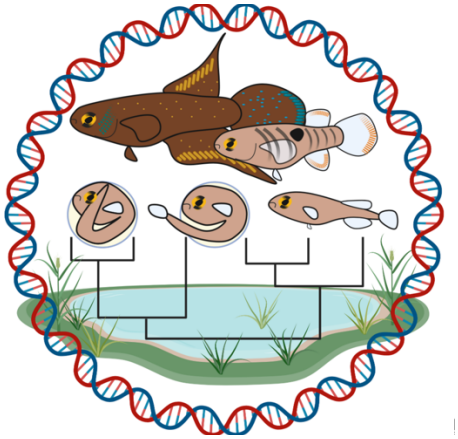
How to Run MAKER



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Before you start:

**This guide assumes you are working on a server with all prerequisites and modules installed and that you have an assembled genome in fasta file format. You will also need a basic understanding of Linux/Unix command terminal operations, bash scripting, grep, awk, and sed.*

1.) First sort your contigs largest to smallest in Geneious or with sorting options on Unix/a sorting script.

2.) Use the batch rename function in Geneious or other available script/sed function to remove the current name and give a different consistent, but meaningful prefix like this:

ScDou1A_293;HRSCAF=313 SPECIESID_scaf
ScDou1A_312;HRSCAF=333 SPECIESID_*scaf*
ScDou1A_1939;HRSCAF=2079 SPECIESID_scaf
……

Then, export the list of sequences as a new fasta file.

3.) Run the following awk command to give the names a number that corresponds to their order in the fasta:

```
awk '/>/{print $0(++i)}!/>/' NAME_OF_YOUR_FASTA > NAME_OF_NEW_FASTA.fasta
```

Submit your fasta file to NCBI, labeling mitogenome as so:
> Fish_name _scaf_1950 [location=mitochondrion]
AACAGTAC……..

NCBI genome submissions are started here:
https://submit.ncbi.nlm.nih.gov/subs/genome/
(you will need to register an account at NCBI and login)

*You should do this before you start annotation. This is because NCBI will do an initial scan for contamination and you will want to remove that before proceeding.

1.) Making a custom repeat library

It is best to make a custom library for your newly sequenced fish if one doesn't already exist for that species. If you use a library from a related species or those available from other model organisms you will likely underestimate the percentage of repetitive elements in the genome. Repeat Modeler can do this and it is easy to use:

```
#! /bin/bash
module load RepeatModeler/1.0.8
BuildDatabase -name NAME_OF_YOUR_DB -engine ncbi PATH_to_FASTA
nohup RepeatModeler -database NAME_OF_YOUR_DB >& seqfile.out
```

Repeat Modeler will output a file called "consensi.fa.classified" You can then use this as the input library to Repeat Masker when running MAKER, as well as combining it with other repeat libraries to use for MAKER.
You can also do this with RepeatScout like so:

```
#! /bin/bash
module load RepeatScout/1.0.5
build_lmer_table -sequence PATH_to_FASTA -freq output_lmer.frequency
RepeatScout -sequence PATH_to_FASTA -output output_repeats.fas  -freq
output_lmer.frequency
```

Cleaning up your fastas:
If necessary, remove ambiguities and all non-ATCGN characters from your sequences in your repeat library. If these are present, MAKER will give an error telling you this and will not run. There are a couple simple commands you can run on your repeat fasta to remove these unwanted characters:

```
sed '/^[^>]/ s/[^AGTC]/N/gi' < NAME of INPUT FASTA >> NAME of OUTPUT FASTA
```

-OR- (depending on your environment)

```
perl -pe 's/[^AGTC]/N/gi unless m/>/;'  NAME of INPUT FASTA > NAME of OUTPUT
FASTA
```

For more information see:
http://weatherby.genetics.utah.edu/MAKER/wiki/index.php/Repeat_Library_Construction--Basic

2.) Getting Control Files:

The first part of running maker is obtaining and establishing the options you want in your control files:

After loading your MAKER module, type: "maker –CTL" This creates control file templates: maker_bopts.ctl maker_exe.ctl maker_opts.ctl in the working directory.

