbp) representing poly(A)-selected RNA from six *Eucalyptus* tissues and varying in lengths from 36 bp to 60 bp, were generated in 14 lanes on Illumina GA and GAII instruments. Following a sequence filtering process to exclude low quality and ribosomal RNA-derived reads, we assembled 36 million paired-end reads (3.93 Gbp, Additional file 1 - Table S1 and Figure S1, NCBI Sequence Read Archive accession SRA012408) of non-normalized mRNA sequence, using the Velvet short-read assembler (version 0.7.30, [29]). In total, 18,894 RNA-derived contigs were assembled (comprising 22.1 Mbp of transcriptome sequence) that were greater than 200 bp in length (mean = 1170 bp, Figure 1 and Additional file 2), with a median coverage per base (CPB) per contig of 37×, ranging from 8× (minimum coverage cut-off for assembly) to 5,262× (Additional file 1 Figure S2).

We performed *ab inito* CDS prediction using GENSCAN [30] and found that 15,713 contigs (83.2%) contained a predicted CDS (Additional file 1 Table S3). Analysis of the predicted coding sequences using Anaconda [31] identified 6,208 contigs that contained

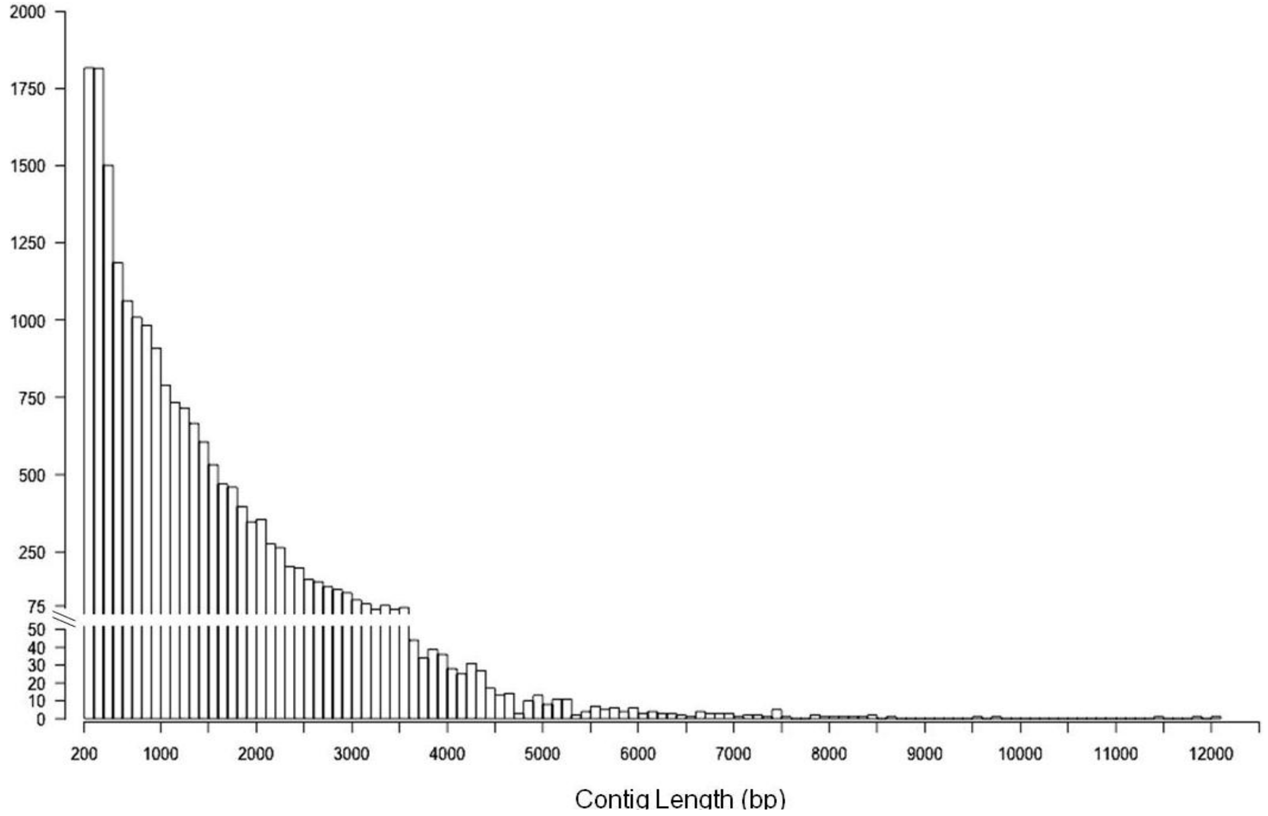

**Figure 1 Summary distribution of the lengths of the 18,894 assembled contigs (> 200 bp, mean length = 1170 bp, N50 = 1,640 bp, Q3 = 1,573 bp, Max = 12,053 bp)**.

putatively full-length CDSs (i.e. containing start and stop codons), 4,610 predicted to contain a start but no stop codon, 4,874 predicted to contain a stop but no start codon, and only 21 with neither. To ascertain the quality of Velvet assembly of short reads into long contiguous coding sequences, we compared a subset of 35 of our transcript-derived contigs to corresponding Sanger-sequenced, full-length, cloned *Eucalyptus grandis* mRNA sequences in NCBI (Figure 2 and Additional file 3). Paired reads were independently mapped to each Sanger reference sequence, the *de novo* assembled Velvet contig and its corresponding predicted CDS. A Needleman-Wunsch alignment of these three sequences was used for contiguity validation of the assembled contigs. Independently, each sequence had 100% coverage validation across the contig, except in cases of low quality assembly ('N's inserted by Velvet), which occurred in regions of coverage lower than 8× per base. Of the 35 transcript-derived contigs evaluated, 25 (71%) assembled completely with a 5' UTR, 3' UTR, as well as a contiguous coding sequence matching that of the reference mRNA sequence. We found several cases where, despite high coverage, our transcript-derived contigs differed from the Sanger reference sequence due to indels, but these were generally in the UTR regions and likely represent allelic differences between the F1 hybrid individual and the reference sequences (Additional file 3).

