Lies, damn lies, and .... genomics

you, your data, your perceptions and reality

Christopher West Wheat

Goal of this lecture

- Present a critical view of ecological genomics
- Make you uncomfortable by sharing my nightmares
- Encourage you to critically assess findings and your expectations in light of publication biases

Disclaimer

I'm a positive person

I love my job and the work we all do

I'm just sharing scrumptious food for thought

What if .....

50% of your favorite studies had conclusions that were just wrong?

How would that affect your expectations and work?

If the biomedical science has the most money and oversight, then ....

Their findings should be robust:

- Repeatable effect sizes
- The same across different labs
- The same across years

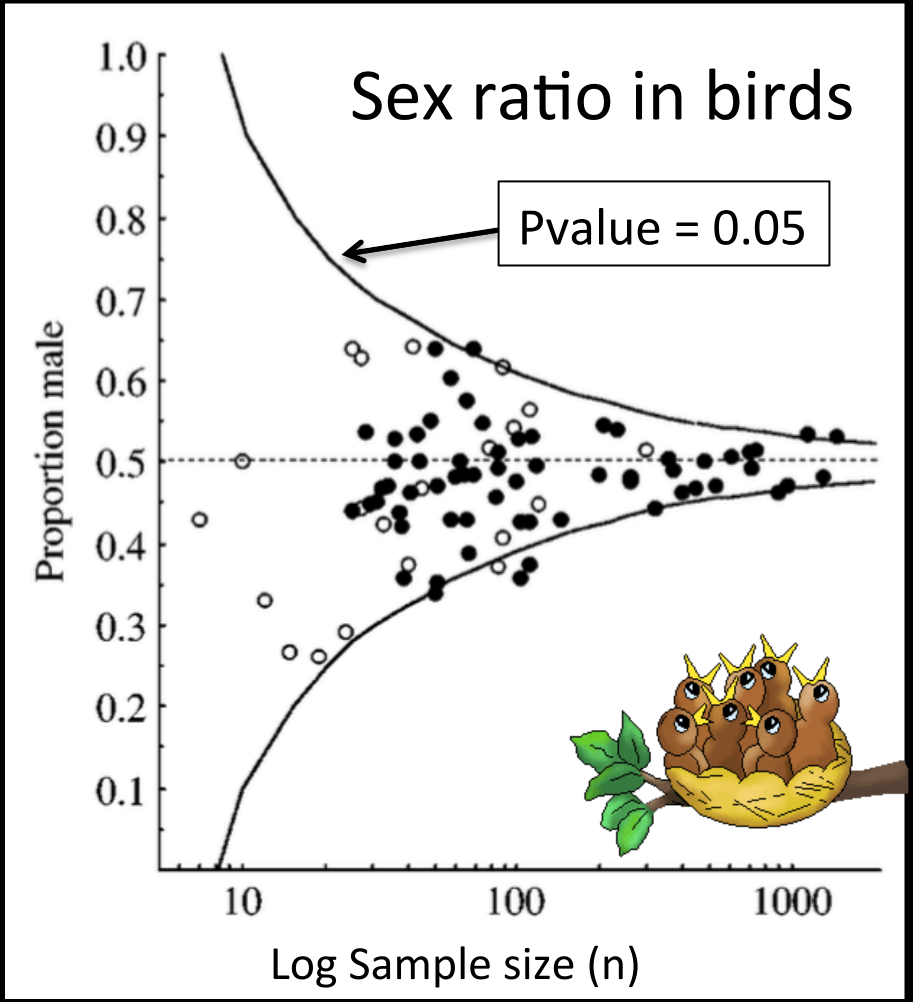
Publication replication failures

- Biomedical studies
  - Of 49 most cited clincal studies, 45 showed intervention was effective
  - Most were randomized control studies (robust design)

- Mouse cocaine effect study, replicated in three cities
  - Highly standardized study

Ioannidis 2005 *JAMA;* Lehrer 2010

Assessing reality using funnel plots



Small sample sizes affect measurement accuracy

Each dot = a study and has error

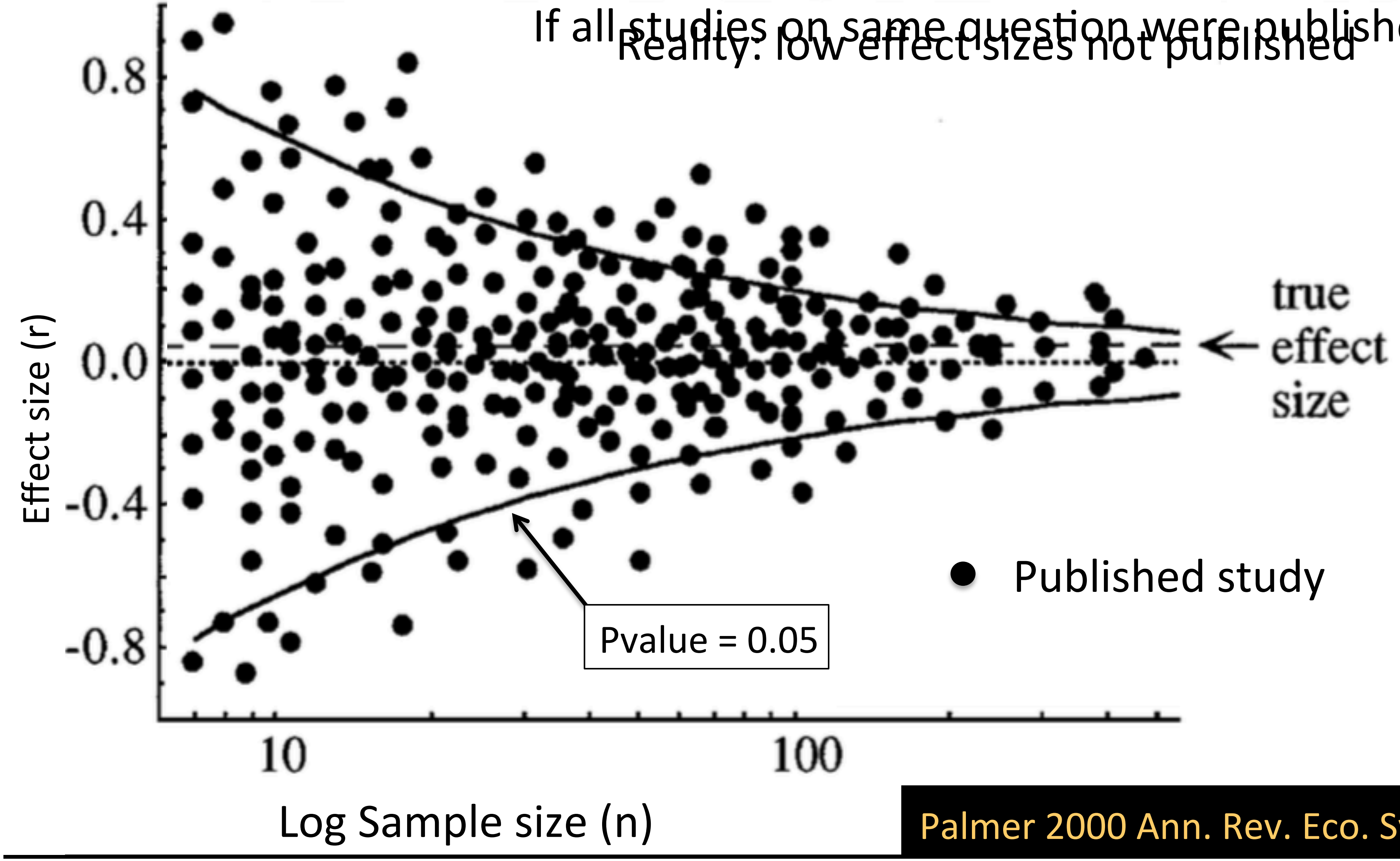
Study estimates are randomly distributed about the real value

Your study is just a random estimate of some idealized value

Publication bias increases effect size

If all studies on same question were published

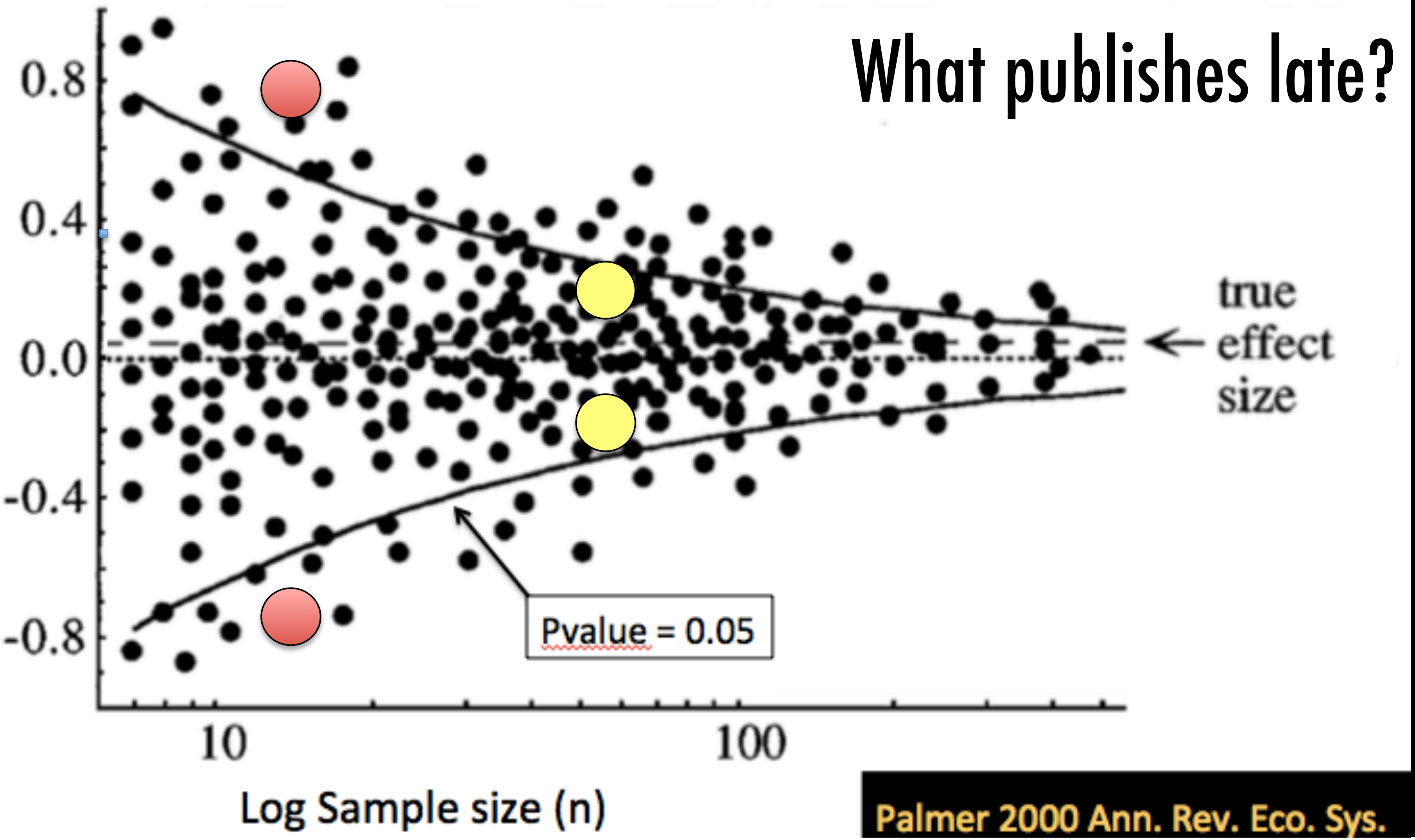
Reality: low effect sizes not published



Palmer 2000 Ann. Rev. Eco. Sys.

What if there is no replication?

What is most likely to publish first & where?



What publishes late?

Palmer 2000 Ann. Rev. Eco. Sys.

Why Most Published Research Findings Are False

A research finding is less likely to be true when:

✓ the studies conducted in a field have a small sample size

✓ when effect sizes are small

✓ when there is a greater number of tested relationships using tests with *a priori* selection

✓ where there is greater flexibility in designs, definitions, outcomes, and analytical modes

✓ when there is greater financial and other interest and prejudice

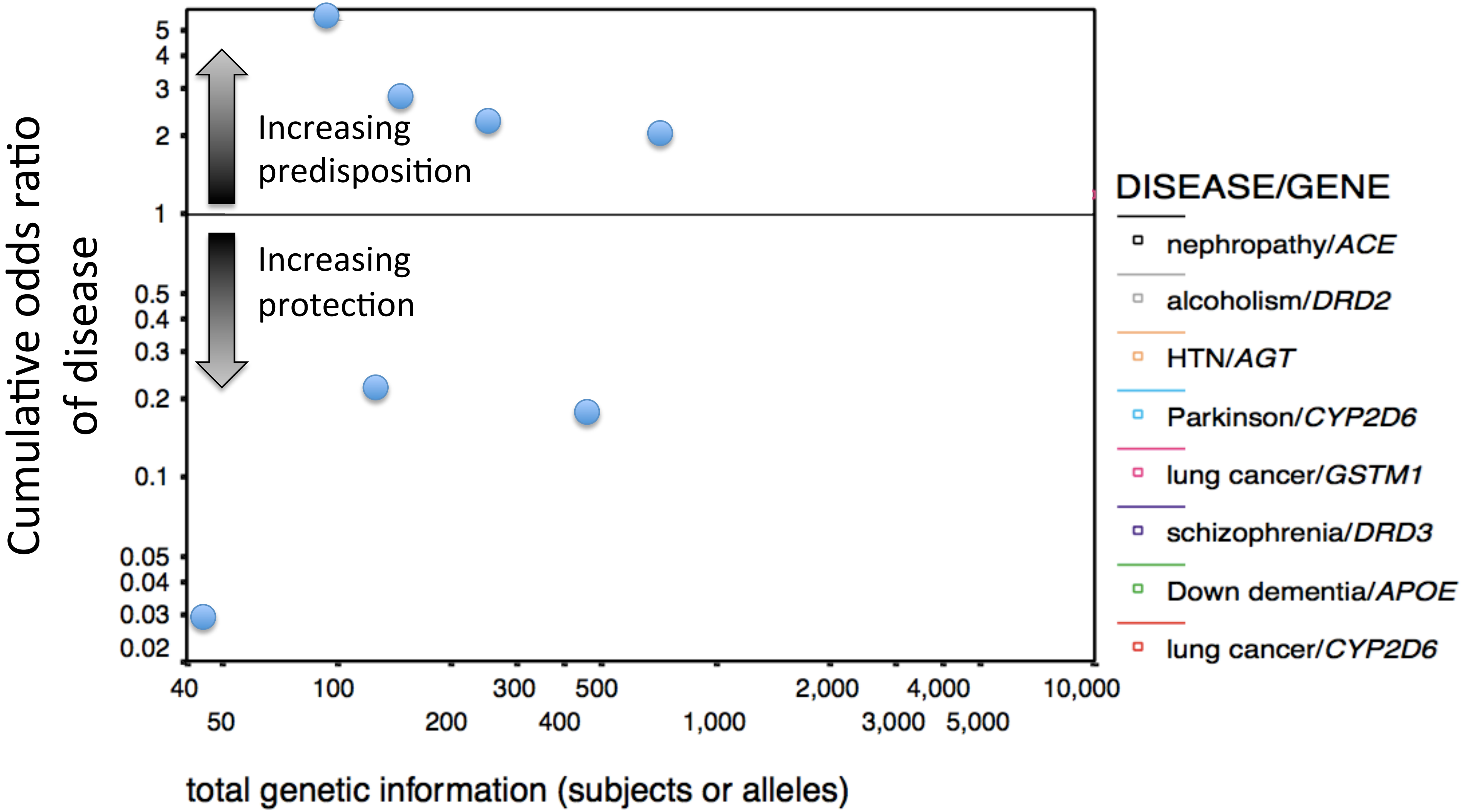
✓ when more teams are involved in a scientific field, all chasing after statistical significance by using different tests

Ioannidis 2005 Plos Med.

But surely, this doesn’t apply to genomics ….

Or does it?

8 topics first reported with P < 0.05



Ioannidis, J. P., E. E. Ntzani, T. A. Trikalinos, and D. G. Contopoulos-Ioannidis. 2001. Replication validity of genetic association studies. Nat Genet 29:306–309.

There are lies, damn lies, and ....

But wait, is that fair?

Are these really lies?

Where does this bias come from?

- Population heterogeneity
  - Space and time
- Publication bias
  - Large & significant effects publish fast and with high impact
  - Small & non-significant effects publish slow with low impact

Where does this bias come from?

YOU!!

And me …. All of us

Its arises from humans doing science

The way we think

The way our institutions work

Apophenia

A universal human tendency to seek patterns in random information and view this as important

- Similar to Type 1 error
  - false positive
- Opposite from Type 2 error
  - false negative

Outline

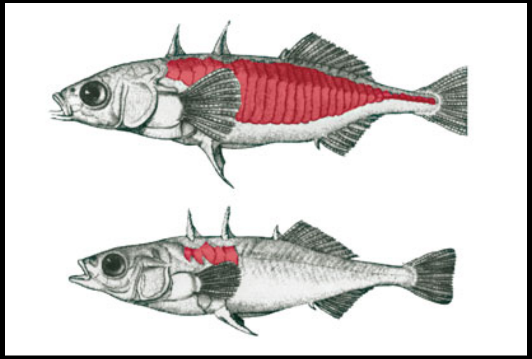
- What is the genomic architecture of phenotypes?
- What is the power of molecular tests of selection?
- What does the dissection of some classic comparative genomics study reveal?

Non – adaptive

disease, aging, height, etc.

1000’s of loci, each of small effect size

Adaptive



salinity, color, resistance, etc.

One or several loci of large effect

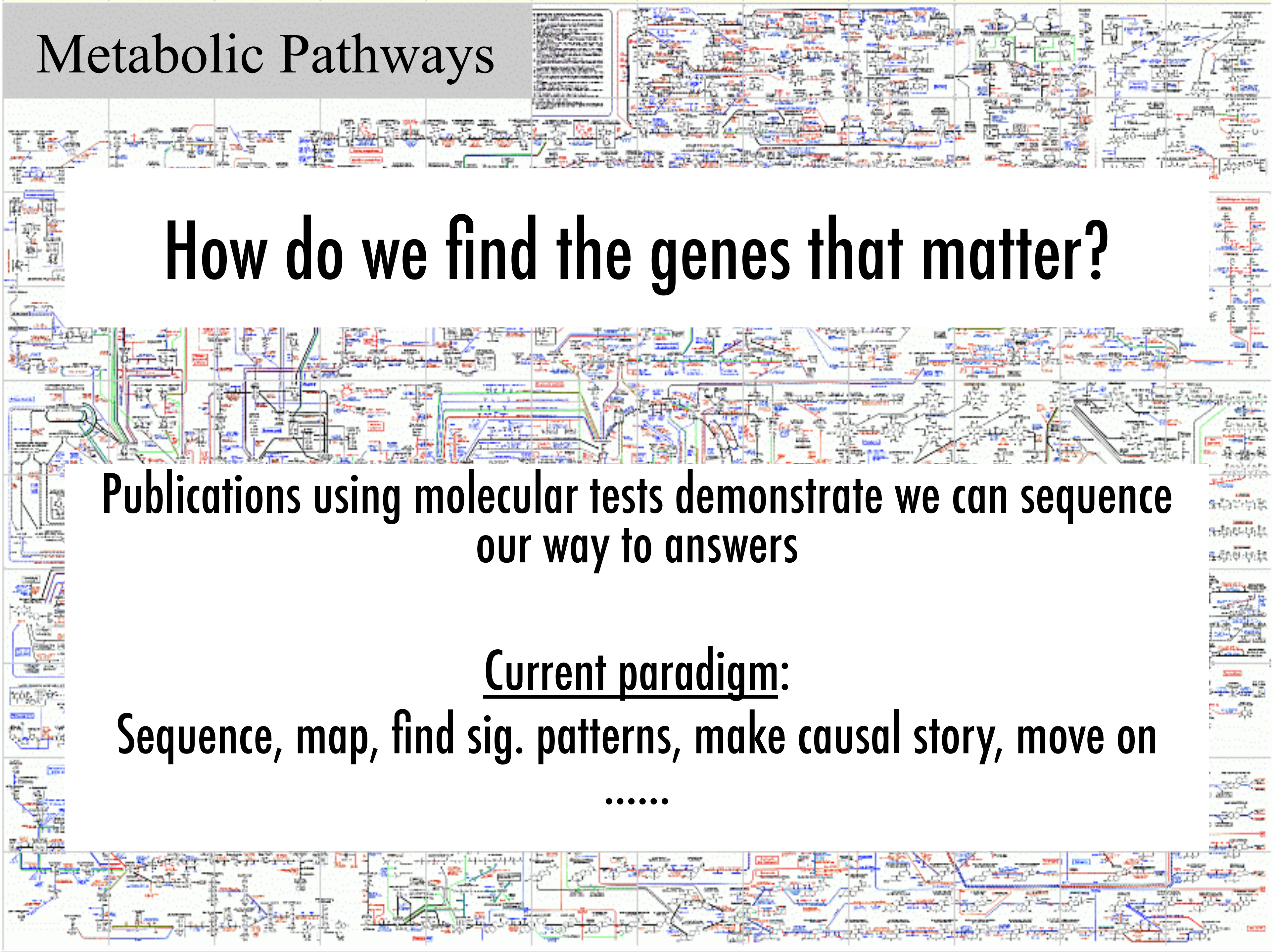
generally …

Is this a publication bias?