Now you need to fill in the options you want for each control file. The main file is maker_opts.ctl. I usually start by leaving the options in the other two files set as default. for more info go to:
http://weatherby.genetics.utah.edu/MAKER/wiki/index.php/The_MAKER_control_files_explained

3.) Setting up the Initial Run:

If you are running Maker for the first time on your favorite newly sequenced fish, you need to use the following options as so in "maker_opts.ctl":

#-----Re-annotation Using MAKER Derived GFF3
maker_gff= This is blank because you haven't run it previously to get an initial gff file. This is useful when re-running maker as everything does not need to be realigned, masked, etc. Set remaining options in this section to "0."

#-----EST Evidence (for best results provide a file for at least one)
est= This is where you put the full path to the EST dataset(s) you are using as evidence. Note that this should be the same species you are annotating. If you are using multiple files, the paths can be separated by "," and no spaces. The rest of the options here I leave blank as they are only for alternate sets from different species other than the one you are annotating, or if you already have a gff file of the ESTs

#-----Protein Homology Evidence (for best results provide a file for at least one)
protein= This is where you put the full path to the protein datasets you are using from this species as well as related species.
protein_gff= Again, I leave this option blank as they are only for if you already have a gff file of the proteins

#-----Repeat Masking (leave values blank to skip repeat masking)
model_org=all This option takes advantage of the dataset of repetitive elements from multiple species that come with the MAKER package.
-OR-
rmlib= Path_to_FASTA This is where you can give the path for your species-specific repeat library that you created as described in the previous section (If you do this, leave model_org= blank).
repeat_protein= I leave this blank.
rm_gff= Again, I leave this option blank as they are only for if you already have a GFF file repetative elements
prok_rm=0 There is no reason to change this.
softmask=1 I use soft masking

#-----Gene Prediction
snaphmm= On your first run you have to leave this blank. You will make a SNAP model based on results from this first run you will use later.
gmhmm= leave this blank-I usually use SNAP and AUGUSTUS for gene models
augustus_species= On your first run you have to leave this blank. You will make a AUGUSTUS model based on results from this first run you will use later.
fgenesh_par_file= I leave this blank.
evm_weights= I leave this blank.
pred_gff= Again, I leave this option blank as they are only for if you already have a gff file.
model_gff= Again, I leave this option blank as they are only for if you already have a gff file.
est2genome=1 Turn this on with "1". This causes MAKER to infer "MAKER genes" directly from your EST evidence. This is necessary because you haven't created any models yet and the ESTs for this species are all you have.
protein2genome=0 Leave this off if your proteins are from different species from the one you are annotating. Leave the remainder of the options in this section on default.

#-----Other Annotation Feature Types (features MAKER doesn't recognize)
leave as defaults

#-----External Application Behavior Options
leave as defaults

#-----MAKER Behavior Options
leave as defaults

4.) Run MAKER
Make a shell script to run maker as so:

```
#! /bin/bash
#$ -cwd
export TMP=/data/run/tmp/
module load MAKER/r1128
module load MPICH2
mpiexec -n 10  maker -q -fix_nucleotides
```

Run MAKER via the above shell script like this:

```
/PATH_to/the_shell_script > maker_text.txt  2>&1
```
Note that the shell script and the .ctl files should all be in the same directory.

Now, you wait for it to run! Once MAKER has finished, run the following regular expressions on the "*datastore_index.log" to make sure there were no failures:

```
grep -c 'START' *_datastore_index.log
```

```
grep -c 'FINISH' *_datastore_index.log
grep -c 'FAIL' *datastore_index.log
```

If there are no fails, run gff_merge on *datastore_index.log as so:

```
module load MAKER/r1128
gff3_merge -d /PATH_to/*datastore_index.log
```

This will search through a directory tree of each contig or scaffold and merge the maker results from the first run into a single gff called "*.all.gff".

5.) Checking percent of repeat masking (optional):

```
module purge
module load GNU/4.4.5
module load BEDTools/2.24.0
grep "repeat" *.all.gff >> repeats.gff
bedtools sort -i repeats.gff >> sorted.gff
bedtools merge -i sorted.gff >> merged.gff
```

This will give you a GFF of all of the regions in the genome that were repeat masked, accounting for any overlap. You can total the number of masked nucleotides from this file and divide by total bases in the genome.