Of the 18,894 assembled contigs, 18,606 (98.48%) exhibited significant similarity (BLASTN, -10, [31]) to the preliminary draft 8X DOE-JGI *E. grandis* genome assembly (http://eucalyptusdb.bi.up.ac.za/) consistent with the origin of the mRNA contigs (an F1 hybrid of *E. grandis* and *E. urophylla*). We further characterized the assembled contigs by high stringency BLASTX analysis (-10 confidence, minimum 100 bp high scoring pair (HSP) match length) to protein datasets from three reference sequenced angiosperm genera (*Arabidopsis*, *Populus* and *Vitis*). Cumulatively, 15,055 contigs (79.68%) exhibited high similarity to *Arabidopsis* (14,235 contigs), *Populus* (14,769 contigs) or *Vitis* proteins (14,833 contigs, Additional file 1 Figure S3). Of the 15,055 contigs with high similarity to *Arabidopsis*, *Populus* or *Vitis* proteins, 13,806 (91.70%) also contained predicted coding sequences (Figure 3A), while 1,249 (8.30%) did not (Figure 3B), possibly due to low expression of these transcripts which would have resulted in lower coverage and shorter contigs that represented only a fraction of the open reading frame (or mostly

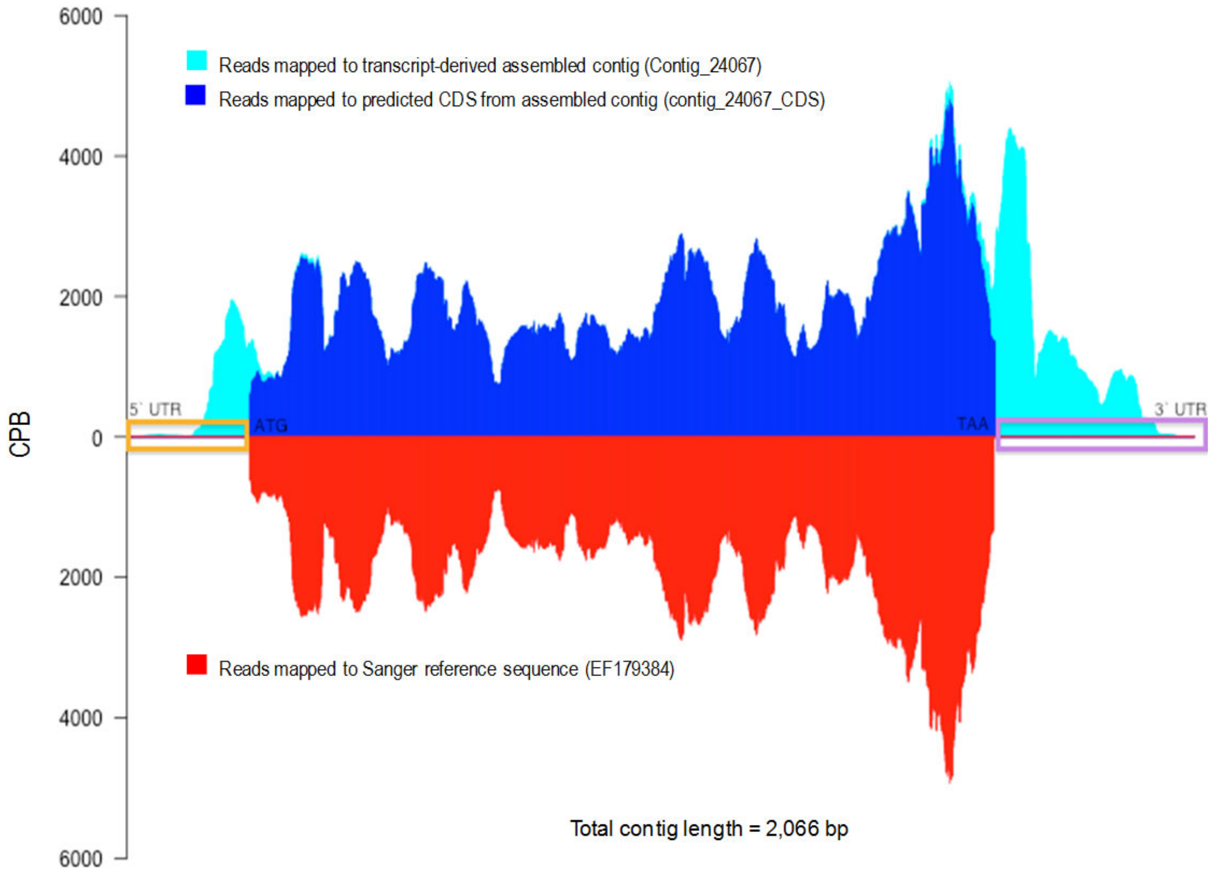

**Figure 2 Comparison of the *de novo* assembled contig of the *Eucalyptus grandis* UDP-glucose dehydrogenase (*UGDH*) transcript to a reference Sanger-based sequence (Genbank** EF179384**) for the same gene**. Peak height indicates coverage per base (CPB) of mapped short-reads across each sequence. CPB of the fully assembled contig is shown in cyan. CPB of the predicted CDS region is shown in dark blue. CPB of the Sanger reference sequence is shown in red. 5′ UTR (orange box) and 3′ UTR (purple box) regions are indicated.