Will your trait have 1000’s of small effect genes, or a few genes of large effect?

Sear (2010) … Is bigger always better?

Rockman (2011) … All that’s gold does not glitter



Metabolic Pathways

How do we find the genes that matter?

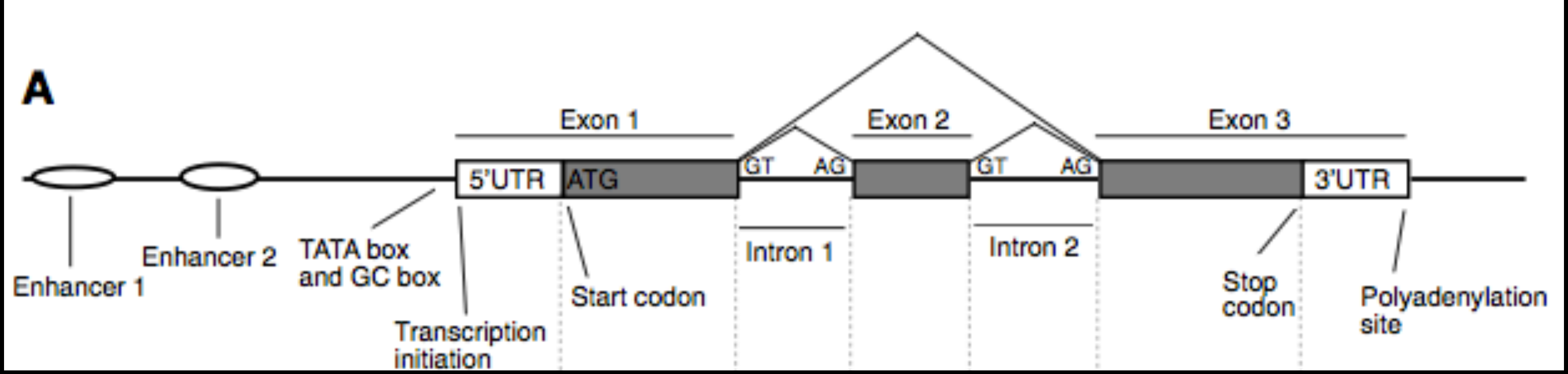
Publications using molecular tests demonstrate we can sequence our way to answers

<u>Current paradigm</u>:

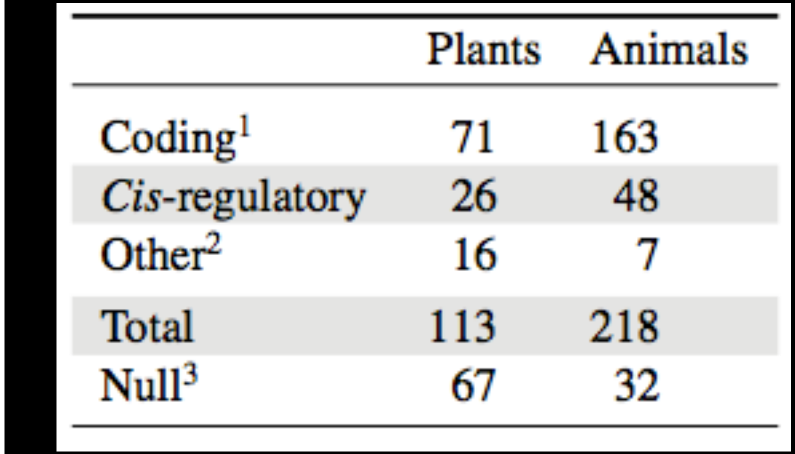
Sequence, map, find sig. patterns, make causal story, move on

……

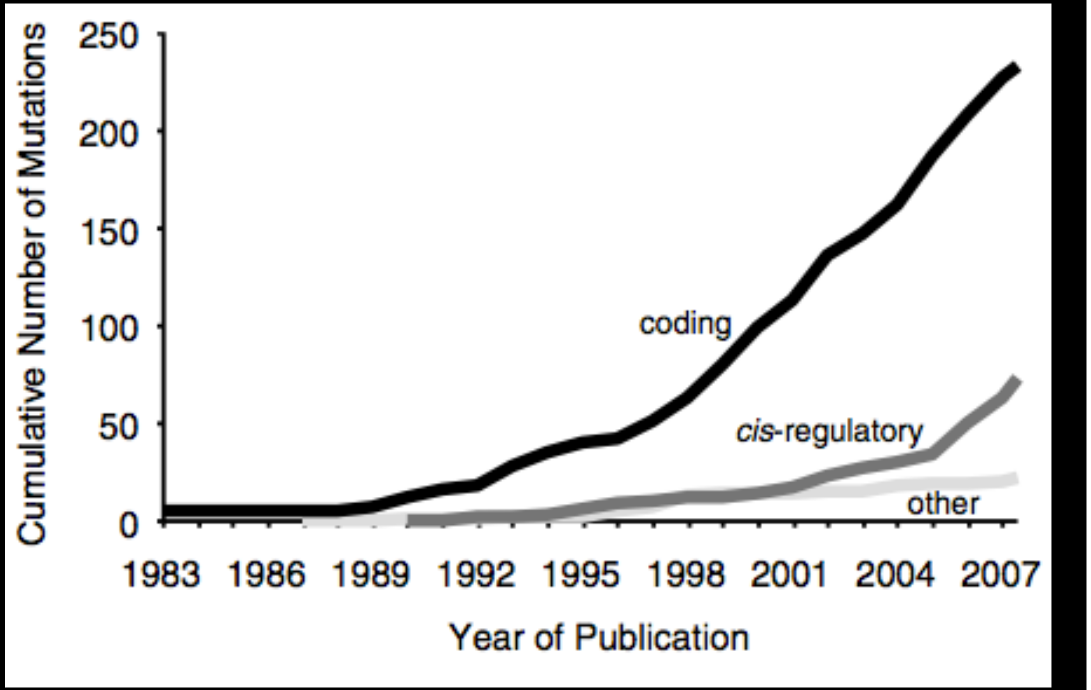
What is the architecture of a causal variant?

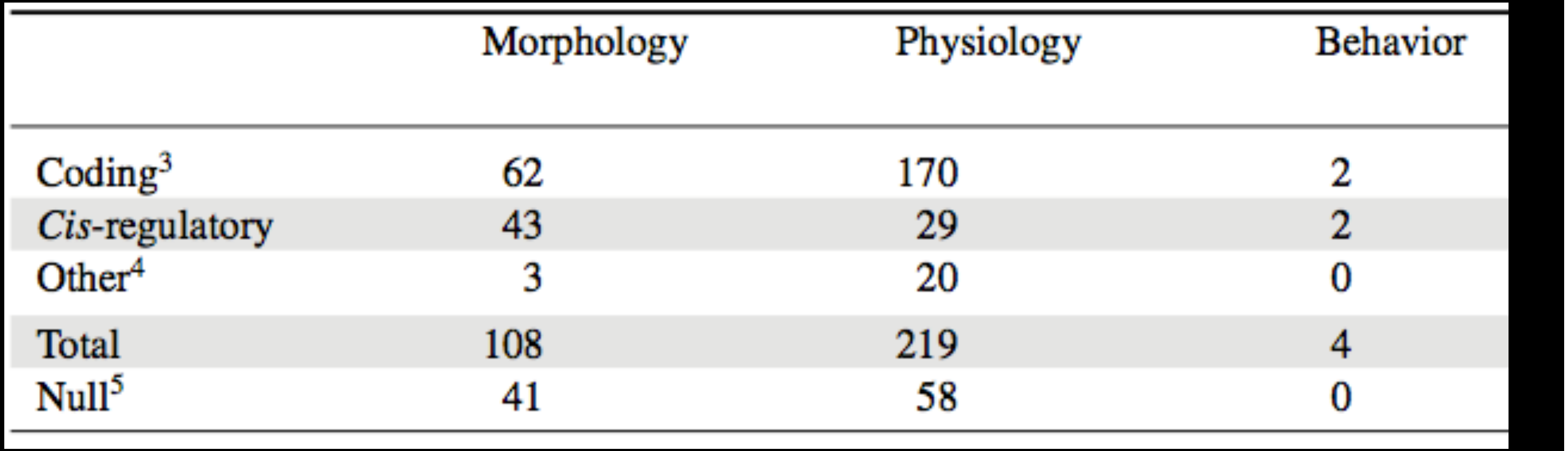


How predictable are adaptations?



	Plants	Animals
Coding[1]	71	163
Cis-regulatory	26	48
Other[2]	16	7
Total	113	218
Null[3]	67	32





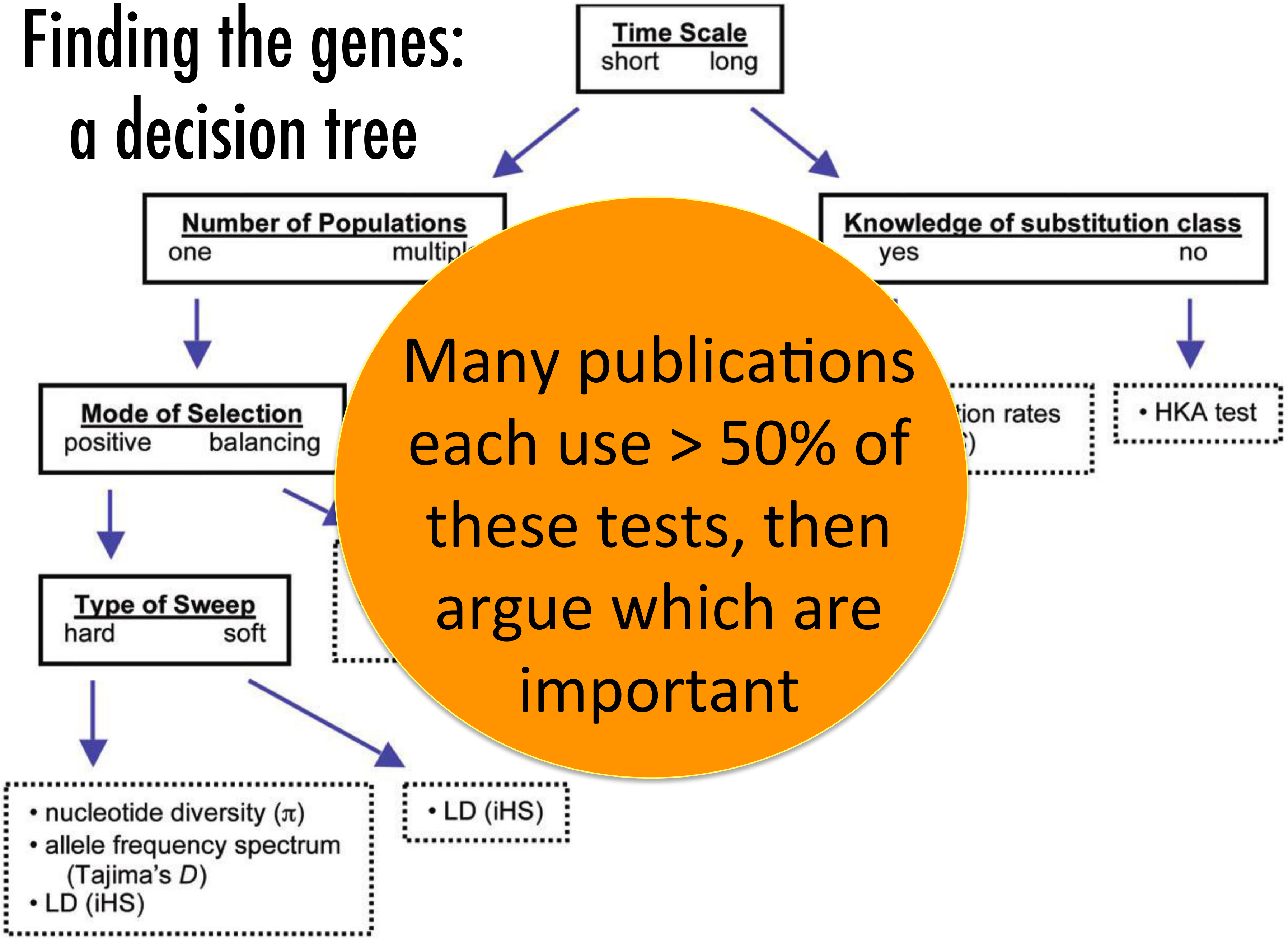
	Morphology	Physiology	Behavior
Coding[3]	62	170	2
Cis-regulatory	43	29	2
Other[4]	3	20	0
Total	108	219	4
Null[5]	41	58	0

Stern & Orgogozo 2008 Evolution

How do we identify the genes that matter?

- Molecular tests of selection are popular, but …
  - What are their assumptions and power?
- What are these tests detecting?
  - What is a footprint of selection?
    - How are they formed?
    - How large are they?
    - How long do the last?

Finding the genes: a decision tree



Time Scale
short long

Number of Populations
one multipl

Knowledge of substitution class
yes no

Mode of Selection
positive balancing

tion rates
)

- HKA test

Type of Sweep
hard soft

- nucleotide diversity (π)
- allele frequency spectrum (Tajima's *D*)
- LD (iHS)

- LD (iHS)

Many publications each use > 50% of these tests, then argue which are important

Hohenlohe et al. 2010 Int. J. Plant Science

What power do we have to detect evolution by natural selection?

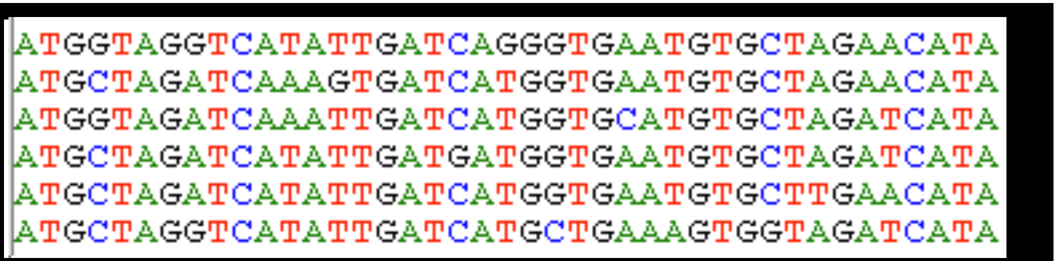
What is statistical power?

Power is the probability that the test will reject the null hypothesis when the alternative hypothesis is TRUE

Using a t-test, you would want power > 90% at reasonable sample size, right?

Directional selection:

an example of the expectations of hard selection



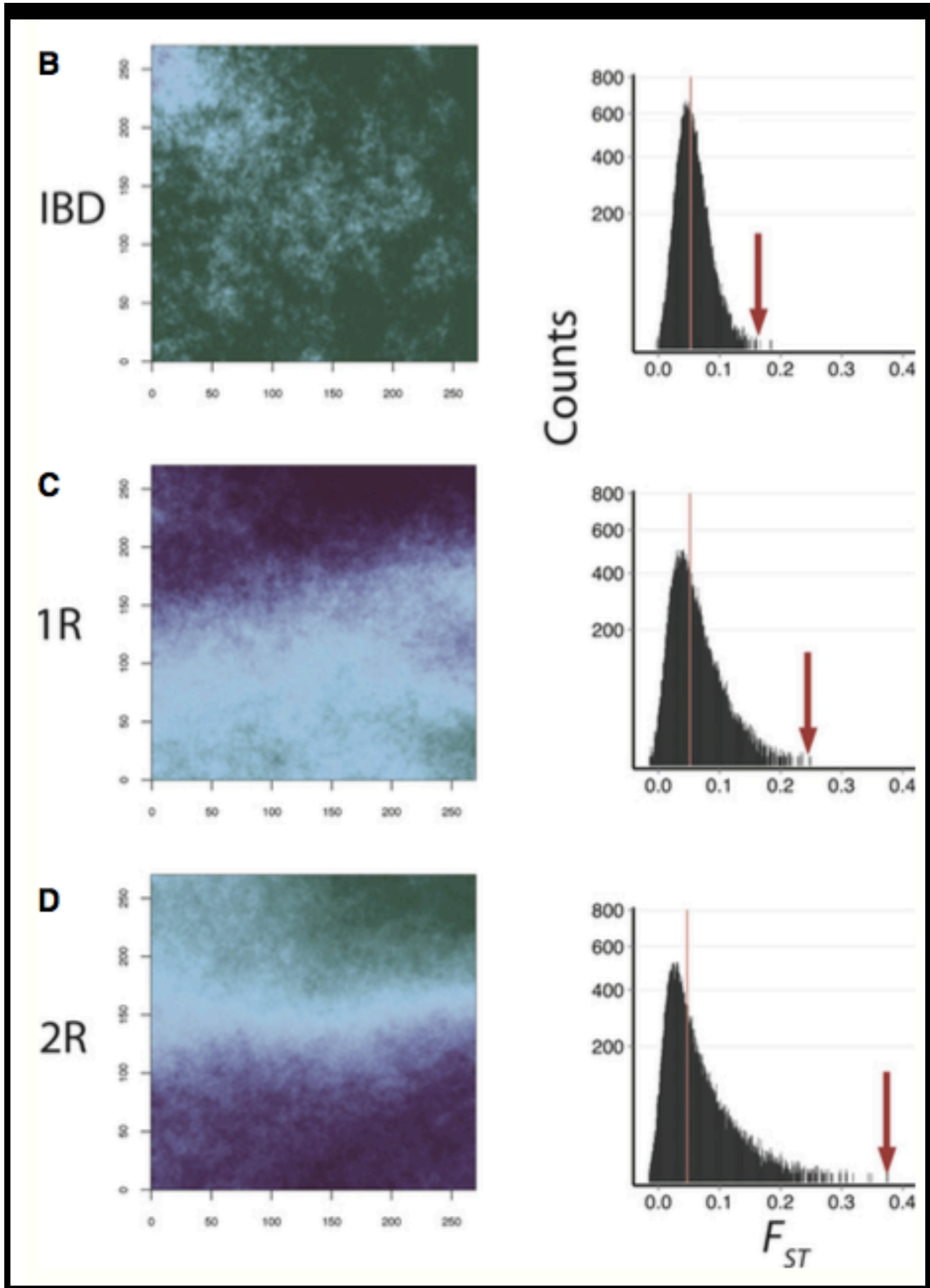
```
ATGGTAGGTCATATTGATCAGGGTGAATGTGCTAGAACATA
ATGCTAGATCAAAGTGATCATGGTGAATGTGCTAGAACATA
ATGGTAGATCAAATTGATCATGGTGCATGTGCTAGATCATA
ATGCTAGATCATATTGATGATGGTGAATGTGCTAGATCATA
ATGCTAGATCATATTGATCATGGTGAATGTGCTTGAACATA
ATGCTAGGTCATATTGATCATGCTGAAAGTGGTAGATCATA
```

Population genomics has been dominated by developing methods to detect hard sweeps for past two decades

- But a proper ‘null model’ continues to be elusive, resulting in a high false positive rate since their inception

Storz 2005 Mol. Ecology

Fst outlier analysis



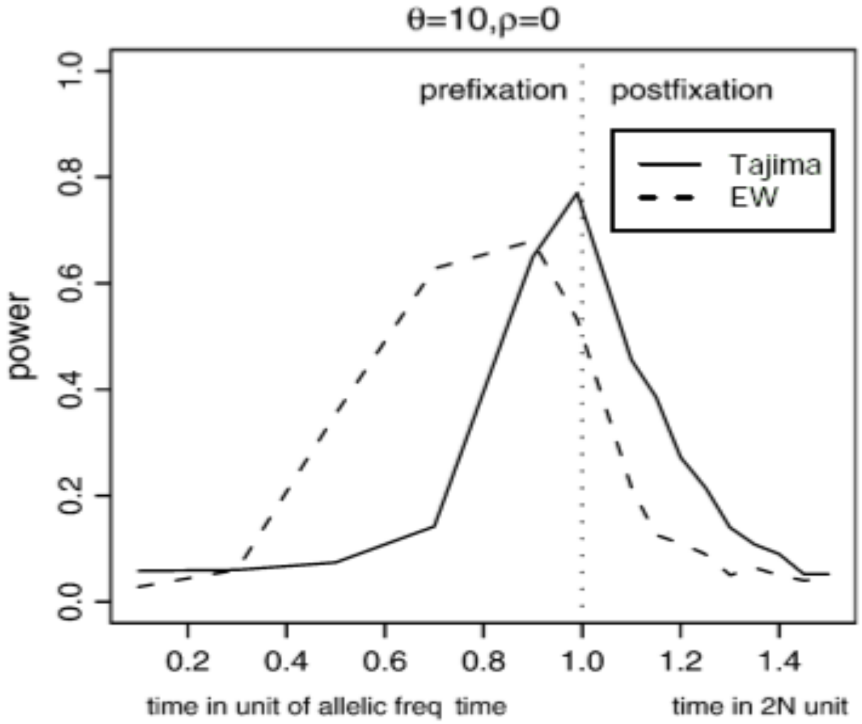
Lotterhos and Whitlock. 2014. Molecular Ecology 23:2178–2192.