6.) Training SNAP:

Make and cd into SNAP directory. Make and run a shell script to execute SNAP model training based on results from your initial MAKER run. This will work by using the "best" "MAKER genes" promoted by EST2genome with an AED cutoff of "0.2" in your first run:

```
#! /bin/bash
module load MAKER/r1128
maker2zff -x 0.2 -l 200 -n *.all.gff #this is the merged gff file
fathom -categorize 1000 genome.ann genome.dna
fathom -export 1000 -plus uni.ann uni.dna
forge export.ann export.dna
hmm-assembler.pl NAME_SNAP1_hmm /PATH_to/SNAP >
/PATH/_to/SNAP/NAME_SNAP1.hmm
```

Run SNAP via the above shell script like this:

```
/PATH_to/the_shell_script > SNAP_text.txt  2>&1
```

7.) Training AUGUSTUS:

Make and cd into an AUGUSTUS directory. Use the train_augustus.sh (available here: https://github.com/Childs-Lab/GC_specific_MAKER) written by the Childs Lab and detailed below to train AUGUSTUS based on results from your initial maker run. This

will work by using the "best" "MAKER genes" promoted by EST2genome with an AED cutoff of "0.2" in your first run. Make sure you have access to fathom_to_genbank.pl get_subset_of_fastas.pl randomSplit.pl scripts in the path for this to run (available here: https://github.com/Childs-Lab/GC_specific_MAKER and here: https://github.com/nextgenusfs/augustus/blob/master/scripts/randomSplit.pl) Execute AUGUSTUS training with a shell script as follows:

```
#! /bin/bash
bash /PATH_to/train_augustus.sh /PATH_to/AUGUSTUS /PATH_to/*.all.gff
"Species_Name" /PATH_to*_EST.fasta
```

Run AUGUSTUS via the above shell script like this:

```
/PATH_to/the_shell_script > AUGUSTUS_text.txt  2>&1
```

8.) Re-Run MAKER with Gene Models from SNAP and AUGUSTUS:

Now you are ready to re-run MAKER a final time. This time, MAKER will use alignments from ESTs and Proteins to guide the models that you trained in SNAP and AUGUSTUS with the best scoring (AED score) MAKER genes from your initial MAKER run with the EST2genome option. You don't want to re-align evidence or re-run repeat masker. Start by making a new directory outside of your first MAKER output and copy into it, your control files. Change the "maker_opts.ctl" as described below so you can give MAKER the gff output from your initial run.

```
#-----Re-annotation Using MAKER Derived GFF3
maker_gff=/PATH_to/*.all.gff #MAKER derived GFF3 file  (This will be the output of the
gff3_merge command)
est_pass=1 #use ESTs in maker_gff: 1 = yes, 0 = no
altest_pass=0 #use alternate organism ESTs in maker_gff: 1 = yes, 0 = no
protein_pass=1 #use protein alignments in maker_gff: 1 = yes, 0 = no
rm_pass=1 #use repeats in maker_gff: 1 = yes, 0 = no
model_pass=0 #use gene models in maker_gff: 1 = yes, 0 = no
pred_pass=0 #use ab-initio predictions in maker_gff: 1 = yes, 0 = no
other_pass=0 #passthrough anyything else in maker_gff: 1 = yes, 0 = no
```

You **DO NOT** need to change the following:

```
#-----EST Evidence (for best results provide a file for at least one)
est=  #set of ESTs or assembled mRNA-seq in fasta format
est_gff=  #aligned ESTs or mRNA-seq from an external GFF3 file

#-----Repeat Masking (leave values blank to skip repeat masking)
rmlib=  #provide an organism specific repeat library in fasta format for RepeatMasker
rm_gff= #pre-identified repeat elements from an external GFF3 file

#-----Protein Homology Evidence (for best results provide a file for at least one)
```

protein= #protein sequence file in fasta format (i.e. from mutiple oransisms)
protein_gff= #aligned protein homology evidence from an external GFF3 file

These other options are for other ways of getting the data into MAKER.
But you **DO** need to make the additional following changes to the maker_opt.ctl file:

Change est2genome=0
Change snaphmm=/PATH/SNAP/NAMESNAP1.hmm #SNAP HMM file
Change augustus_species=Species_Name #Augustus gene prediction species model"
Make sure keep_preds=1 This will retain predictions supported by models only -if evidence is lacking.
Also, if you have an idea of what the maximum intron length could be, you may want to adjust split_hit=10000 #length for the splitting of hits (expected max intron size for evidence alignments).