UTR sequence). Predicted codon usage and amino acid frequencies in the proteome represented by the *Eucalyptus* expressed gene catalog were very similar to those of expressed gene catalogs from *Arabidopsis* and *Populus* (Additional file 1 Figure S4 and Figure S5).

To compare the completeness of our expressed gene catalogue to that of all publicly available gene sequence data for *Eucalyptus*, we generated a separate dataset, termed EucALL, containing all publicly available *Eucalyptus* gene sequence data to date (March 2010). This included all NCBI unigenes and ESTs, assembled 454 EST data from *E. grandis* leaf tissue (DOE-JGI, http://eucalyptusdb.bi.up.ac.za/), assembled 454 EST data produced by Novaes and colleagues [15], and the EucaWood contig dataset [33]. We compared the representation of *Arabidopsis* genes in the EucALL dataset and in our assembled *E. grandis* × *E. urophylla* (EGU) transcript dataset by BLASTX at significance levels of < $1e^{-05}$, < $1e^{-10}$ and < $1e^{-20}$ (Additional file 1 Table S2). While the overall numbers of hits were higher in the EucALL dataset, these were mostly in the lower size ranges. For our *de novo* assembled contigs, a much higher number of significant hits in contigs larger than 2000 bp in size (6,602 compared to 1,940 at significance < $1e^{-10}$) suggested that a greater proportion of our contigs represent full-length gene models than the publicly available *Eucalyptus* gene sequence set (EucALL).

### Functional annotation of the expressed gene catalog

The transcript-derived contig sequences were annotated according to several functional annotation conventions, including Gene Ontology (GO - http://www.geneontology.org/), KEGG (http://www.genome.jp/kegg/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). The numbers and assortment of allocated GO categories provides a good indication of the large diversity of expressed genes sampled from the *Eucalyptus* transcriptome (Figure 4). This was also reflected in the diversity of InterProScan categories identified (Additional file 1 Figure S6 and Figure S7), as well as the