9900 Neutral, 100 selected sites

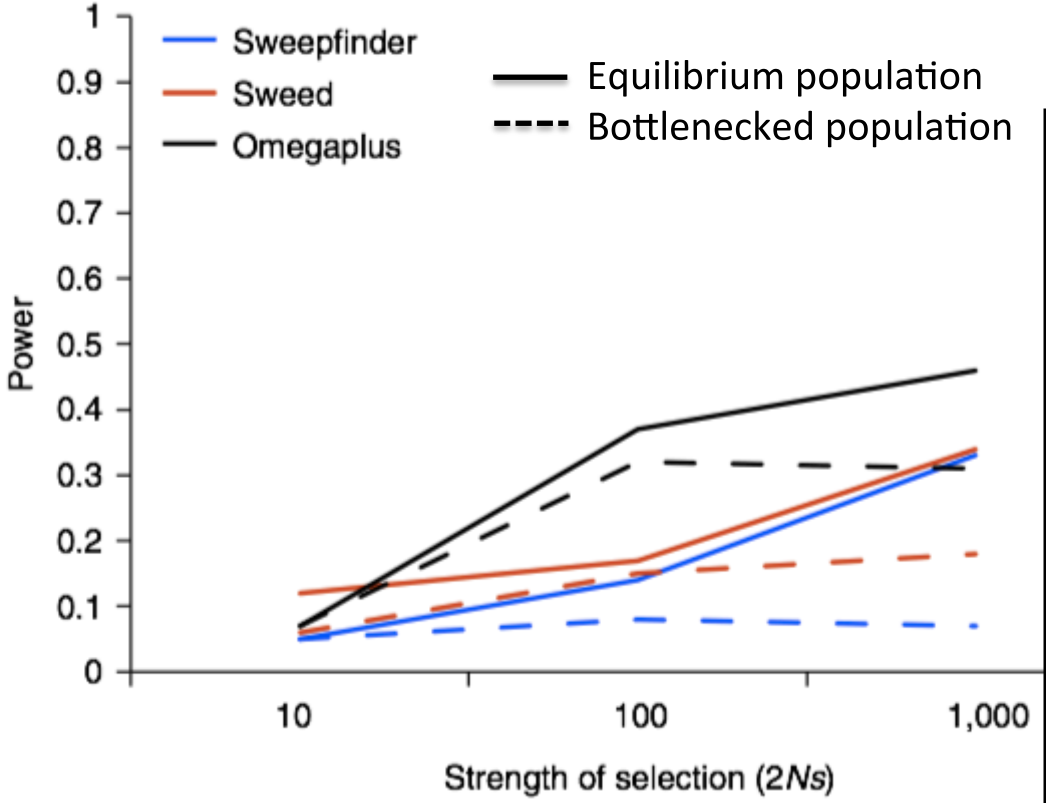
N=1500 (20 ind. per 75 populations)

■ Bayescan 🟩 FDIST2 🟧 FLK

What is our power to detect hard sweeps within a population?



Zhai, Nielsen & Slatkin 2008 MBE

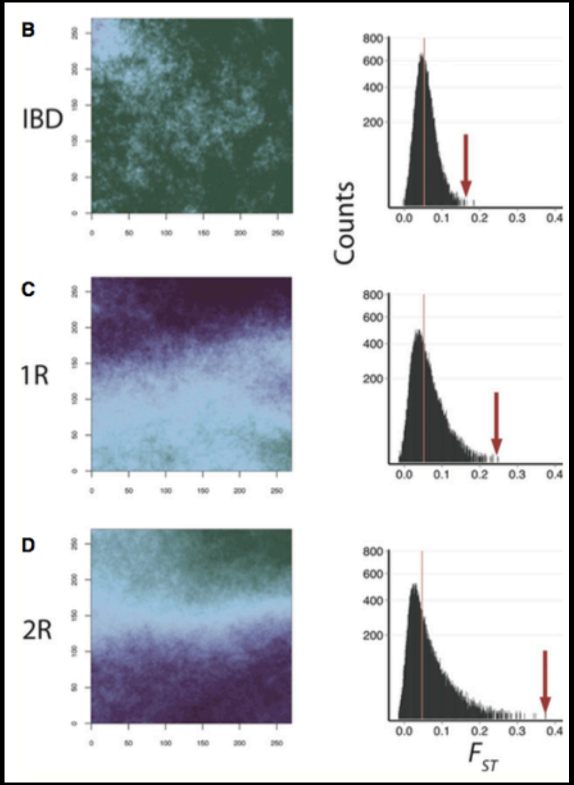


When did selection act on your phenotype?

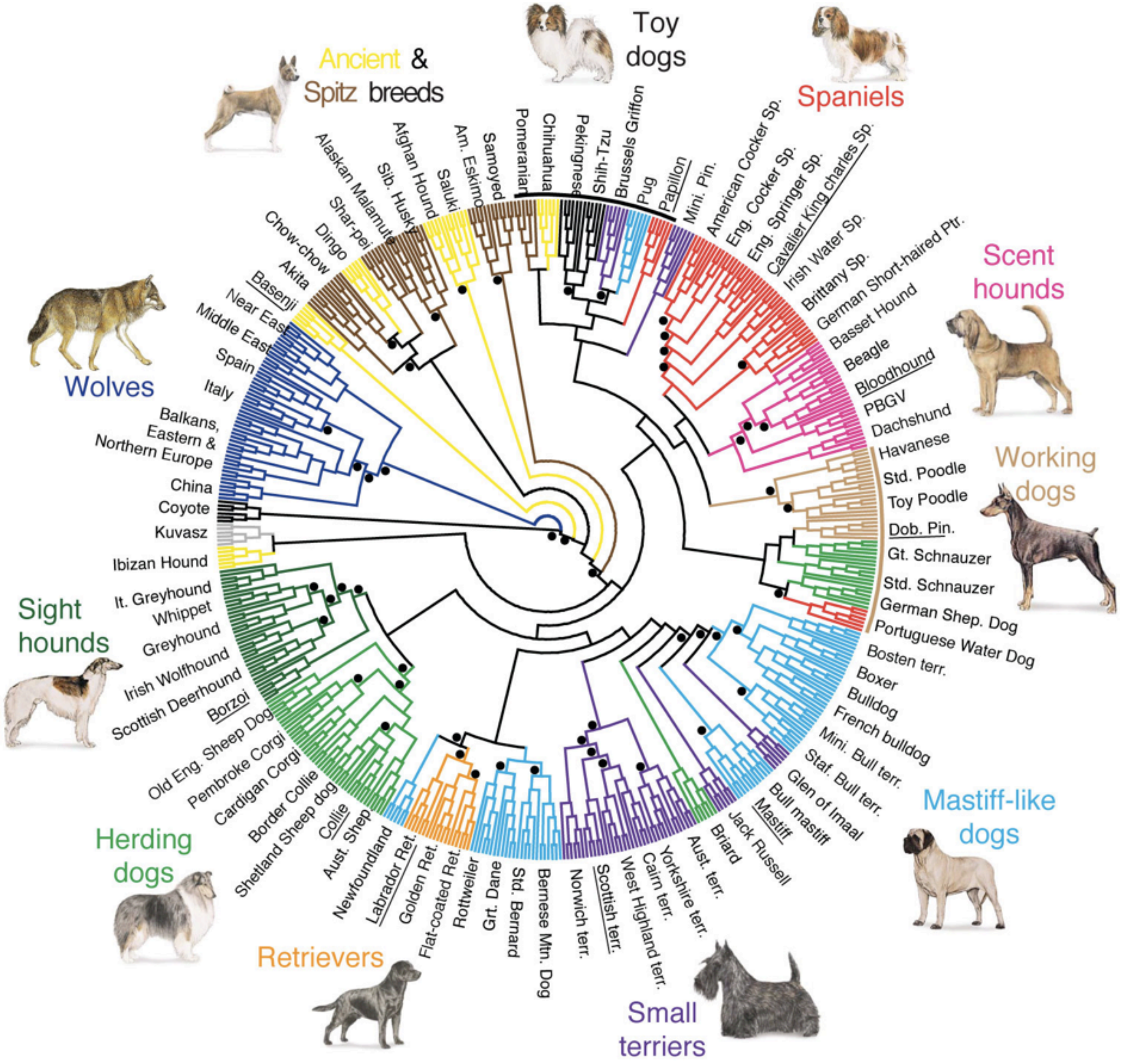
What's the demographic history of your population?

Jensen 2014. Nature Communications 5:1–10.

What's a good way to assess molecular tests?



- Computer simulations of evolution
  - Across range of demographic scenarios
- What else?
- Testing them on real data where we know the targets of selection = real world validation
  - Which ones work and when
  - We could then use this to make better tests, right? (very rare)



von Holdt et al. 2010. Nature

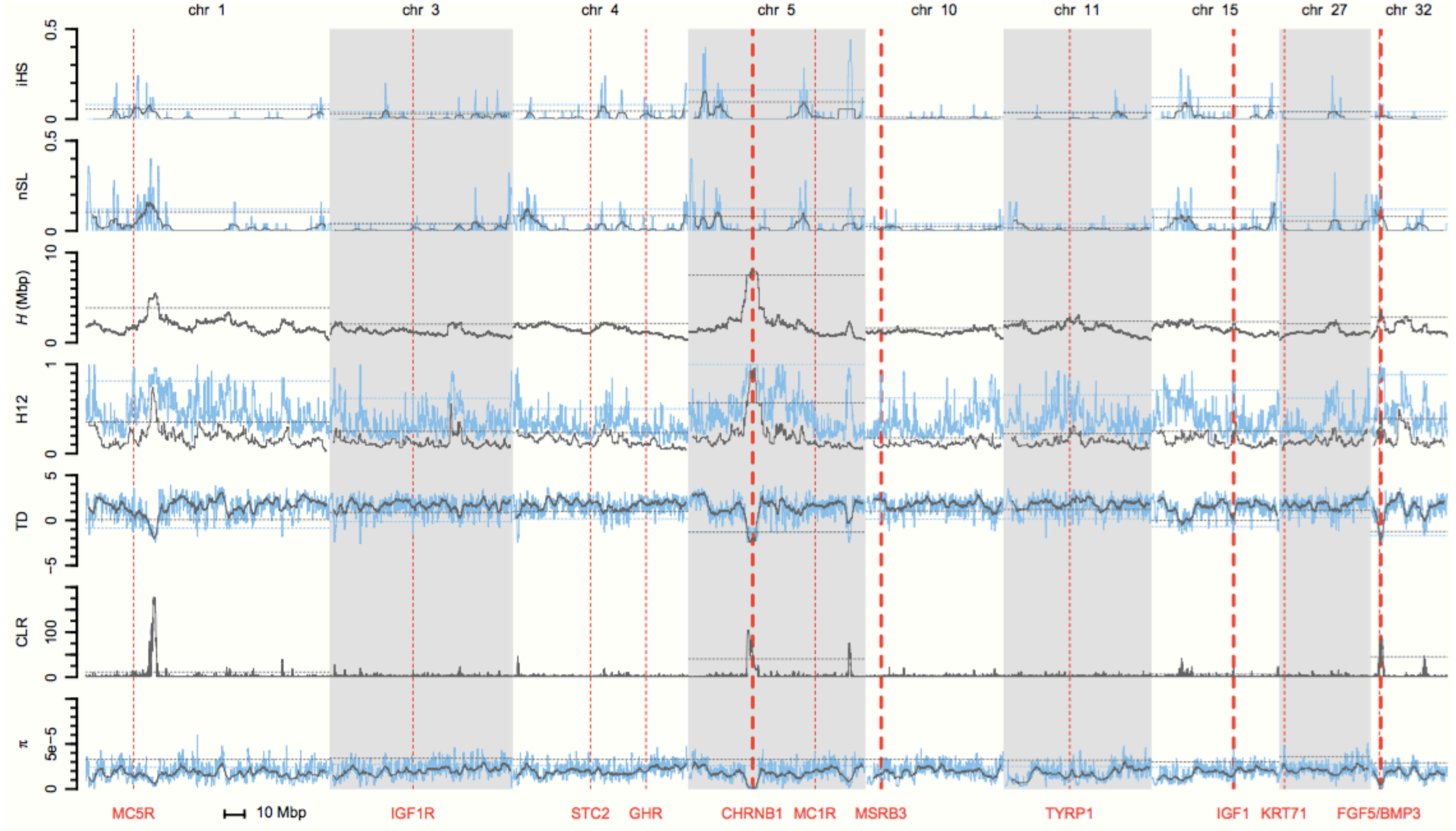
Breed specific morphologies

Test set of Schlamp et al. 2016:

- 25 breeds
- 12 causal loci
- N = 25 / breed
- 7 tests of selection
  - – iHS,nSL,H,TajD, etc.

What can state of the art molecular tests of selection detect?

French Bulldog sample: low power, high type I & II error



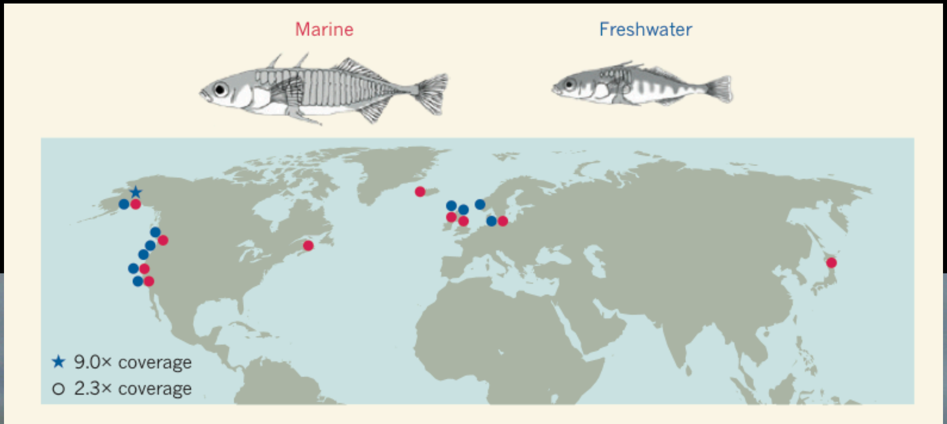
Schlamp et al. 2016. Evaluating the performance of selection scans to detect selective sweeps in domestic dogs. Molecular Ecology 25:342–356.

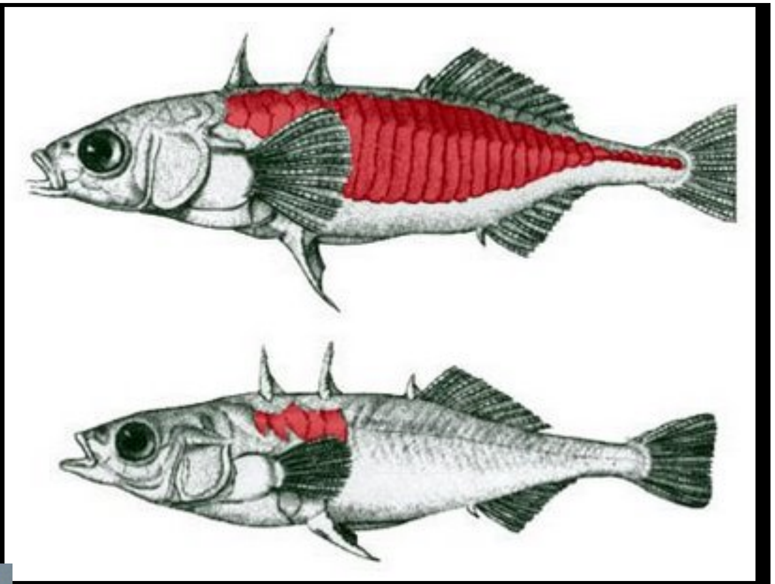
Molecular tests ...

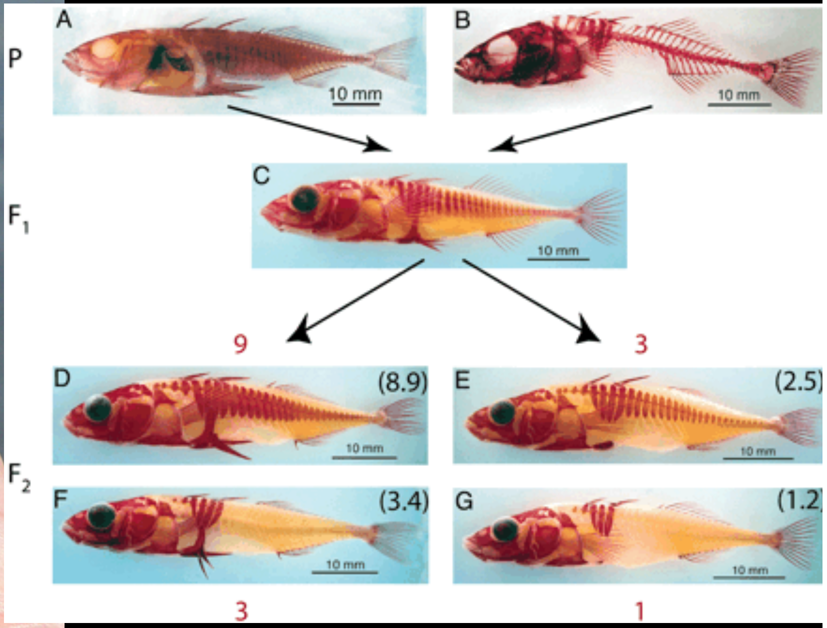
BASED ON 20 YEARS OF PUBLICATIONS

- Are still chasing an elusive null model ....
  - Each performs better than previous ones under a specific set of conditions, all have poor null model
- But ... under realistic biological conditions, they all
  - Have very low power
  - Have high false positive rates