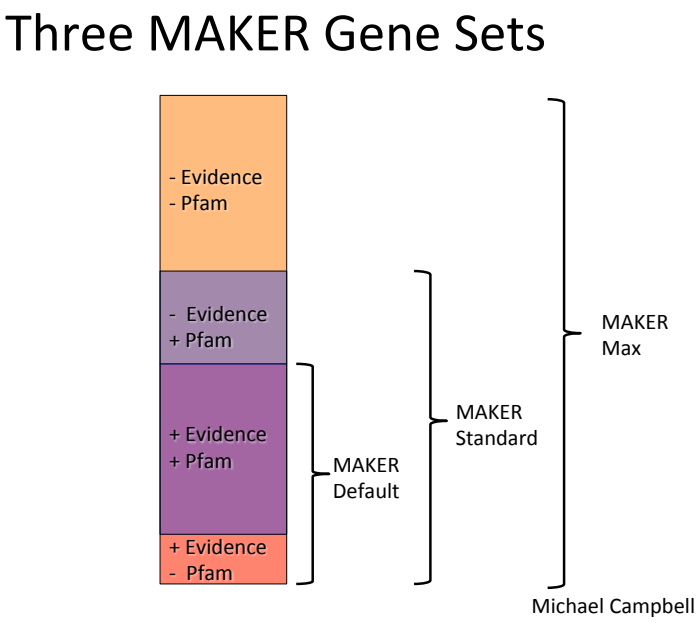
Now, run MAKER and GFFMerge as you did before

9.) Getting "MAKER Standard": genes:

First, run Fasta_merge like so:

module load MAKER/r1128
fasta_merge -d /PATH/*datastore_index.log

This has given you the "MAKER Max" gene set. The next step is to run a Pfam search for genes with known protein domains. The goal is to get a "MAKER Standard" gene set that only keeps genes with EST or protein evidence or that have been detected by models but also contain Pfam domains. See figure below:



Perform pfam search using hmmscan from the iprscan package:

```
module load iprscan
PATH_to/hmmscan --domE 1e-3 -E 1e-5 --cpu 12 --acc --tblout Pfam_out.txt PATH_to/
pfam.hmm PATH_to/.all.maker.proteins.fasta > PFam_output.txt 2>&1
```

Then you identify the maker standard genes using a number of scripts provided by the Childs Lab (https://github.com/Childs-Lab/GC_specific_MAKER):

```
perl /PATH_to/generate_maker_standard_gene_list.pl --input_gff /PATH_to/*.all.gff --
pfam_results Pfam_out.txt --pfam_cutoff 1e-10 --output_file
NAME_complete_w_single_exons_standard_gene_list.txt
```

Generate the transcript and protein fasta files for the maker standard gene set.
Generate the gff file for the maker standard gene set.

```
module load BioPerl
perl /PATH_to/get_subset_of_fastas.pl  -l
/PATH_to/complete_w_single_exons_standard_gene_list.txt  -f
/PATH_to/*.all.maker.proteins.fasta  -o
/PATH_to/complete_w_single_exons_maker_standard_set.proteins.fasta

perl /PATH_to/get_subset_of_fastas.pl   -l
/PATH_to/complete_w_single_exons_standard_gene_list.txt  -f
/PATH_to/*.all.maker.transcripts.fasta  -o
/PATH_to/complete_w_single_exons_maker_standard_set.transcripts.fasta

#options: -l list_gene_ids  -f multifasta_file  -o output_fasta_file

perl /PATH_to/create_maker_standard_gff.pl --input_gff /PATH_to/*.all.gff --output_gff
/PATH_to/complete_w_single_exons_maker_standard_gene_set.gff --
maker_standard_gene_list /PATH_to/complete_w_single_exons_standard_gene_list.txt
```

Congrats you ran MAKER and should have an annotated genome of your newly sequenced fish!
**For another, detailed description see: http://gmod.org/wiki/MAKER_Tutorial_2013*

Other Helpful tips for downstream Analyses:

1.) You might want to hard and/or soft mask the repeats in your genome and get a quick estimate of repeat content, as found by repeatmasker in MAKER. This is how you do it. You will need a basic understanding of using regular expressions in the command terminal and in a text editor like BBedit.

grep “repeatmasker” /PATH_to/*maker_standard_gene_set.gff | sortBed | gff2bed | bedtools merge >> merged_repeats.bed

This gives you a bed file of repeats you can use to mask your genome as follows:

bedtools maskfasta -soft -fi /PATH_to/*genome.fasta -bed /PATH_to/*merged_repeats.bed -fo softMASKED_genome.fasta

bedtools maskfasta -fi /PATH_to/*genome.fasta -bed /PATH_to/*merged_repeats.bed -fo HARDMASKED_genome.fasta

You can then calculate repeat content like so:

grep -v "^>" softMASKED_genome_.fasta | tr -cd [a-zA-Z] | wc -c

This gives you the total number of nucleotides in the softmasked genome

grep -v "^>" softMASKED_genome_JAAMPK000000000.fasta | tr -cd [a-z] | wc -c

This gives you the total number of lowercase nucleotides in the softmasked genome- using this and the total you can calculate percent repeat content.

2.) You might want to rename genes annotated by MAKER in the order they appear by scaffold. This is how you do it:

bedtools sort -i /PATH_to/*maker_standard_gene_set.gff > sorted_maker_standard_gene_set.gff

This sorts the maker standard gff file by coordinate.

grep "maker" /PATH_to/*sorted_maker_standard_gene_set > maker.gff

This pulls out maker annotated regions into a gff without repeats and blast hits.

Next, make and run a bash script like below to get the "maker_standard_reduced.gff" To make this script, you grep out sequence names in maker standard fasta files from the reduced gff you made above like so:

“grep “>” /PATH_to/*transcripts or proteins.fasta >> name.output”
then remove the “>” characters and add “>> maker_standard_reduced.gff” to each line to get:

#!/bin/sh
grep "maker-scaf_15265-snap-gene-0.2" maker.gff >> maker_standard_reduced.gff

```
grep "augustus_masked-scaf_14540-processed-gene-0.0" maker.gff >>
maker_standard_reduced.gff
grep "augustus_masked-scaf_269-processed-gene-0.0" maker.gff >>
maker_standard_reduced.gff
grep "maker-scaf_698-snap-gene-0.4" maker.gff >> maker_standard_reduced.gff
grep "snap_masked-scaf_4994-processed-gene-0.1" maker.gff >>
maker_standard_reduced.gff
…..
```

Execute this script with bash This pulls out only maker standard genes from the gff.

Note: You should also reduce to root names and remove duplicates in this output to test whether the number of lines matches number of genes in the maker standard set protein and transcript fasta files.

Then coordinate sorts the above output.

```
bedtools sort -i maker_standard_reduced.gff > sorted_maker_standard_reduced.gff
```

Then, pull out gene names from sorted_maker_standard_reduced.gff using regular expressions. Remove duplicates, and number them in that order in a tsv map files for renaming. See mapfile example below for new gene name scheme. I like to make mine similar to Ensembl stable IDs:

```
SPECIESG00000001        maker-Nwh_scaf_1-augustus-gene-1.2
SPECIESG00000002        maker-Nwh_scaf_1-snap-gene-4.2
SPECIESG00000003        snap_masked-Nwh_scaf_1-processed-gene-6.0
SPECIESG00000004        snap_masked-Nwh_scaf_1-processed-gene-8.0
SPECIESG00000005        maker-Nwh_scaf_1-snap-gene-6.2
…..
```

There are a number of scripts available in repositories like Github that can use this mapfile to rename headers in fastas and names in gffs. Run these scripts to rename genes in the gffs (Max and Standard if you like) and fastas (proteins and transcripts) using this mapfile. For proteins I use SPECIESP00000001… and for transcripts I use SPECIEST00000001…

To produce a final, renamed, reduced maker standard gff, you can grep out the root of renamed genes like “SPECIESG” from the renamed MAKER standard gff file. This gives you a gff with only the maker standard genes without repeats and blast hits. This whole process will also rename your genes in the order they appear in the genome starting with the first gene in scaf_1. Proteins and transcripts will also have the same ID number as their parent gene.