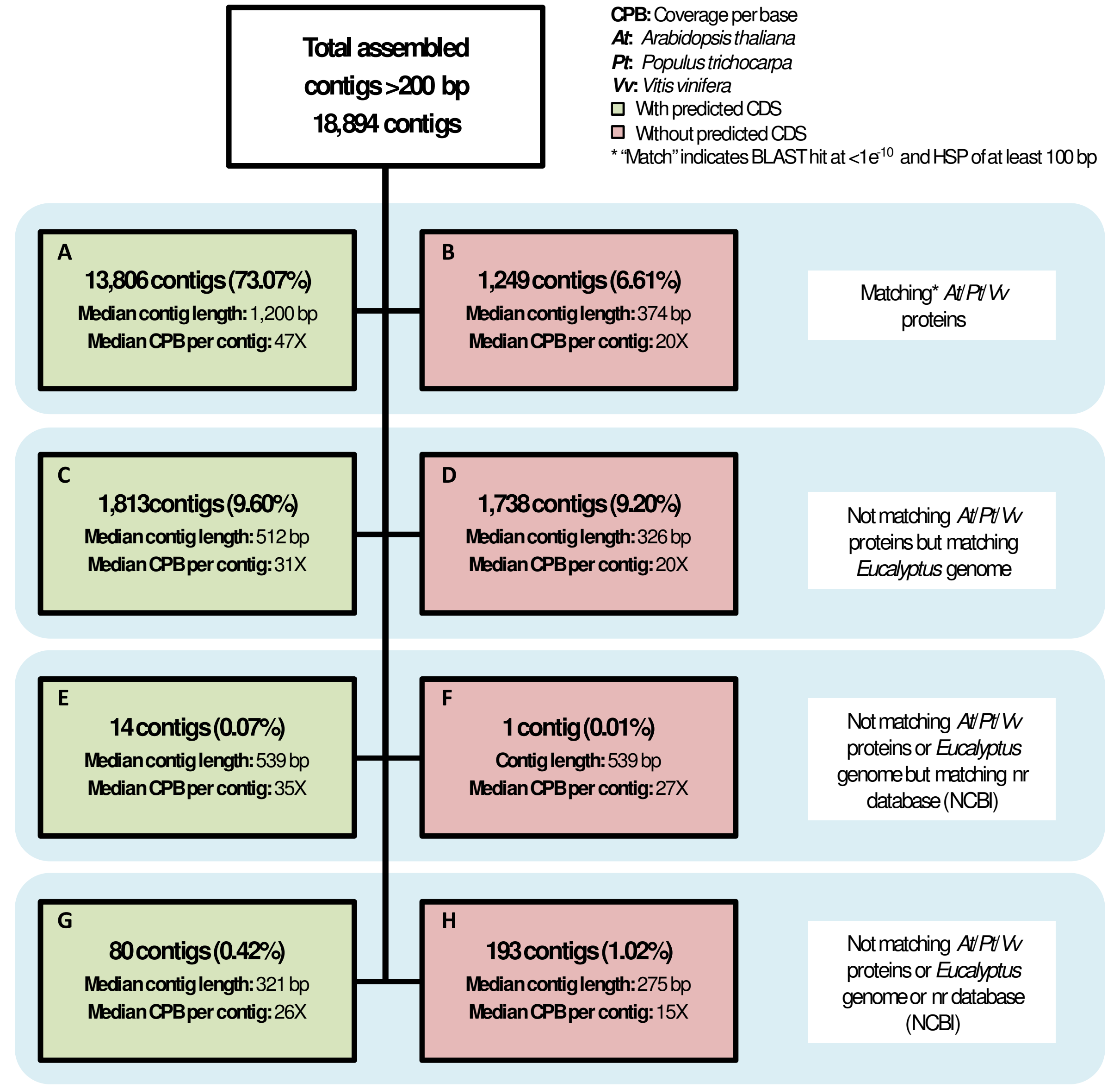

**Figure 3 Breakdown of annotation categories for all 18,894 transcript-derived contigs**. A large proportion (98.5%) of assembled contigs (A-D) had significant BLAST hits (< $1e^{-10}$ confidence, minimum 100 bp HSP match length) to the draft *Eucalyptus* genome assembly (http://eucalyptusdb.bi.up.ac.za/), 80% of which (A, B) also exhibited significant similarity (BLASTX, < $1e^{-10}$, > 100 bp HSP) to coding sequences of *Arabidopsis*, *Populus* or *Vitis*.

comprehensive coverage of biochemical processes by KEGG annotation, which was similar to that of the entire *Arabidopsis* gene catalog (Additional file 1 Figure S8).

## Digital expression profiling

An accepted method of identifying large scale differences in gene expression is to use EST abundance as an indicator of transcript abundance. This method has been implemented and validated in numerous studies using Sanger-derived ESTs [34,35], as well as 454-pyrosequencing methods [13,36-39]. Quantitative transcriptome analysis using ultra-high-throughput sequencing technologies such as Illumina and SOLiD has been shown to be accurate and highly correlated with other quantitative methods such as RT-qPCR and microarray analysis [1,5]. To quantify tissue-specific transcript abundance reflected in our short-read dataset, we combined data (multiple lanes in most cases) generated from the same tissues and mapped six tissue-specific datasets (Additional file 1 Table S1) to the assembled gene catalog using Bowtie [40]. Following this, we used

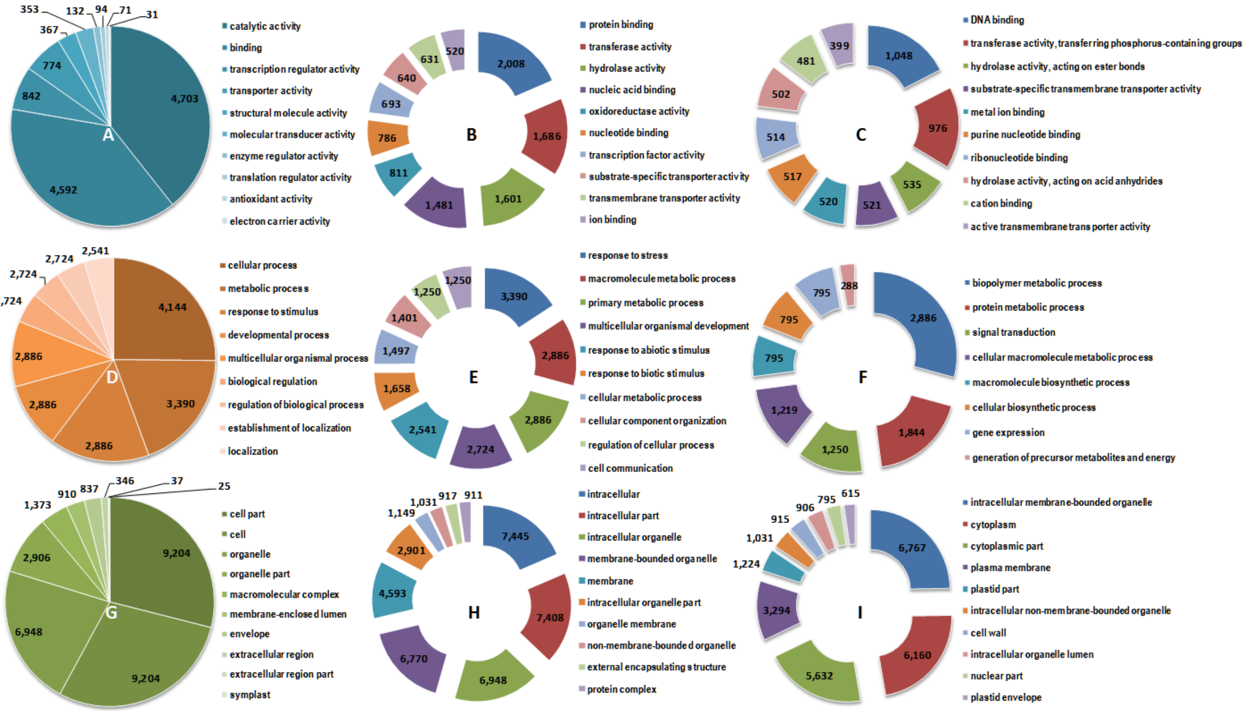

**Figure 4 Top ten most represented GO categories under the "Molecular Function" (A-C), "Biological Process" (D-F) and "Cellular Compartment" (G-I) categories in level 2 (A, D and G), 3 (B, E and H) and 4 (C, F and I).** The numbers and proportions in all categories reflect the diversity and complexity of genes expressed in multiple tissues sampled to make up the *Eucalyptus* gene catalog.