Hard selection case example: threespine stickleback fish



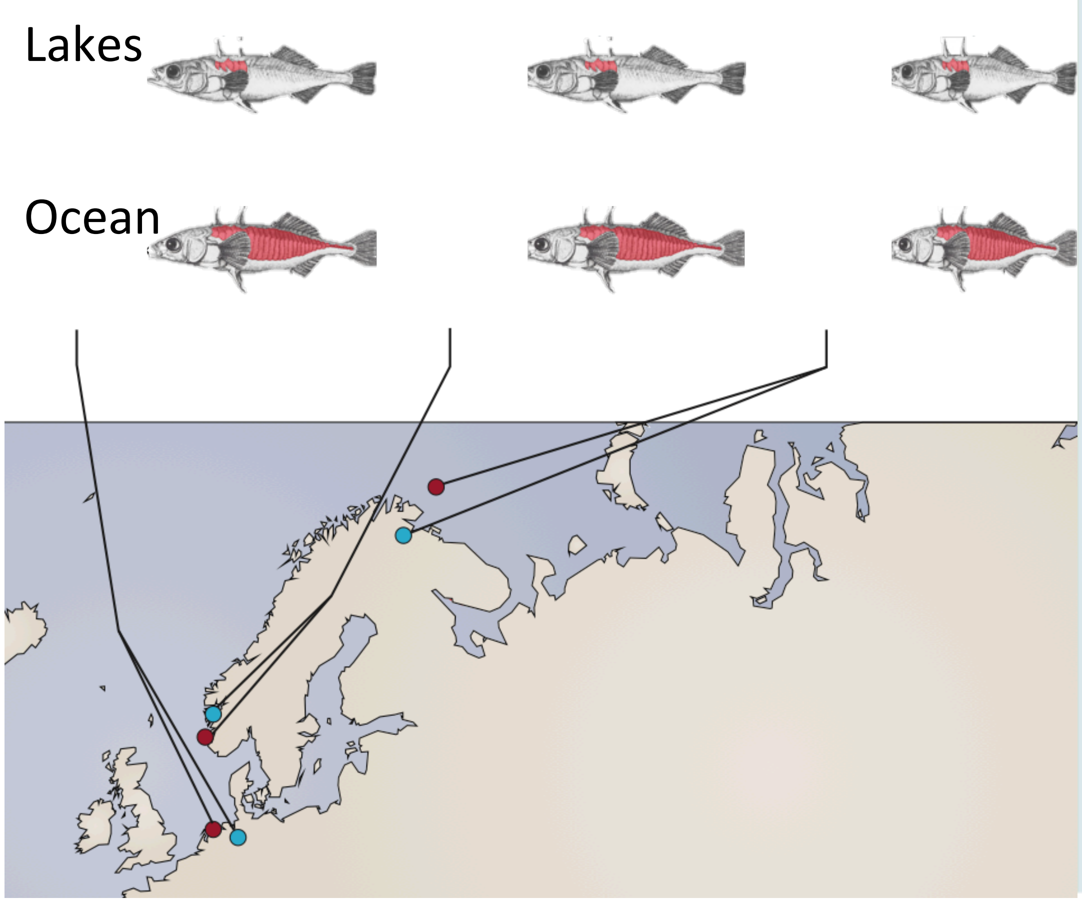


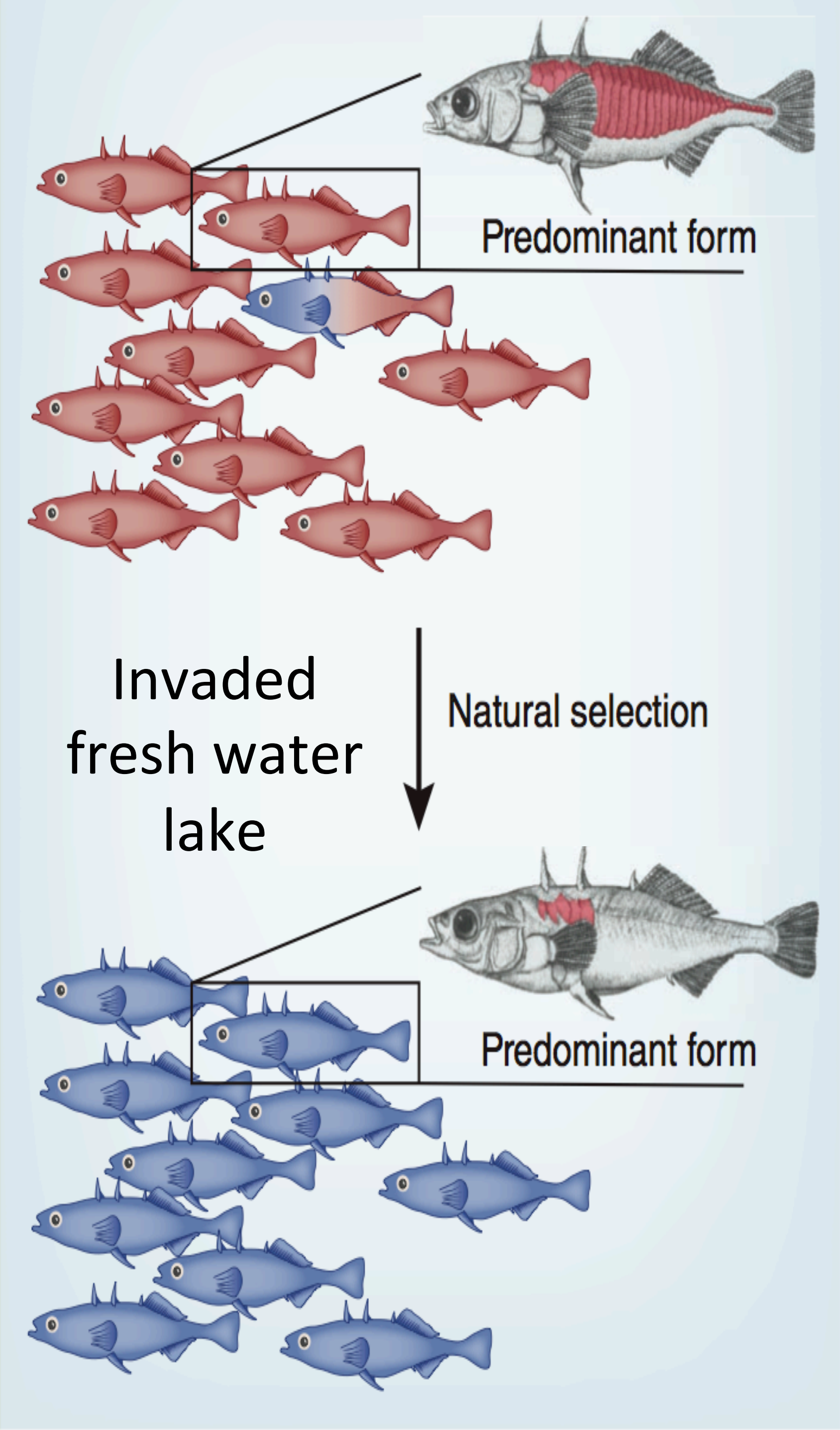


Threespine stickleback fish

(Gasterosteus aculeatus)

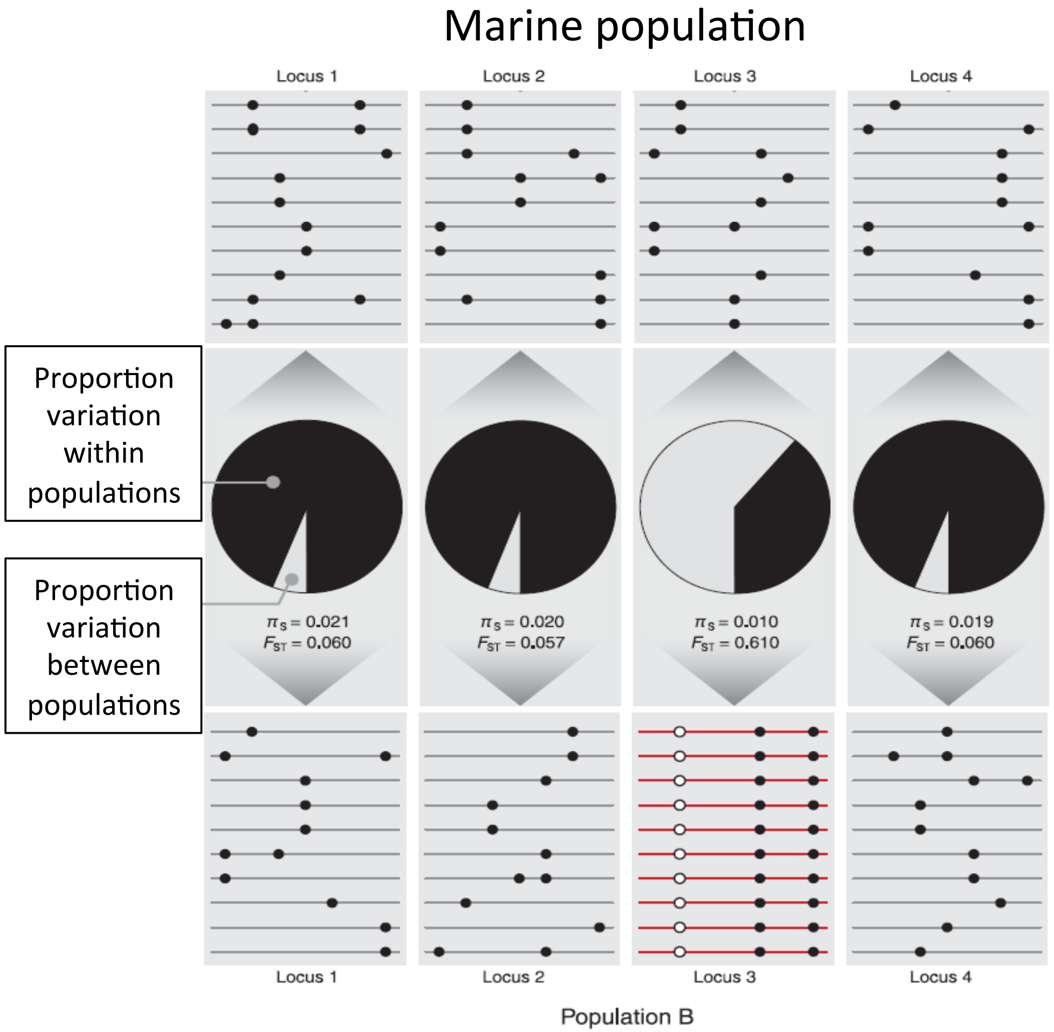
- Has body armor in the ocean
- Loses almost all armor in lakes





Invaded fresh water lake

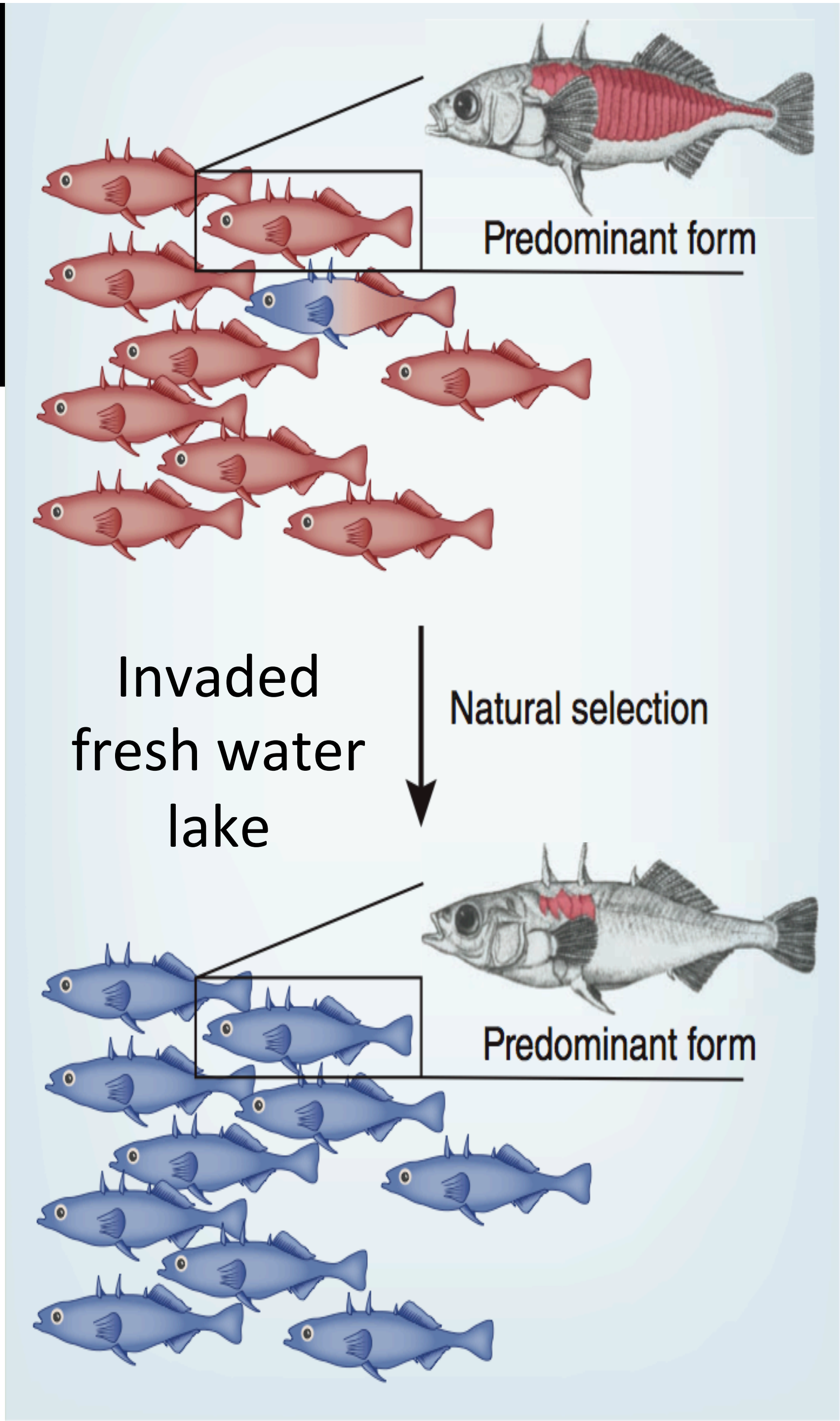
Parallel adaptation in fresh water lakes via hard sweeps


Marine population

Proportion variation within populations

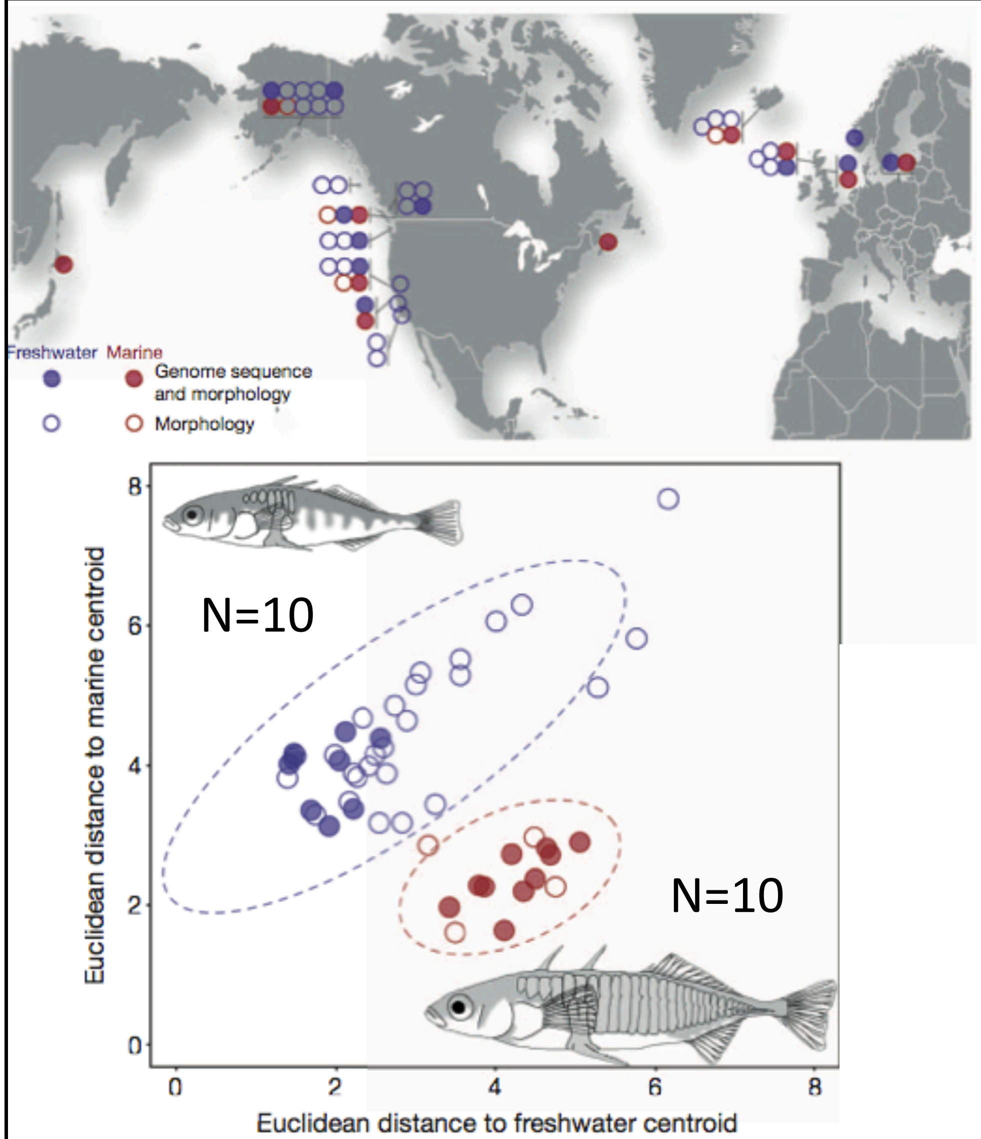
Proportion variation between populations

Population B



Invaded fresh water lake

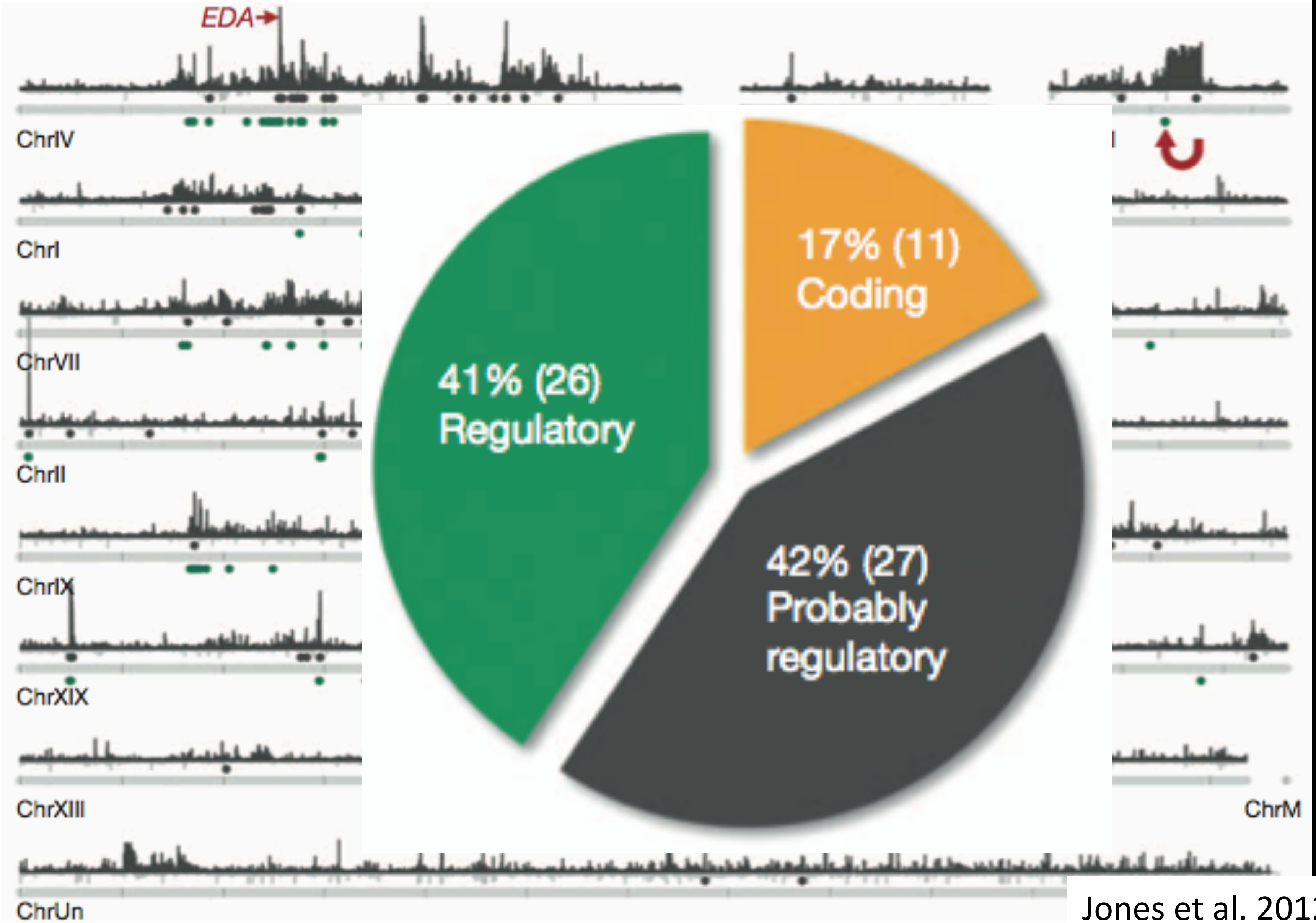
Individual genome sequencing: powerful insights



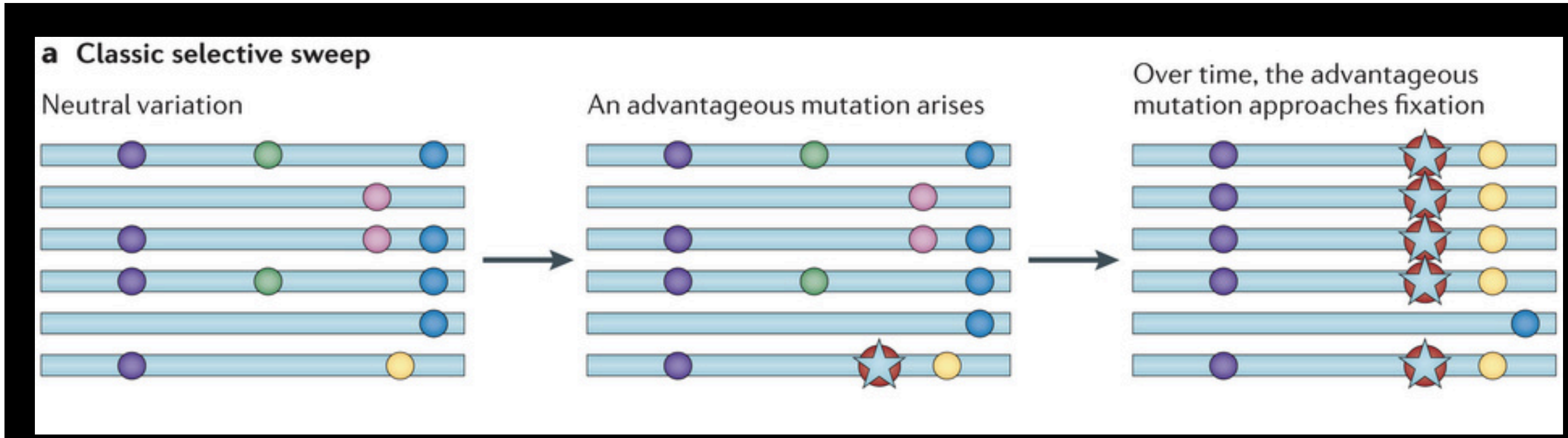
2-5 X per individual, sliding 2500 bp window, 500 bp step

Jones et al. 2012 Nature

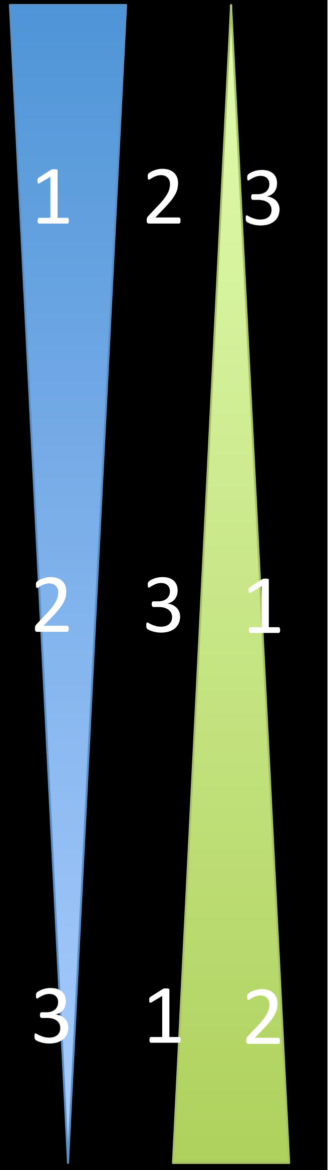
Which regions are more important? Coding or expression?