the Cufflinks [41] program (http://cufflinks.cbcb.umd.edu), which provides relative abundance values by calculating Fragments Per Kilobase of exon per Million fragments mapped (FPKM) as validated previously [2]. This enabled the allocation of a tentative digital expression profile for each transcript-derived contig (Additional file 4).

To compare between two general tissue types that are of interest for woody biomass production, we evaluated groups of genes whose FPKM values were greater than two-fold higher in woody (xylogenic) tissues (average FPKM of immature xylem and xylem: 1,897 annotated contigs) or leaf (non-xylogenic) tissues (average FPKM of shoot tips, young leaves and mature leaves: 1,531 annotated contigs). GO categories over-represented in the xylem-upregulated set compared to the leaf set (Figure 5A) was representative of developing woody tissues, with significant enrichment ($p < 0.05$) in signalling ("kinase activity"), carbohydrate metabolism, and genes associated with the Golgi, cytoskeleton and the plasma membrane - consistent with an emphasis on delivery of biopolymers to the cell wall. In contrast, gene categories significantly enriched ($p < 0.05$) in leaf tissue compared to woody tissue (Figure 5B) were associated with photosynthesis ("plastid", "thylakoid", "photosynthesis"), growth and energy production (precursor metabolites, "lipid biosynthesis", "amino acid metabolism").

We also interrogated our transcriptome data using the "core xylem gene set" identified in *Arabidopsis* by Ko and colleagues [42]. Of the 52 genes identified by the authors as markers of secondary xylem formation in *Arabidopsis*, 33 had putative homologues in the *Eucalyptus* transcriptome (BLASTX, < $1e^{-10}$) and in total 43 contigs were identified. Of these, 40 (93%) showed greater than two-fold "Xylem" to "Leaf" digital expression profile ratios and six were only detected in xylem tissues (Additional file 1 Table S4). Most of the expression profiles were also highly correlated with that of secondary cell wall-specific *Eucalyptus* cellulose synthase genes, similar to the patterns previously observed in *Arabidopsis*. These results are comparable to the 80% (51 out of 63 genes) reported recently for the same set of *Arabidopsis* homologs in *Populus* [43], which provided further support for the biological validity of the short-read-based digital expression profiles associated with the *Eucalyptus* expressed gene catalog.

## Public data resource

We constructed a public data resource, Eucspresso (http://eucspresso.bi.up.ac.za), which provides a

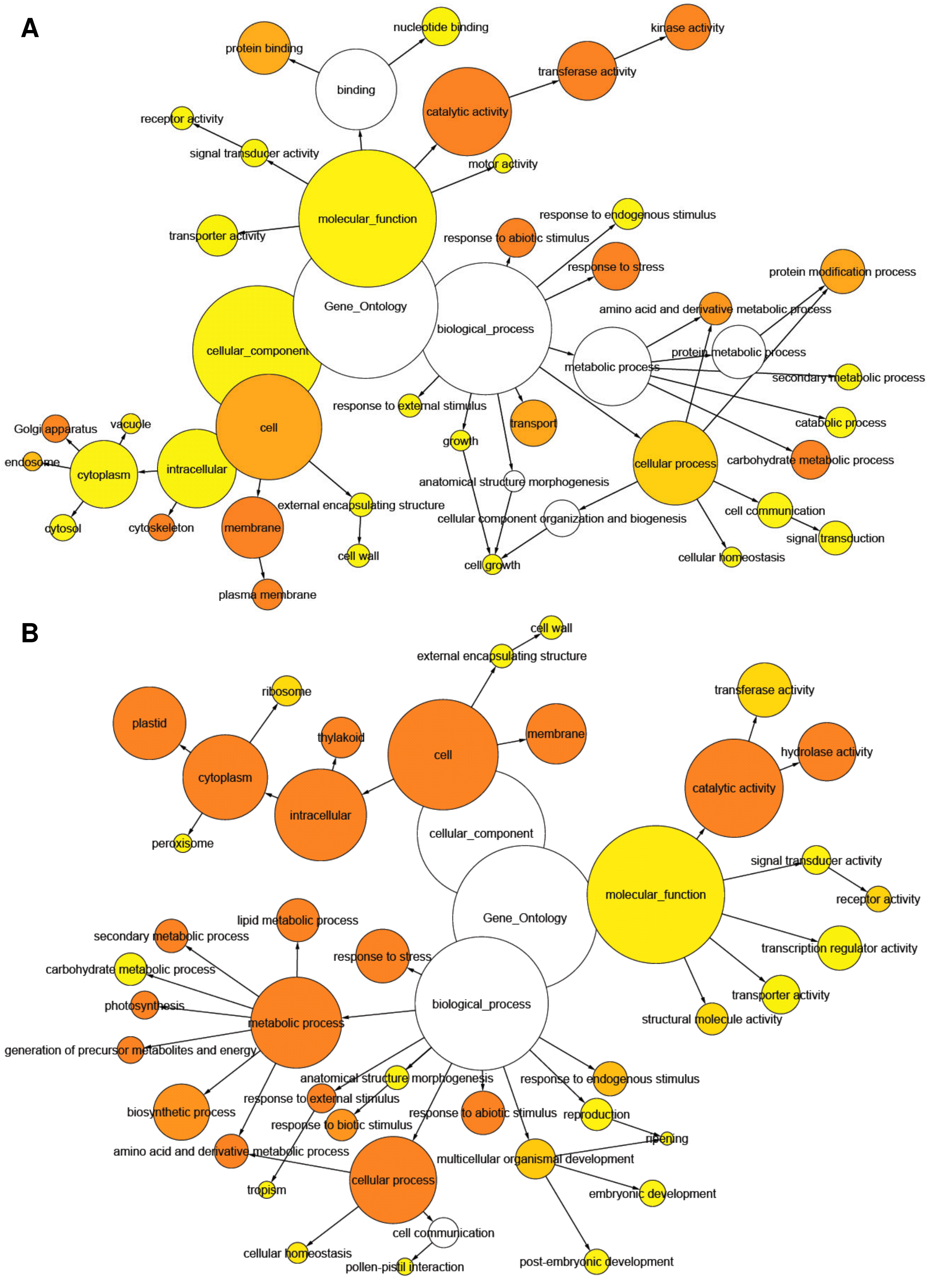

**Figure 5 Over-represented GO categories in xylem (A - 1,897 annotated contigs) and leaf (B - 1,531 annotated contigs) tissues**. All genes with a FPKM value more than two-fold higher in one tissue type versus the other were considered for the analysis. Data were analyzed using BiNGO (Maere et al. 2005). Node size is proportional to the number of genes in each category and colors shaded according to significance level (white - no significant difference, yellow - FDR = 0.05, Orange - FDR < 0.05).

searchable interface to the assembled contigs. The database can be queried based on closest homologous entry in the *Arabidopsis thaliana* (TAIR9), *Populus trichocarpa* (Version 2.0) and *Vitis vinifera* (Sept 2009 build) sequence data sets. Simple and compound keyword searches can be performed based on all of the functional annotation terms and the predicted coding and protein sequences can be obtained for all contigs. Finally, the tissue-specific (FPKM) digital expression profile and the location of each contig in the draft 8X *E. grandis* genome assembly (http://eucalyptusdb.bi.up.ac.za/) can be viewed from within Eucspresso.

## Discussion

We have assembled nearly 19,000 expressed gene sequences from xylogenic and non-xylogenic tissues of an actively growing *Eucalyptus* plantation tree using only Illumina mRNA-Seq technology and *de novo* short-read assembly. Quality control comparisons to full-length, cloned, Sanger-derived transcript sequences from *Eucalyptus*, as well as multiple lines of evidence such as CDS prediction and Pfam prediction showed that the transcript assemblies are robust and that thousands of full-length coding sequences and their respective 5' and/or 3' UTR regions were successfully assembled. Comparison of assembled gene models to gene catalogs of other angiosperm species by BLAST analysis and functional annotation (GO, InterProScan and KEGG category numbers and proportions, Figure 4 and Additional file 1 - Figure S6, Figure S7 and Figure S8) indicate that we have sampled an expansive and diverse expressed gene catalog representing a large proportion of the genes expressed in mature *Eucalyptus* trees across a variety of woody and non-woody tissues. Comparison to all publicly available *Eucalyptus* DNA sequence suggests that we have sampled a more comprehensive set of genes, which is also more complete in length (Additional file 1 - Table S2) from a single eucalypt tree genotype than has been available to date for the entire genus. Additionally, using a validated approach to quantify mRNA-Seq data we have produced an informative database of transcript abundance across six *Eucalyptus* tree tissues, which, due to the depth of sequencing, results in higher sensitivity and wider dynamic range than Sanger or 454-derived EST counts usually associated with this type of analysis.

A concern associated with *de novo* assembly of transcript sequences, be it Sanger derived [33] or 454 sequence derived [15] assemblies, is the contiguity of assembled sequences. This concern intuitively increases as the read length decreases, and may be one of the main reasons why most transcriptome *de novo* assembly approaches have utilized technologies with longer read lengths to date. We provide several lines of evidence which jointly support the contiguity of transcript sequences assembled in our study using short-read data. First, a high proportion of the contigs exhibited high-confidence BLASTX similarity to protein sequences from annotated gene catalogs of three angiosperm species *Arabidopsis*, *Populus* and *Vitis* (Figure 3). Second, a large proportion of the contigs contained long, near full-length, predicted CDSs (Figure 3). Third, InterproScan analysis predicted 45,687 protein domains, which is indicative of contiguous, in-frame predicted protein sequences (Additional File 1). Finally, a random subset of the contigs, which represented a variety of length and read coverage, were validated by direct alignment to previously published, Sanger sequenced, full-length *Eucalyptus* genes that were directly cloned from cDNA (Additional File 3).