Jones et al. 2012 Nature


a Classic selective sweep
Neutral variation
An advantageous mutation arises
Over time, the advantageous mutation approaches fixation

Test power


1 2 3

2 3 1

3 1 2

Freq. in nature

Scheinfeldt & Tishkoff. 2013. Nat Rev Genet 14:692–702.

How common are hard sweeps in nature?

- "we argue that soft sweeps might be the dominant mode of adaptation in many species"

Messer and Petrov 2013 TREE

The lab?

- "Signatures of selection … [are] not associated with 'classic' sweeps … More parsimonious explanations include 'incomplete' [or] 'soft' sweep models."

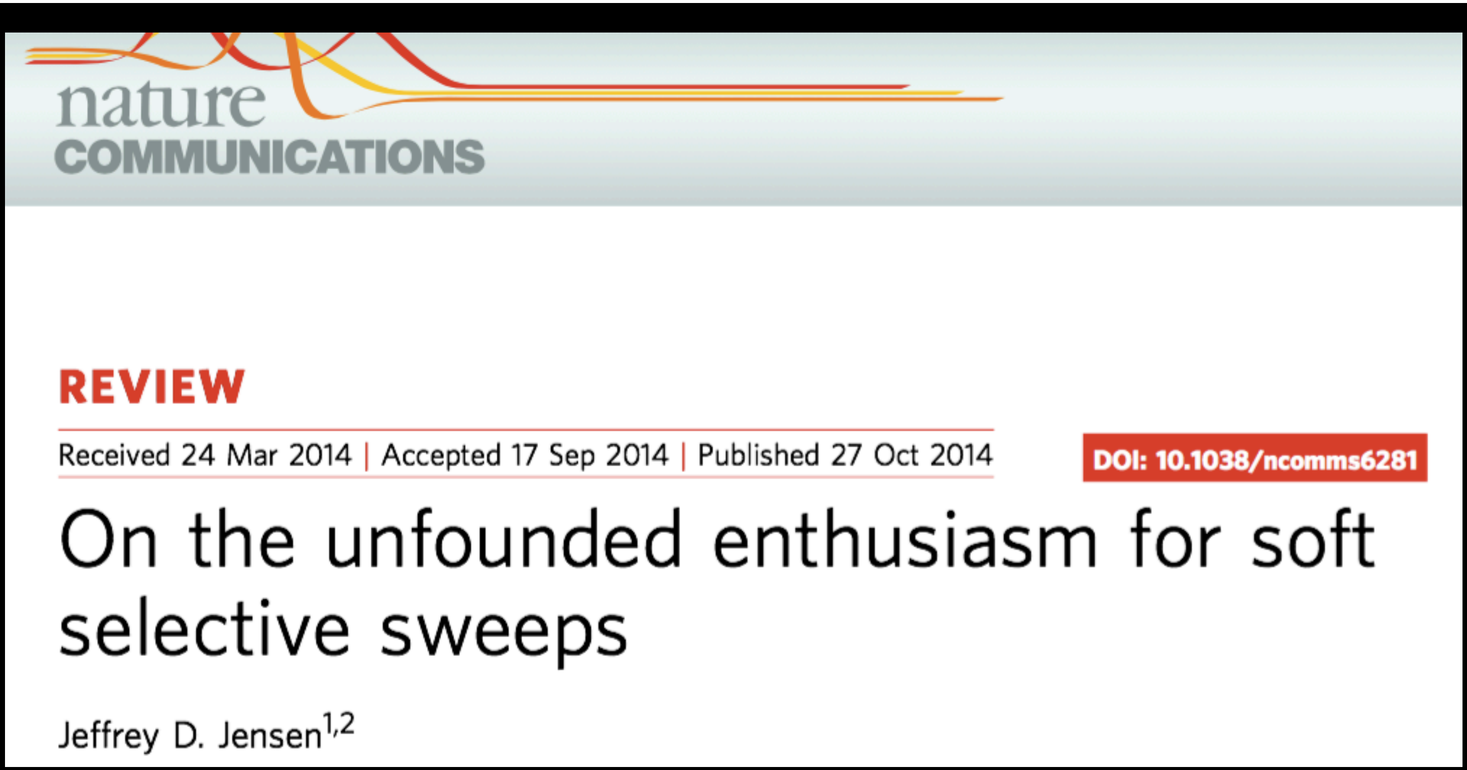
Burke et al. 2010 Nature

How common were hard sweeps in our history?

- "classic sweeps were not a dominant mode of human adaptation over the past 250,000 years"
- "much local adaptation has occurred by selection acting on existing variation rather than new mutation"

1000 Genomes PC 2010 Science
Hernandez et al. 2011 Science

Certainly not everyone agrees ....



nature COMMUNICATIONS

REVIEW

Received 24 Mar 2014 | Accepted 17 Sep 2014 | Published 27 Oct 2014

DOI: 10.1038/ncomms6281

On the unfounded enthusiasm for soft selective sweeps

Jeffrey D. Jensen[1,2]

- This is an important read, critical of
  - assumptions underlying soft sweep
  - low power of molecular tests to detect hard & soft sweeps

How common are soft sweeps in your species?

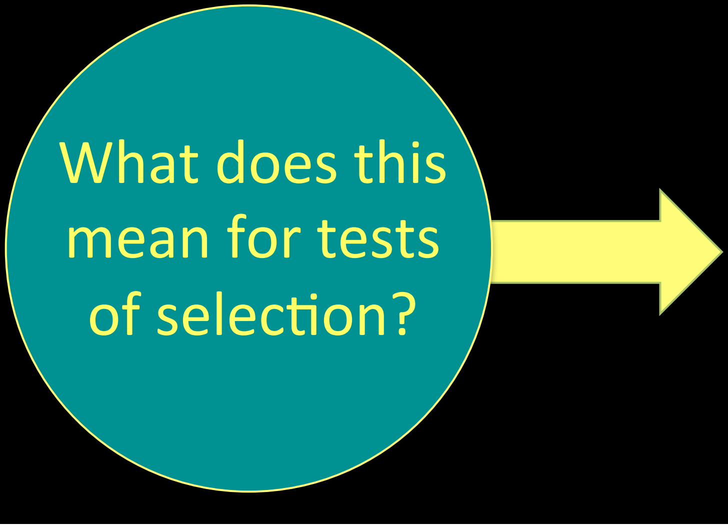
Thought experiment:

What fraction of species respond to selection in the lab?

Why?

If populations have variation, how likely is selection to use it?

What’s likelihood of selection on standing variation in wild?



What does this mean for tests of selection?

We have not been studying the dominant form of selection in the wild & cannot reliably detect it

Age and type of selection matters

- Novel mutation, large effect, hard sweep that goes to fixation
  - Probability of detection 20 – 90%, depending on demography, etc.

- Old mutation and / or polygenetic that does not sweep to fixation
  - Probability of detection close to 0

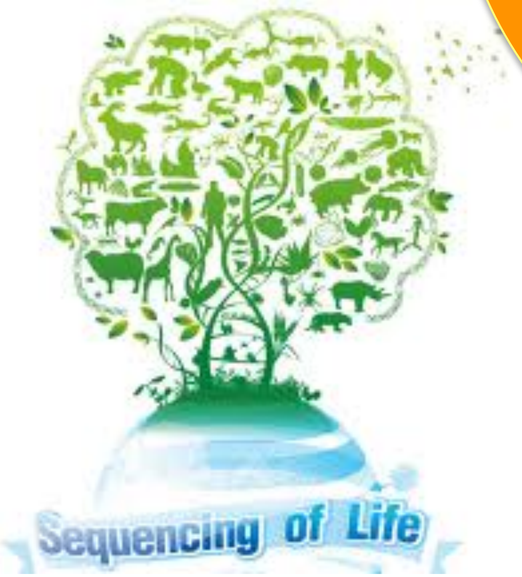
- Finding the causal mechanism
  - Coding > expression (but allele specific expression can be lightening rod for expression)
  - SNPs > more complex mutations (indel, TE, CNV)
  - Ongoing gene flow & grouping by phenotype across replicate populations helps a lot

- What is the relative frequency of these?
  - What will be the architecture of your phenotype?
  - What does your method have the highest power to detect?

Get ready, here come the 1000^n genomes

- Roughly 20 arthropods se to date
  - plans to sequen
- Many other larg

An unprecedented opportunity for large scale errors?

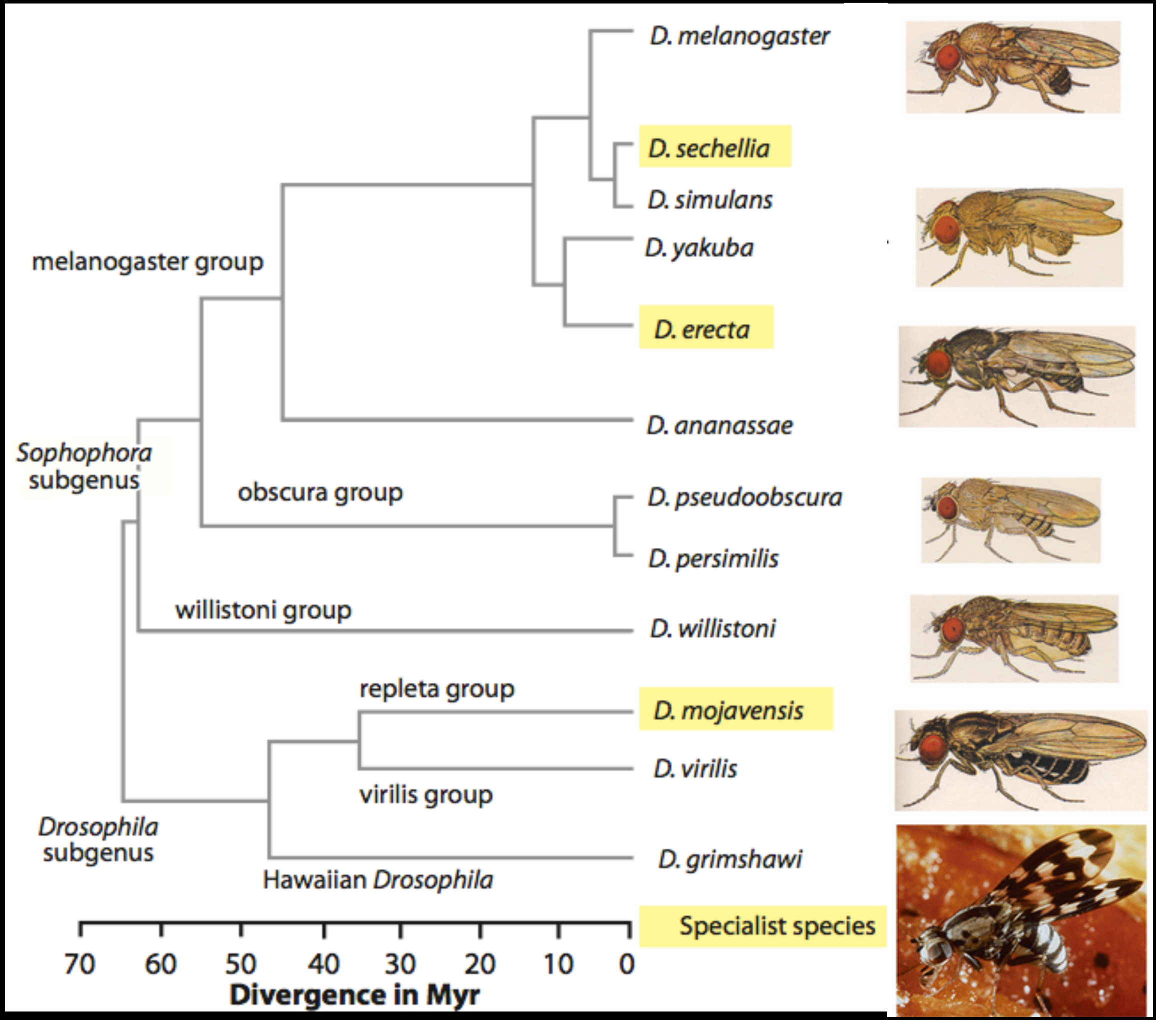


studying:

lationships

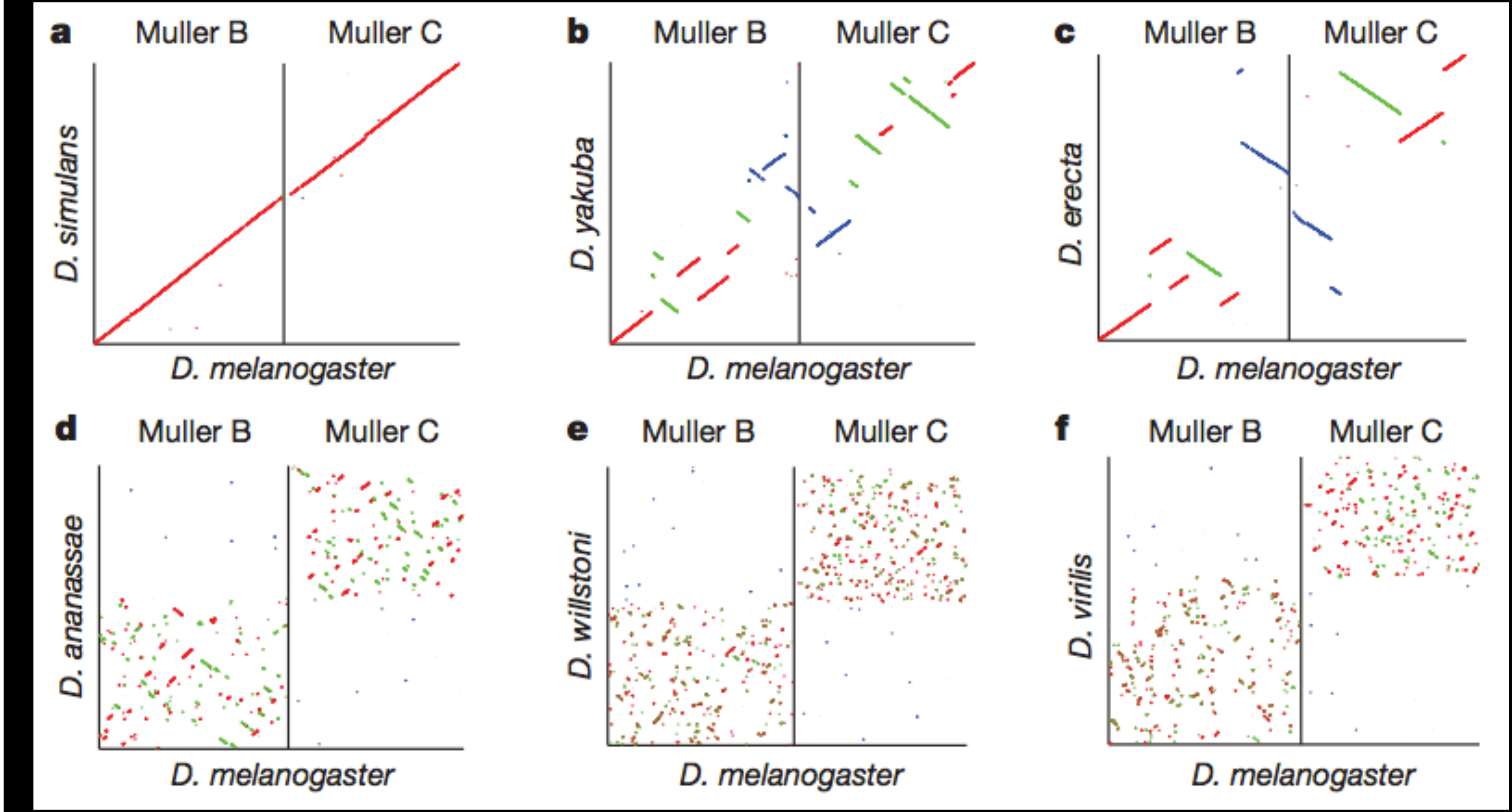
- Genome evolution
- Functional insights into genes and genomic features (e.g. regulation and inheritance)

Classic study: Evolution of genes and genomes on the *Drosophila* phylogeny



Drosophila 12 Genomes Consortium 2007 Nature

Tempo and mode of chromosome evolution



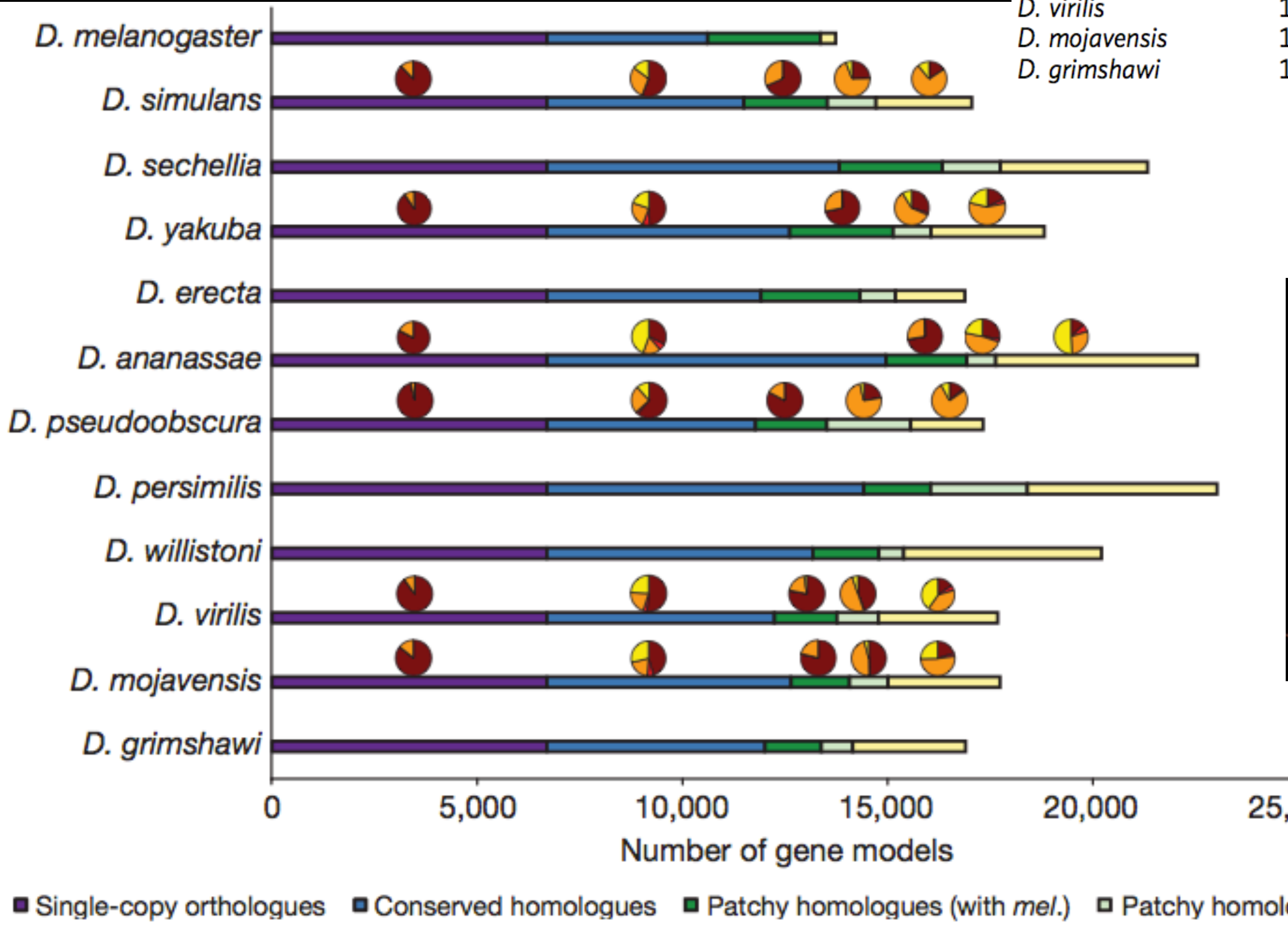
- > 20 My, chromosomal order completely reshuffled in Diptera