Assigning biological significance to *de novo* assembled contigs should be approached with caution. In our study, 13,806 assembled gene models (73.07% of the total assembled contigs, Figure 3A) were considered high confidence annotations due to the presence of a significant high stringency BLAST hit in other angiosperm species, as well as a predicted CDS. These contigs had relatively high coverage per base (CPB) values (median 47X) as compared to contigs lacking a predicted CDS (median CPB of 20× or lower, Figure 3B and 3D and Supplemental Table S3). Thus, a lack of CDS prediction was generally associated with low gene expression level and low CPB, which resulted in 'N's inserted by Velvet in the contig sequences (Figure 3B and 3D and Supplemental Table S3). The assembly quality and annotation of these sequences could be improved in future by even deeper sequencing and the addition of data from new tissue types. Another possible source of error is the spurious prediction of CDSs in long, non-coding RNAs, which has been previously shown to occur [44,45]. It is notable that of the 1,813 *Eucalyptus*-derived contigs with no significant BLAST hit to other angiosperms, but containing a predicted CDS (Figure 3C), only 81 contigs had predicted InterProScan domains. Additionally, the median CDS to contig length ratio was 0.33, as compared to 0.62 in the 13,806 high confidence contigs in Figure 3A, which suggests that many of these CDS predictions may be false positives. *De novo* assembled transcriptome datasets lack the ability to distinguish and classify the lower confidence annotations, an exercise that is beyond the scope of this study, albeit one that can be resolved once a genome-based predicted set of gene models is available.

Validation of the digital expression (FPKM) profiles using the "core xylem gene set" identified in *Arabidopsis* [42] has precedence in similar investigations in conifers [46], cotton [47] and poplar [43]. This analysis, combined with the results shown in Figure 5A and Figure

5B, lend support to the biological significance of digital expression profiles derived from short-read sequencing technology, which will assist in the discovery and annotation of novel *Eucalyptus* genes - and using the genome sequence, promoters - playing key roles in growth and development, and particularly in woody biomass production. The Eucspresso online resource produced from this study, as well as future comparative analysis with other woody species such as *Vitis* and *Populus*, will be valuable for studying the unique biology of woody perennials.

## Conclusions

Taking into consideration the number, length, coverage and quality of assembled gene models, as well as their digital expression profiles, this dataset surpasses several previous *de novo* transcriptome assemblies using Illumina [17,18] or 454 technology [13-16]. This can primarily be attributed to the amount of data generated (3.93 Gbp of non-rRNA derived reads), the diversity of tissues sampled and strategy of paired-end sequencing, as well as read-length (mostly 50-60 bp, compared to only 36 bp in earlier studies). Our dataset was generated using several generations of Illumina GA technology, but considering the current throughput of Illumina sequencing (up to 100 Gbp per flowcell), a gene catalog of this scale can now be produced using a single lane of Illumina mRNA-Seq. Finally, non-normalized short-read data will be extremely useful for downstream applications such as digital gene expression profiling and detection of alternative transcript structure, once reference models are available from the genome.

## Methods

### Plant tissue collection

Tissues from a six-year-old ramet of a commercially grown *E. grandis* × *E. urophylla* hybrid clone (GUSAP1, Sappi Forestry, Kwambonambi, South Africa) were collected in a clonal field trial and immediately frozen in liquid nitrogen, as previously described by Ranik and Myburg [48]. The following tissues were sampled from approximately breast height (1.35 m) on the main stem following bark removal: immature xylem (outer glutinous 1-2 mm layer comprising early developing xylem tissue) and xylem (after removal of the immature xylem layer, 2-mm-deep planing including xylem cells in advanced stages of maturity). Early developing phloem tissue including small amounts of cambial cells was collected by scraping the first 1-2 mm layer from the inner surface of the bark. Additionally, we sampled shoot tips (soft green termini of young crown tip branches containing shoot primordia and apical meristems), young leaves (rapidly-growing leaves in the process of unfolding) and mature leaves (older, fully expanded leaves of the current growth season).

### Paired-end mRNA-Seq library preparation and sequence generation

Total RNA was extracted from the six tissues using the protocol described previously [49]. Total RNA quality and concentration were determined using the Agilent RNA 6000 Pico kit (Agilent, Santa Clara, CA) on a 2100 Bioanalyzer (Agilent). Enrichment of polyA+ RNA was performed using the Oligotex midi kit (Qiagen, Valencia, CA). Two hundred nanograms of polyA+ RNA were fragmented in 1× RNA fragmentation solution (Ambion, Austin, TX) at 70°C for 5 minutes. The fragmented RNA was precipitated with three volumes of ethanol and re-dissolved in water. Double-stranded cDNA was synthesized using the cDNA Synthesis System (Roche, Indianapolis, IN) according to manufacturer's instructions using random hexamers (Invitrogen, Carlsbad, CA) to prime the first strand cDNA synthesis. Paired-end libraries with approximate average insert lengths of 200 base pairs were synthesized using the Genomic Sample Prep kit (Illumina, San Diego, CA) according to manufacturer's instructions. Prior to cluster generation, library concentration and size were assayed using the Agilent DNA1000 kit (Agilent) on a 2100 Bioanalyzer (Agilent). Libraries were sequenced on a Genome Analyzer equipped with a paired-end module (versions I, II and IIx, Illumina).