Drosophila 12 Genomes Consortium 2007 Nature

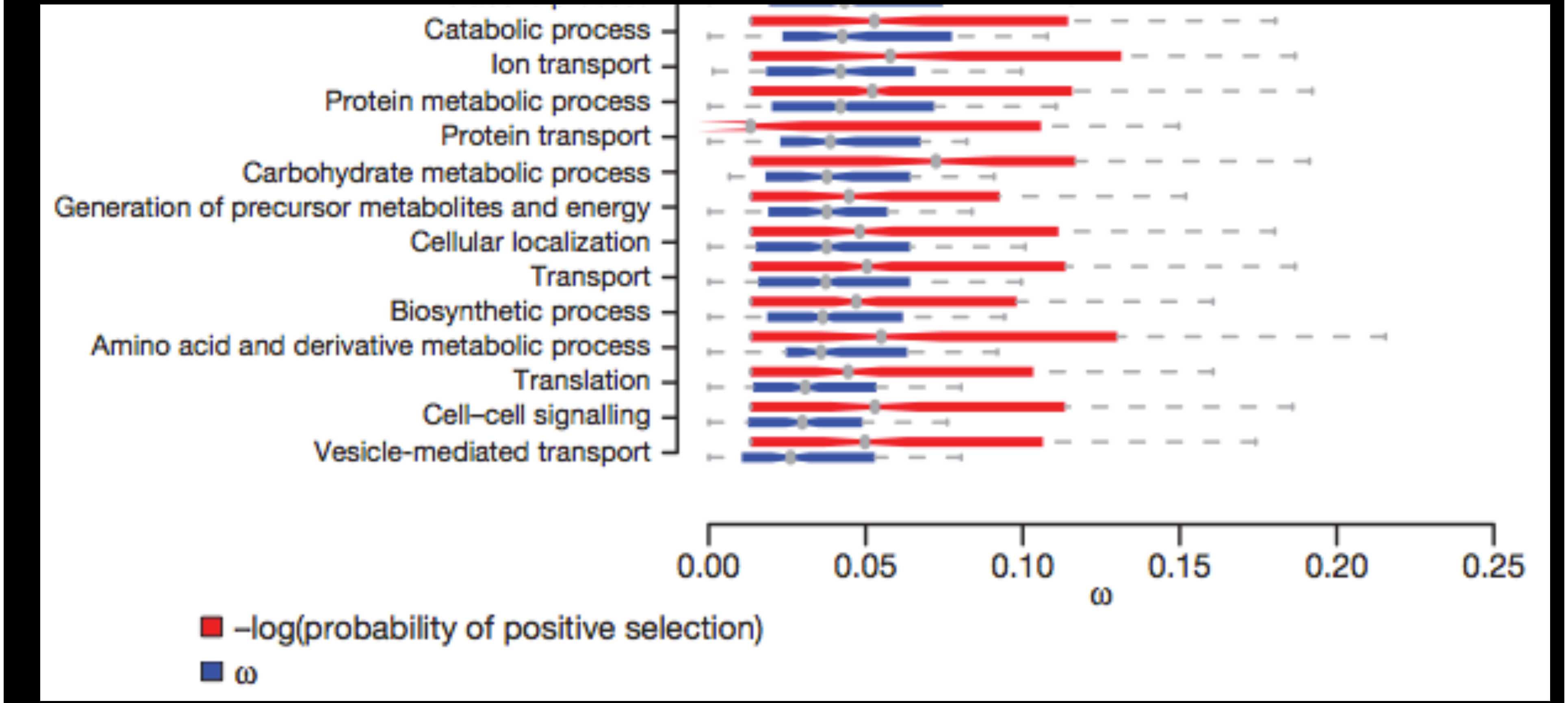
Genome evolution

Drosophila 12 Genomes Consortium 2007 Nature

	Total no. of protein- coding genes (per cent with *D. melanogaster* homologue)	Coding sequence/ intron (Mb)
D. melanogaster	13,733 (100%)	38.9/21.8
D. simulans	15,983 (80.0%)	45.8/19.6
D. sechellia	16,884 (81.2%)	47.9/21.9
D. yakuba	16,423 (82.5%)	50.8/22.9
D. erecta	15,324 (86.4%)	49.1/22.0
D. ananassae	15,276 (83.0%)	57.3/22.3
D. pseudoobscura	16,363 (78.2%)	49.7/24.0
D. persimilis	17,325 (72.6%)	54.0/21.9
D. willistoni	15,816 (78.8%)	65.4/23.5
D. virilis	14,680 (82.7%)	57.9/21.7
D. mojavensis	14,849 (80.8%)	57.8/21.9
D. grimshawi	15,270 (81.3%)	54.9/22.5



Selection dynamics across functional categories



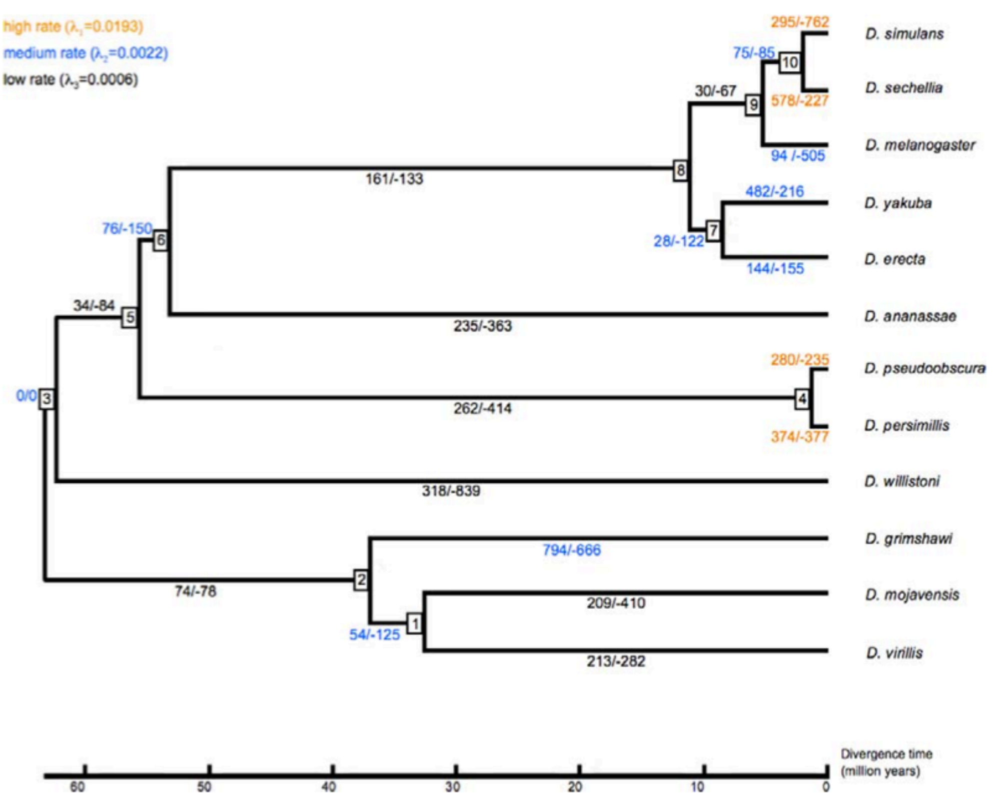
- 33.1% of single-copy orthologues have experienced positive selection on at least a subset of codons.

Drosophila 12 Genomes Consortium 2007 Nature

Gene Family Evolution across 12 Drosophila Genomes

- One fixed gene gain/ loss across the genome every 60,000 yr

- 17 genes are estimated to be duplicated and fixed in a genome every million years



Drosophila 12 Genomes Consortium 2007 Nature
Hahn et al. 2007 Plos Genetics

Comparative Genomics : a house of cards?

- Data scale is too large to thoroughly assess errors ...
  - Perhaps the findings are just .... wrong

- All conclusions, at some stage, rest upon
  - Simple bioinformatics
  - Assumptions that get incorporated into seemingly unbiased methods

Lets exploring two pillars of these studies, their error and repercussions

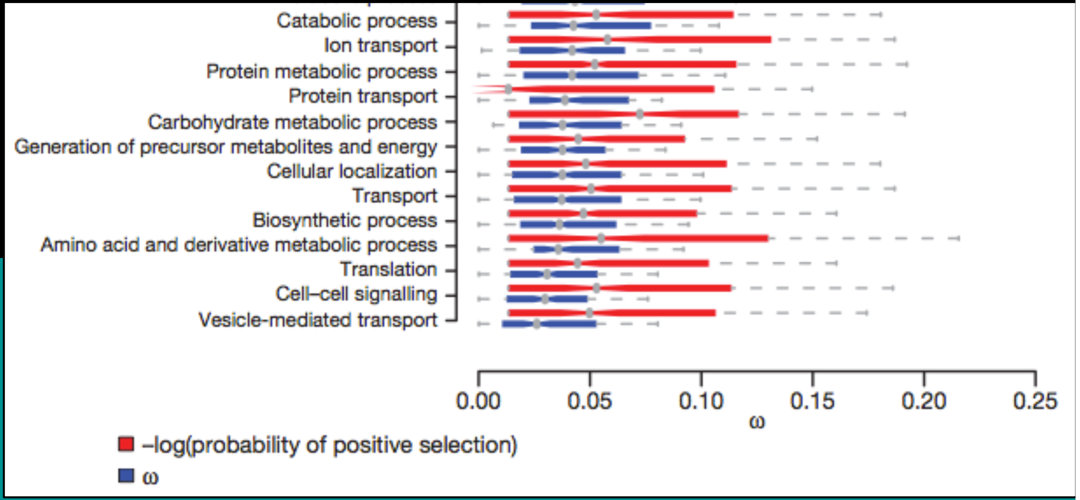
- Gene alignments in detecting positive selection
- Calibrations in temporal analysis

Established studies allow …

Follow up studies to reveal limitations

Robust findings to emerge with age

Inferring selection dynamics:

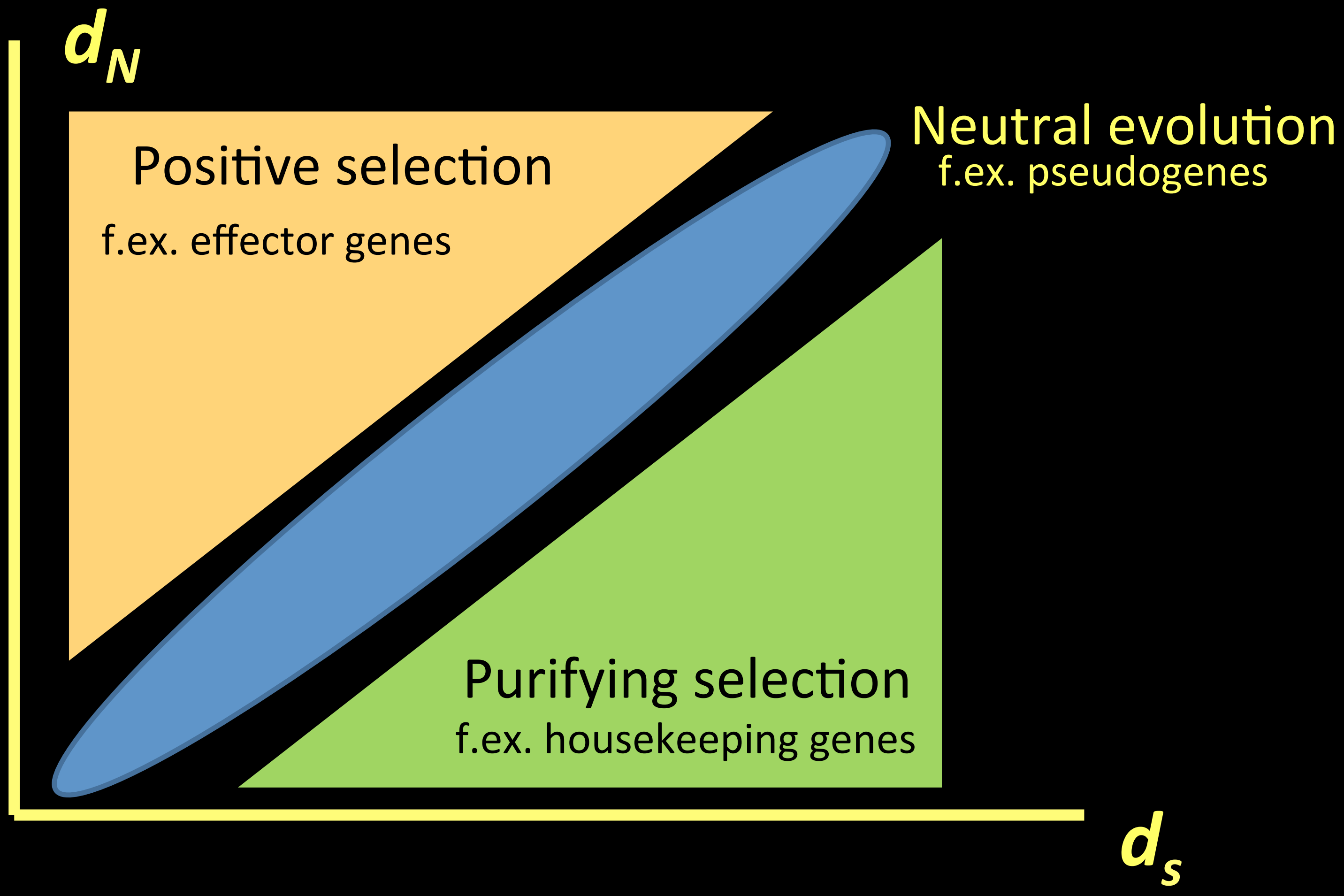


33.1% of single-copy orthologues have experienced positive selection on at least a subset of codons.

s

How robust are these conclusions?

Codon based tests of selection



Positive selection

f.ex. effector genes

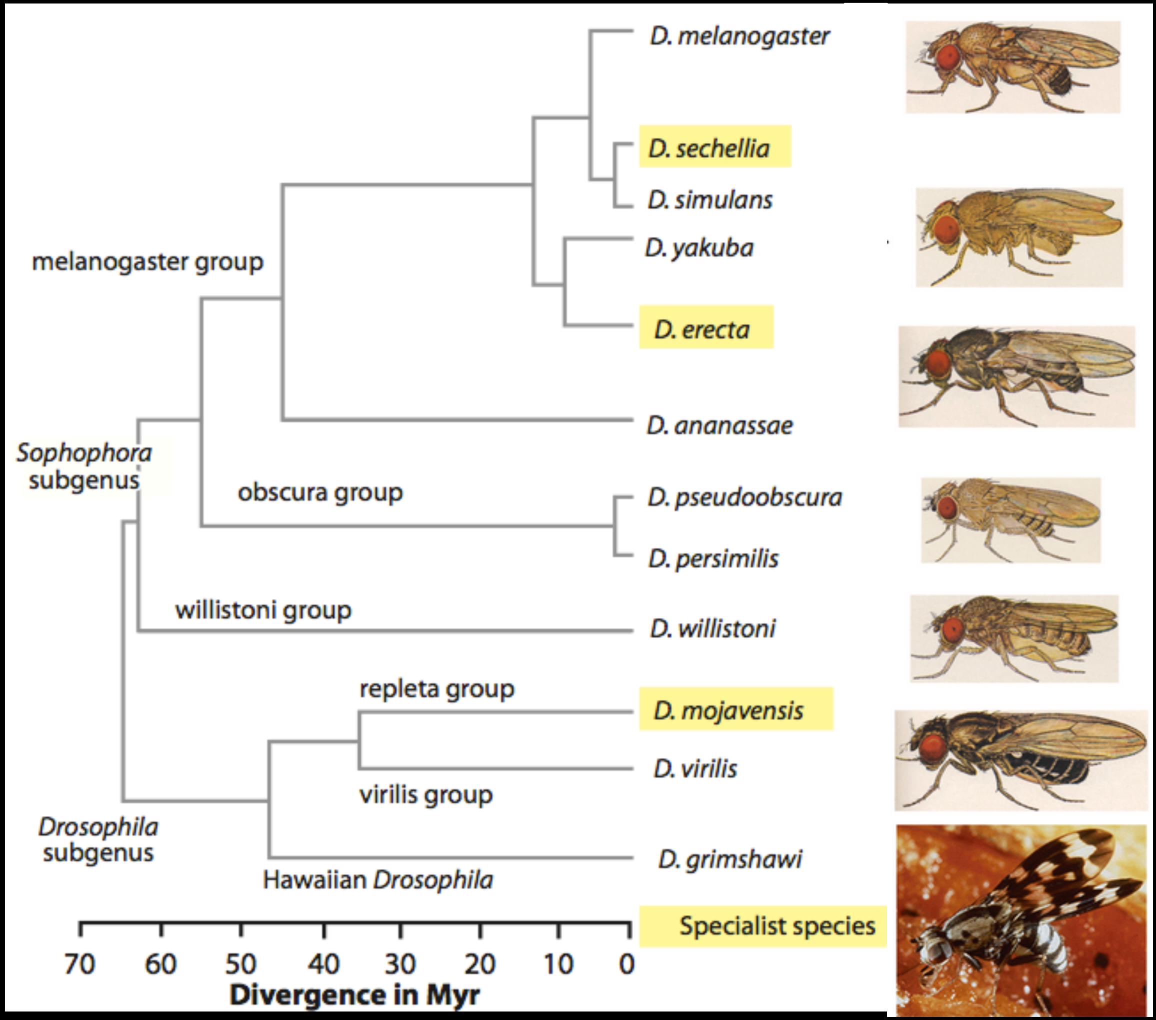
Neutral evolution
f.ex. pseudogenes

Purifying selection
f.ex. housekeeping genes

d_N / d_s
ratio

```
> 1 positive sel.
= 1 neutral
< 1 purifying sel.
```

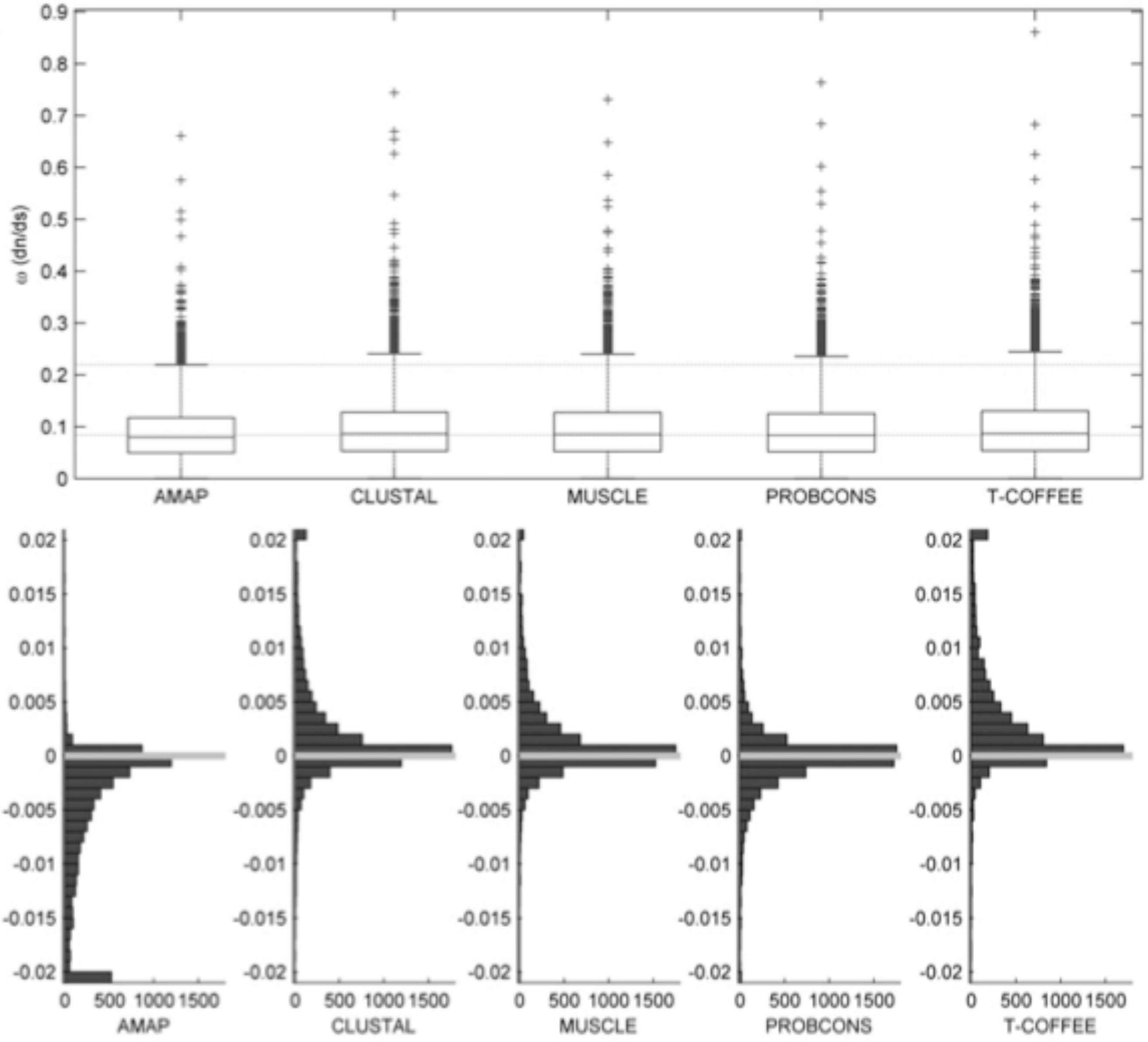
Evolution of genes and genomes on the *Drosophila* phylogeny



Drosophila 12 Genomes Consortium 2007 Nature

dN/dS estimates by aligner

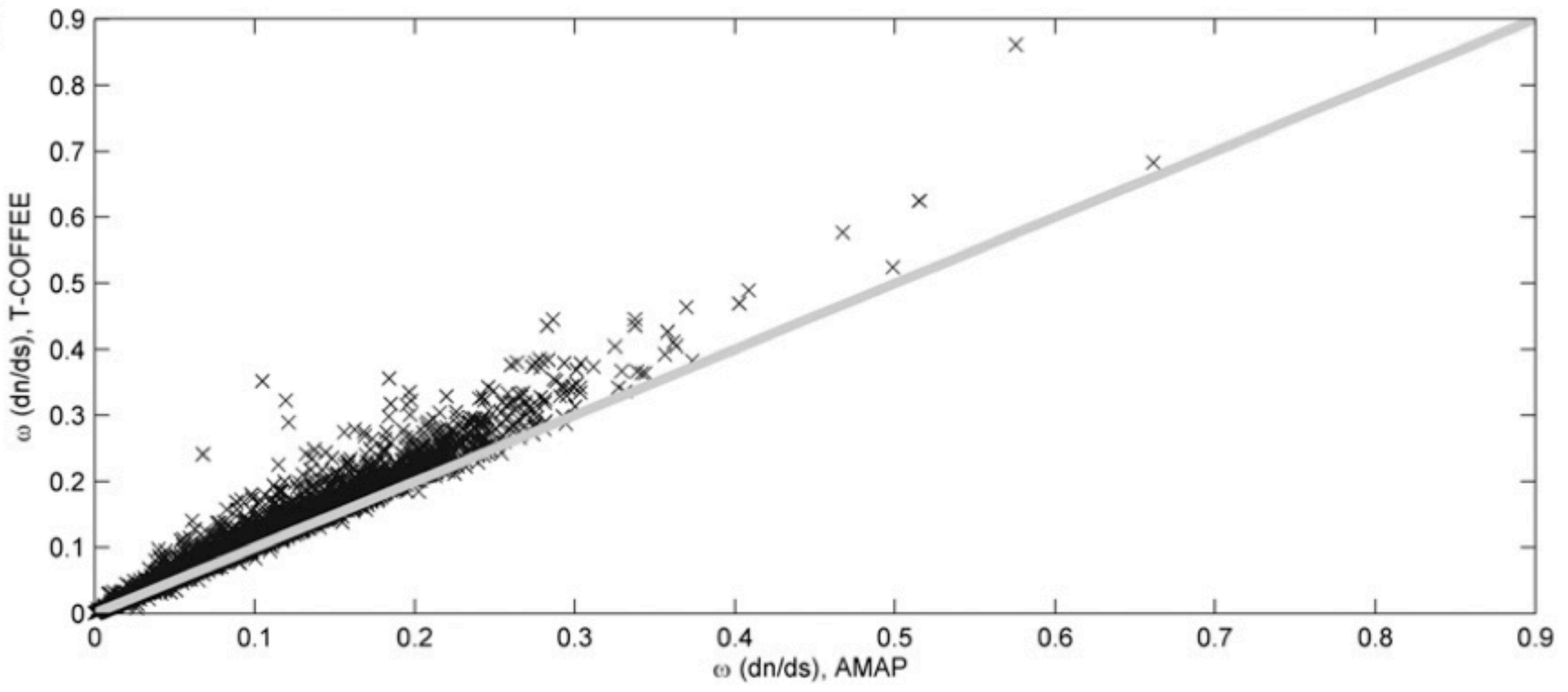
- 6690 orthologs
- 5 alignment methods
- Alignment methods affect dN/dS estimates



Markova-Raina & Petrov 2011 Genome Biology

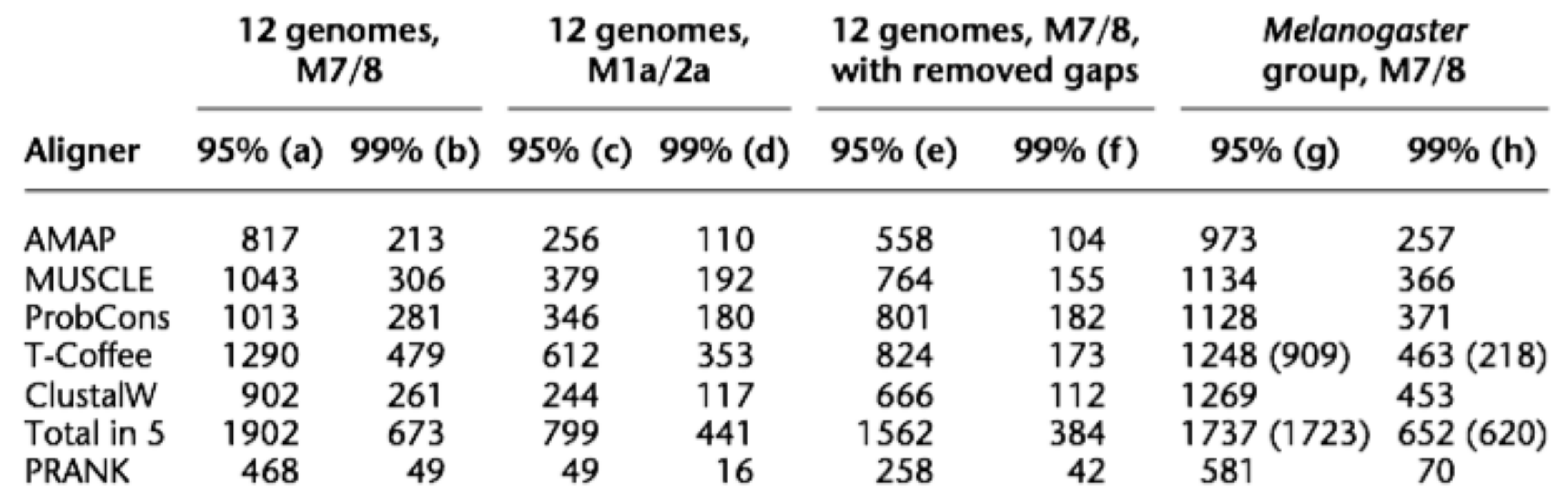
Comparing results across methods is responsible bioinformatics!!!!!

Since we can't look at our data, we need approaches that allow 1st principal assessments



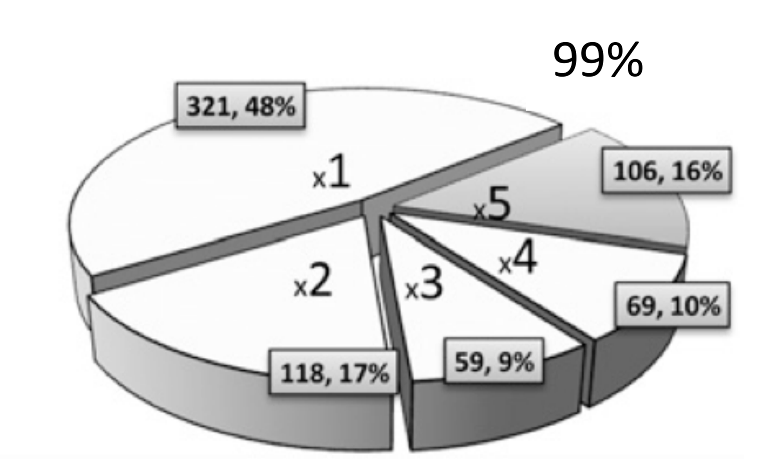
Markova-Raina & Petrov 2011 Genome Biology

Aligner tool has a larger effect than biology



	12 genomes, M7/8		12 genomes, M1a/2a		12 genomes, M7/8, with removed gaps		*Melanogaster* group, M7/8	
Aligner	95% (a)	99% (b)	95% (c)	99% (d)	95% (e)	99% (f)	95% (g)	99% (h)
AMAP	817	213	256	110	558	104	973	257
MUSCLE	1043	306	379	192	764	155	1134	366
ProbCons	1013	281	346	180	801	182	1128	371
T-Coffee	1290	479	612	353	824	173	1248 (909)	463 (218)
ClustalW	902	261	244	117	666	112	1269	453
Total in 5	1902	673	799	441	1562	384	1737 (1723)	652 (620)
PRANK	468	49	49	16	258	42	581	70

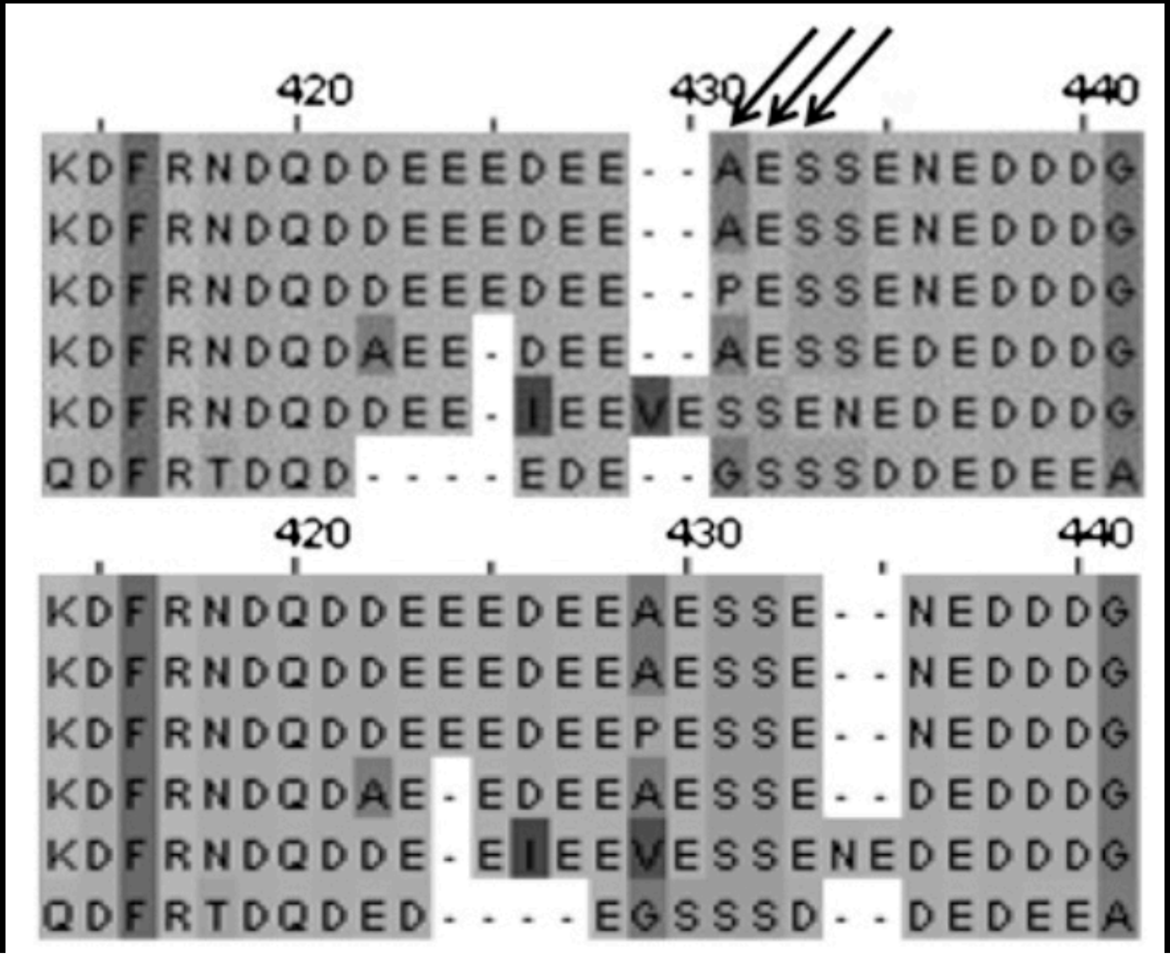
Number of significant genes in common across 1, 2, 3, 4, or all 5 of the alignment methods



Markova-Raina & Petrov 2011 Genome Biology

Alignment results highlight importance of alignment score!

- Tcoffee finds 3 selected sites indicated by arrows
- ProbCons identifies region with low alignment score, not used



Tcoffee

```
KDFRNDQDDEEEDEE--AESSENEDDDG
KDFRNDQDDEEEDEE--AESSENEDDDG
KDFRNDQDDEEEDEE--PESSENEDDDG
KDFRNDQDAEE-DEE--AESSEDEDDDG
KDFRNDQDDEE-IEEVESSENEDEDDDG
QDFRTDQD----EDE--GSSSDDEDEEA
```

ProbCons

```
KDFRNDQDDEEEDEEAESSE--NEDDDG
KDFRNDQDDEEEDEEAESSE--NEDDDG
KDFRNDQDDEEEDEEPESSE--NEDDDG
KDFRNDQDAE-EDEEAESSE--DEDDDG
KDFRNDQDDE-EIEEVESSENEDEDDDG
QDFRTDQDED----EGSSSD--DEDEEA
```

Markova-Raina & Petrov 2011 Genome Biology

What about recent genomes?

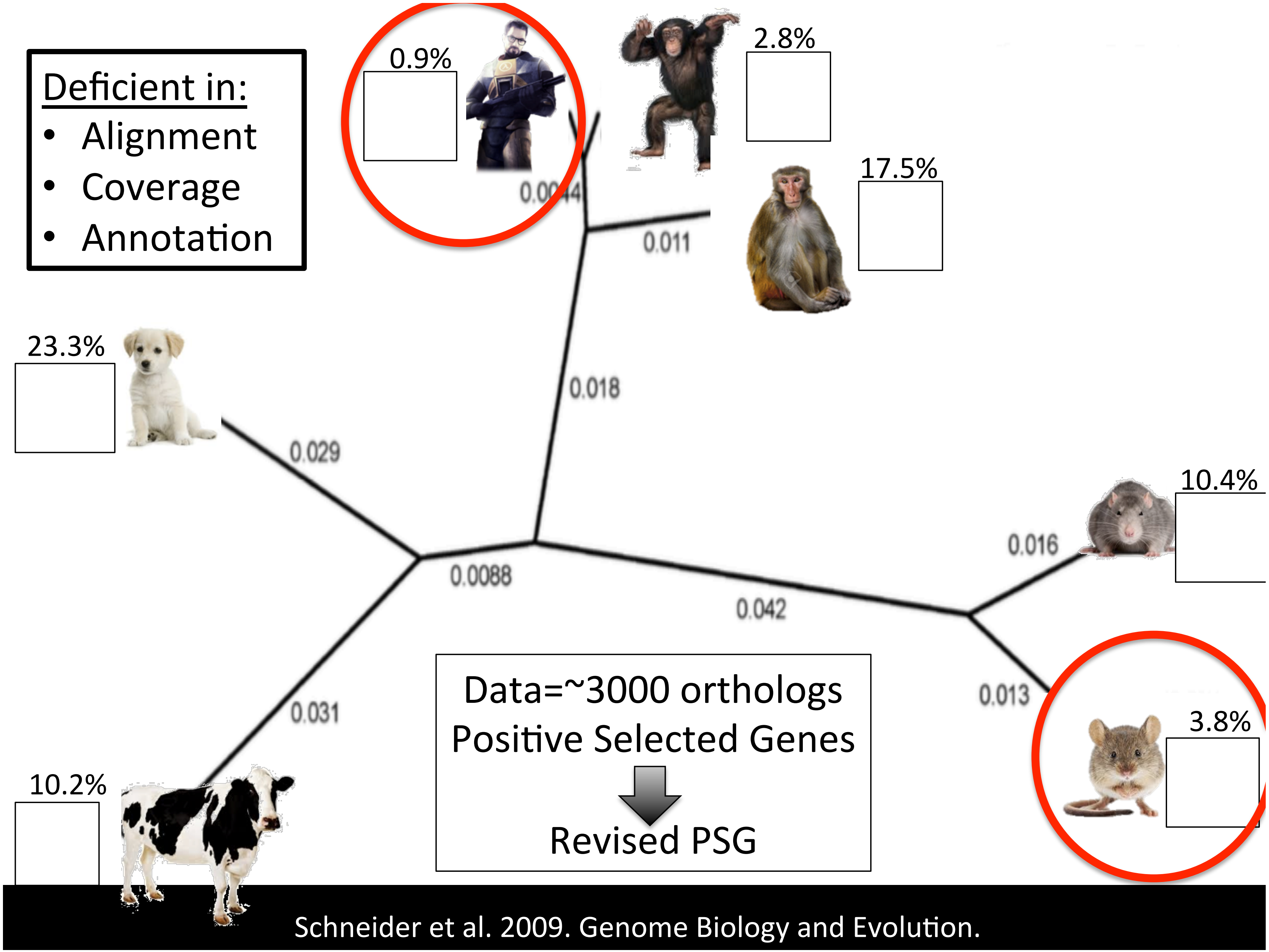
Surely they are better?

and mammals ... they have good genomes

and alignment problems rarely happen

... right?

How did I evolve to be so cute?



Deficient in:

- Alignment
- Coverage
- Annotation

Data=~3000 orthologs

Positive Selected Genes

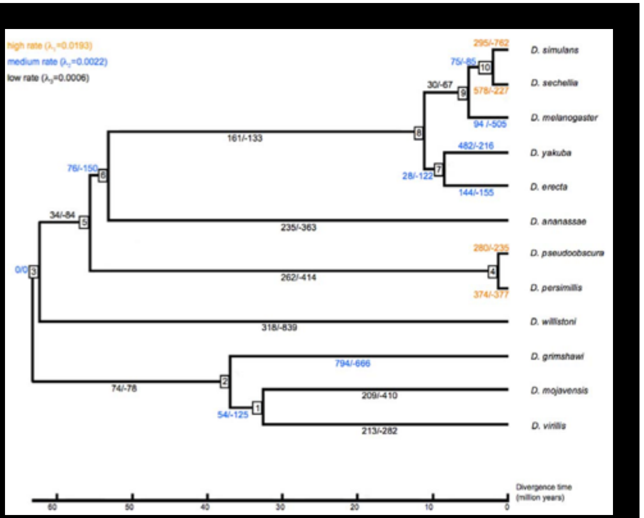
Revised PSG

Schneider et al. 2009. Genome Biology and Evolution.

Temporal inference:

fact or fiction?