### *De novo* assembly of mRNA-Seq data

After removing sequences containing low quality bases ('N's) or single base repeats and ribosomal RNA sequences, the 3.93 Gbp dataset was used for assembly and subsequent coverage per base (CPB) estimation for each assembled contig. We assembled the filtered Illumina paired-end (PE) reads using Velvet version 0.7.30 [29]. Previous studies [1-3,50] have demonstrated that mRNA-Seq technology produces uneven coverage over a transcript, which prompted us to follow a coverage-assisted reference assembly strategy. Using Mosaik (http://bioinformatics.bc.edu/marthlab/Mosaik) to align the filtered Illumina PE sequences to the assembled contigs, the average coverage per contig was calculated. A custom script was then developed to extract the pairs of sequences that mapped to each contig, and using that contig as a template, each contig was re-assembled using Velvet with the associated expected coverage parameter set to the Mosaik average coverage value for that contig.

### Contig validation

The degree to which the assembled contigs represented long, contiguous RNA transcript sequences, was evaluated by aligning 35 Velvet contigs and their respective predicted CDSs to full-length, cloned, Sanger-derived *Eucalyptus* reference sequences present in NCBI. CPB was calculated for the sequences using BWA [51] and a global pairwise alignment of the sequences was performed using the Needle package from EMBOSS [52]. Plots were constructed from the alignments with the CPB on the y-axis of the plot. Zero coverage values were assigned to gaps in the alignments. This revealed where gaps and/or potentially misassembled regions were present in the assembled contigs, and to what depth these contigs were sequenced.

### Coding sequence prediction

Coding sequence predictions were performed using GENSCAN [30] and AUGUSTUS [53], predicting 15,713 and 15,904 proteins respectively. The difference in coding sequences predicted could be attributed to the different training data sets used and inherent difficulty of predicting coding sequences from incomplete genomic sequences. The GENSCAN results (15,713 predicted proteins) were used in downstream analyses.

### Annotation of assembled contigs

Homology searches were performed against public sequence databases. The newest versions as of February 2010 of the protein sequences of *Arabidopsis* (TAIR 9), *Vitis* (Sept 2009 build) and *Populus* (version 2.0, Phytozome) were used to construct the individual BLAST datasets. The *Eucalyptus* public dataset (EucAll) consisted of 45,442 entries in Genbank (downloaded March 2010), 13,930 entries from the *Eucalyptus* Wood unigenes and ESTs [33], *E. grandis* leaf tissue ESTs (120,661 entries from DOE-JGI-produced 454 sequences, http://eucalyptusdb.bi.up.ac.za/) and 190,106 Unigenes and singlets from *E. grandis* 454 data [15]. The BLAST e-value threshold was set at $1e^{-10}$, with a minimum alignment length of 100 nucleotides (33 amino acids). Functional annotation (GO and KEGG) was performed using BLAST2GO [54], using the default annotation parameters (BLAST e-value threshold of $1e^{-06}$, Gene Ontology annotation threshold of 55). InterPro annotations were performed using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

### Coverage and FPKM determination

Sequence depth and base coverage were calculated using BWA (Lin et al. 2009) and the FPKM values estimated by aligning the Illumina reads to the assembled transcriptome using Bowtie [40] and estimating the expression level of each predicted transcript (FPKM value) using Cufflinks (http://cufflinks.cbcb.umd.edu) [41].

## Additional material

**Additional file 1: Supplemental Tables S1-S3 and Supplemental Figures S1-S8 referred to in text.**

**Additional file 2: FASTA formatted sequences of all 18,894 assembled contigs**.

**Additional file 3: Contig validation, Needleman-Wunsch alignment figures**.

**Additional file 4: Table containing all 18,894 contig names and calculated FPKM values for six tissues (immature xylem, xylem, phloem, shoot-tips, young leaves and mature leaves)**. Eucspresso (http://eucspresso.bi.up.ac.za/) - Online database with mRNA contig sequences and their Blast, GO, KEGG, Pfam annotations. The short-read sequence data have been submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession SRA012408.

**Acknowledgements**
The authors would like to acknowledge J. Rees and J.-M. Celton of the University of the Western Cape (Cape Town, South Africa) for assistance with Illumina GA sequencing. Plant materials were kindly provided by Sappi Forestry (Kwambonambi, South Africa). This work was supported through a strategic research grant from the South African Department of Science and Technology (DST) and by research funding from Sappi and Mondi, through the Wood and Fibre Molecular Genetics (WFMG) Programme, the Technology and Human Resources for Industry Programme (THRIP) and the National Research Foundation (NRF) of South Africa.

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### Authors' contributions

EM drafted the manuscript, helped sample the material, prepared the libraries, participated in the *de novo* assembly and data analysis, and helped design Eucspresso. CAH performed the *de novo* assembly and automated annotation, participated in data analysis, designed the database Eucspresso, and helped draft the manuscript. MR prepared the libraries, helped sample the material and participated in data analysis. FJ participated in data analysis and the design of Eucspresso. AAM conceived of the study, and participated in its design and coordination and helped to draft the manuscript and participated in data analysis, and helped design Eucspresso. It is the authors' opinion than EM and CAH contributed equally as first authors to this manuscript. All authors have read and approved the final version of the manuscript.

Received: 30 May 2010 Accepted: 1 December 2010
Published: 1 December 2010

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**Cite this article as:** Mizrachi *et al.*: *De novo* assembled expressed gene catalog of a fast-growing *Eucalyptus* tree produced by Illumina mRNA-Seq. *BMC Genomics* 2010 **11**:681.

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