Timing of divergence



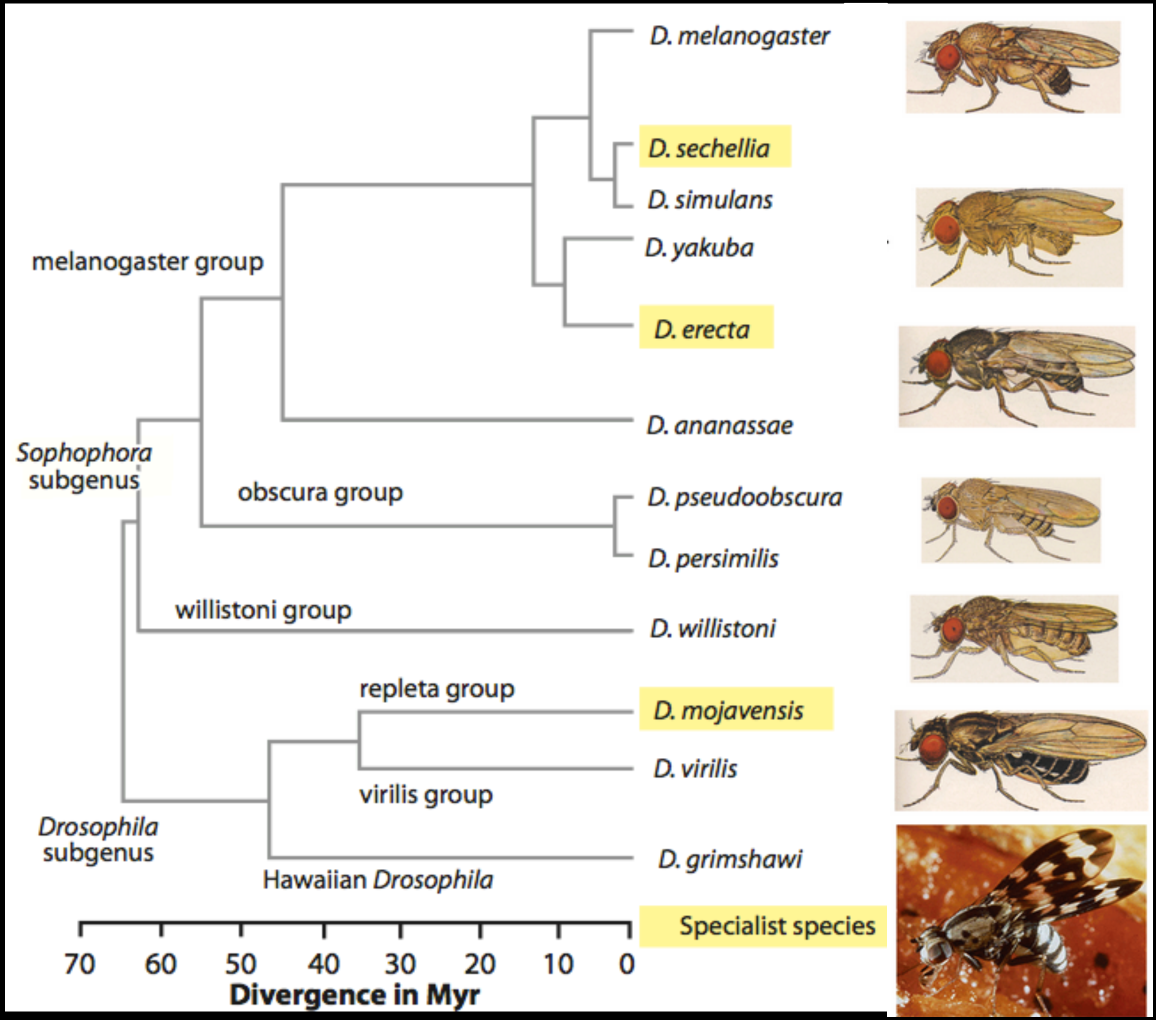
- Directly affects rate estimates

- Deriving unbiased dates from molecular data
  - Large field of software development

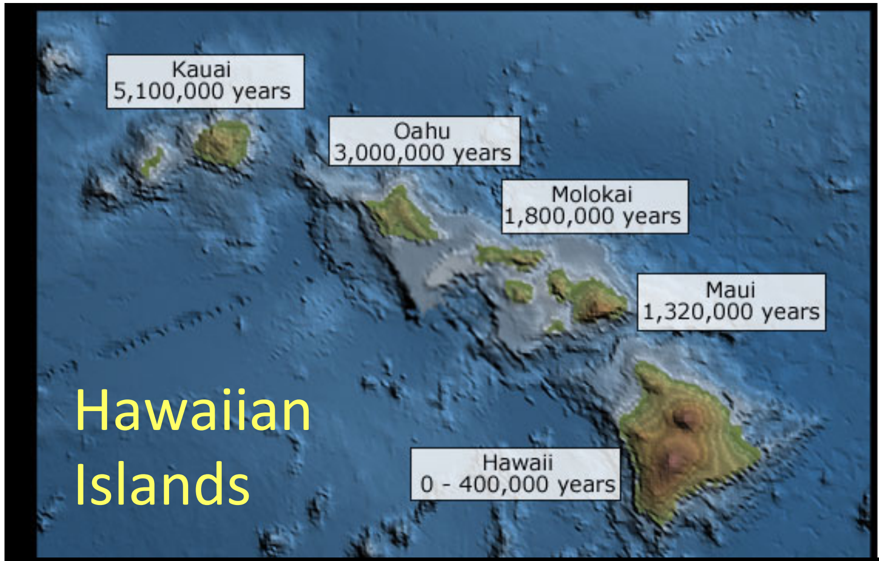
- Bayesian methods, while potentially informative and unbiased
  - Can be easily, and are routinely, abused

Wheat and Wahlberg 2013 TREE

Evolution of genes and genomes on the *Drosophila* phylogeny



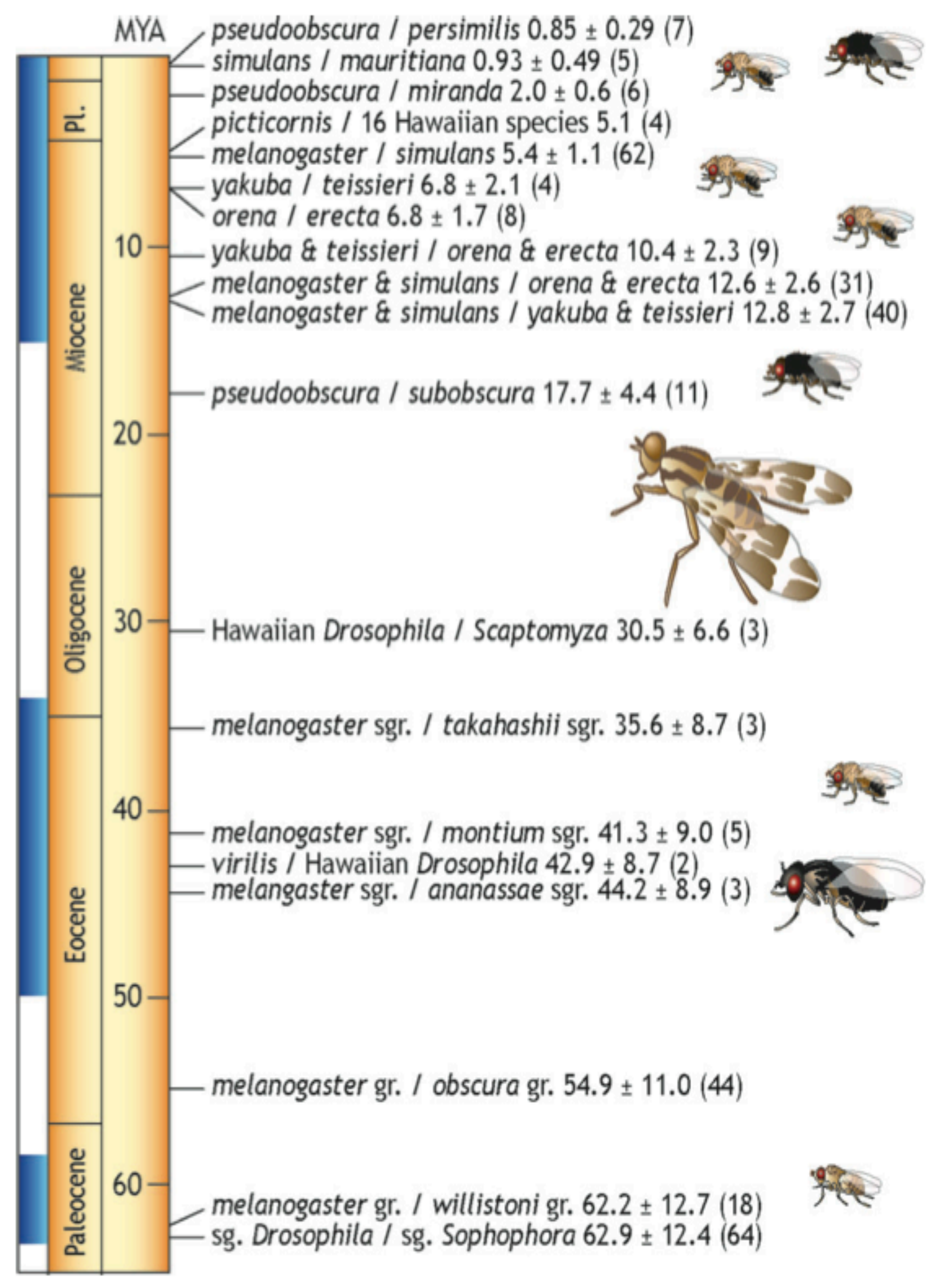
Drosophila 12 Genomes Consortium 2007 Nature



Calibration: Kauai age of 5.1 my for divergence of two Hawaiian species

1. No phylogeny
2. Fixed clock rate
3. Between 3 – 64 genes in pairwise comparisons

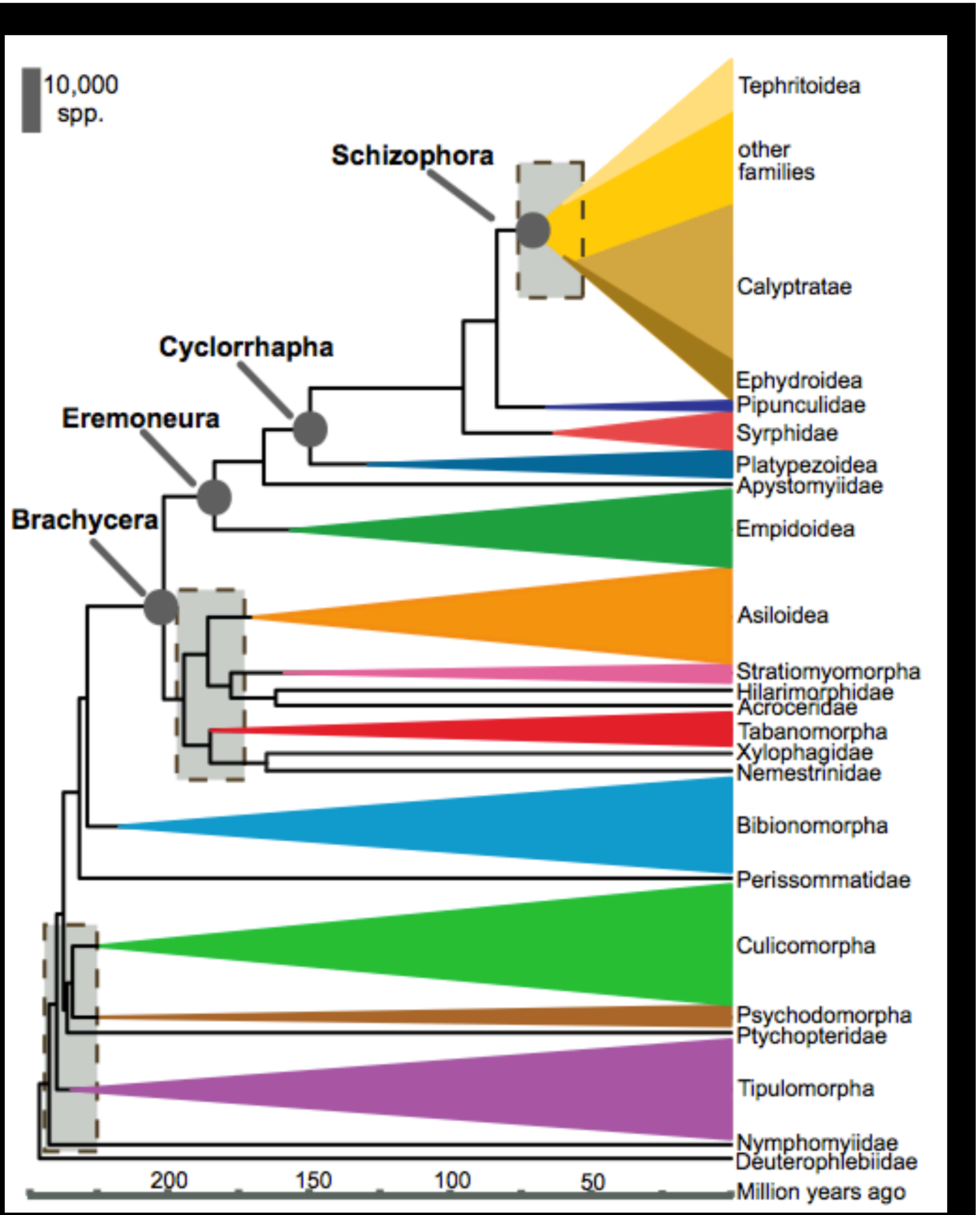
Temporal patterns in fruitflies (Tamura et al. 2004 MBE)



Drosophila clade:

- Schizophora constrained to maximum of 70 Ma
- Without constraint, goes to 115 Ma

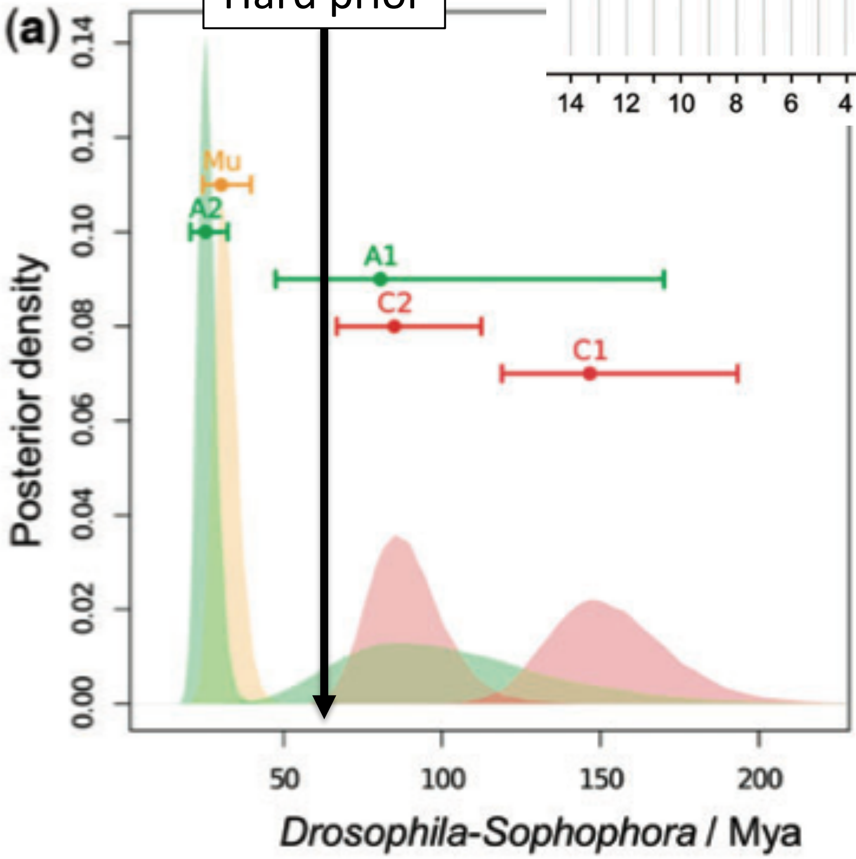
What is reality?

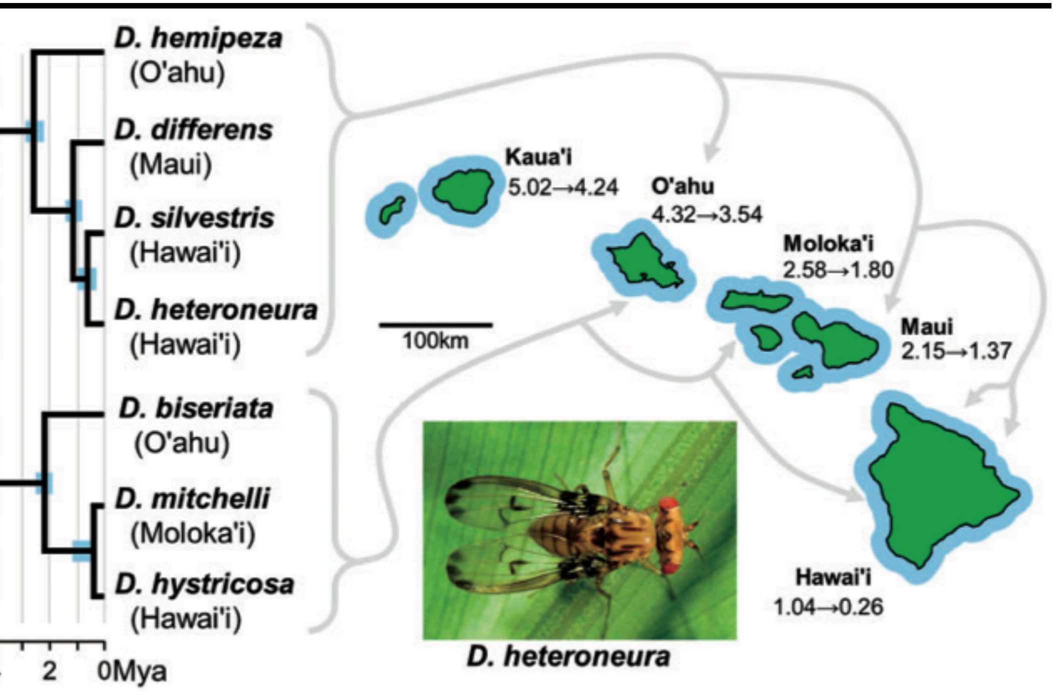


Episodic radiations in the fly tree of life
(Wiegmann et al. 2011 PNAS)

Determining objective priors is challenging

Hard prior





Priors in Bayesian rel. clock analysis:

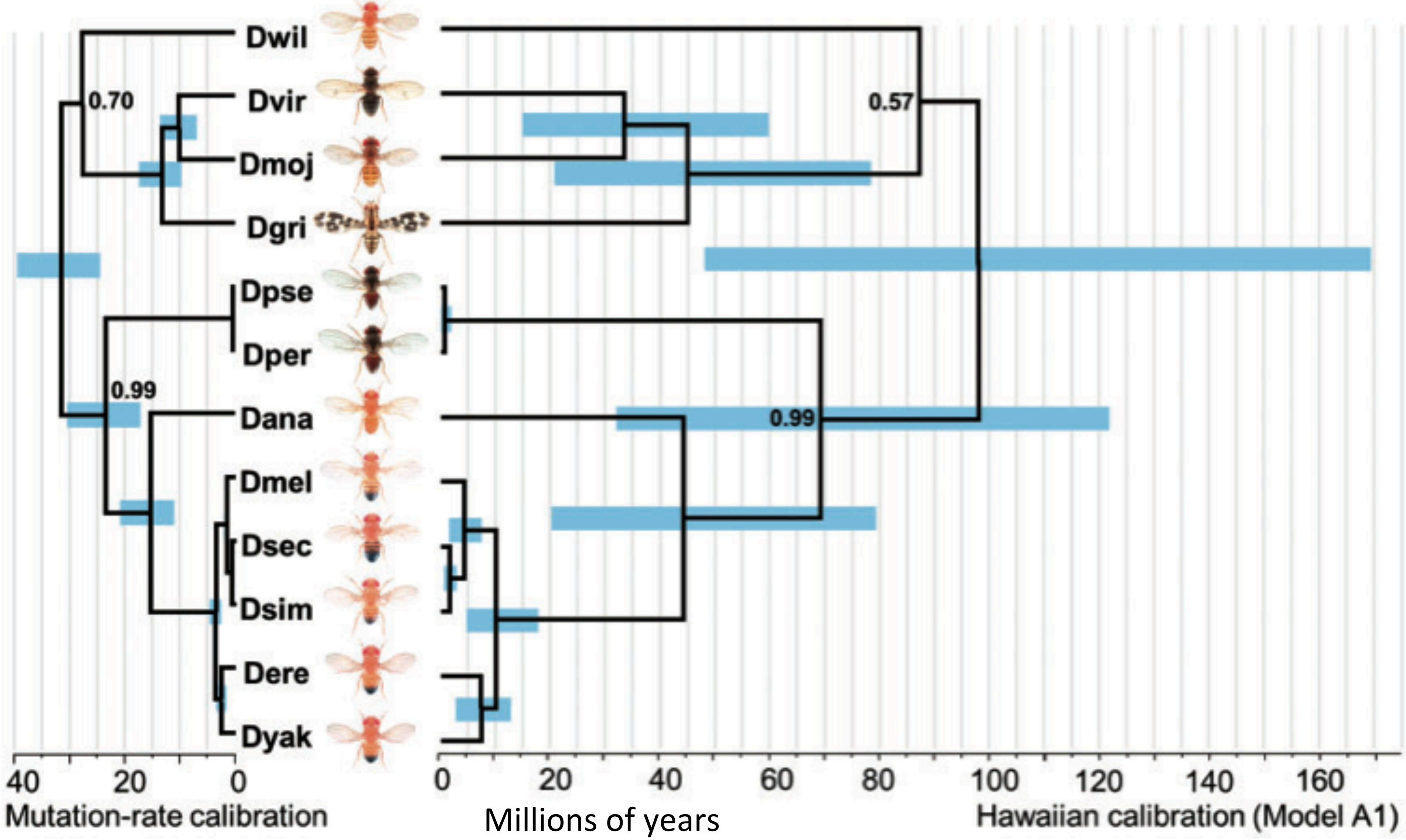
Mu = lab observed mutation rate

A1,2 = geological calibration, small Ne

C1,2 = geological calibration, large Ne

Obbard et al. 2012 Mol. Biol. Evol.

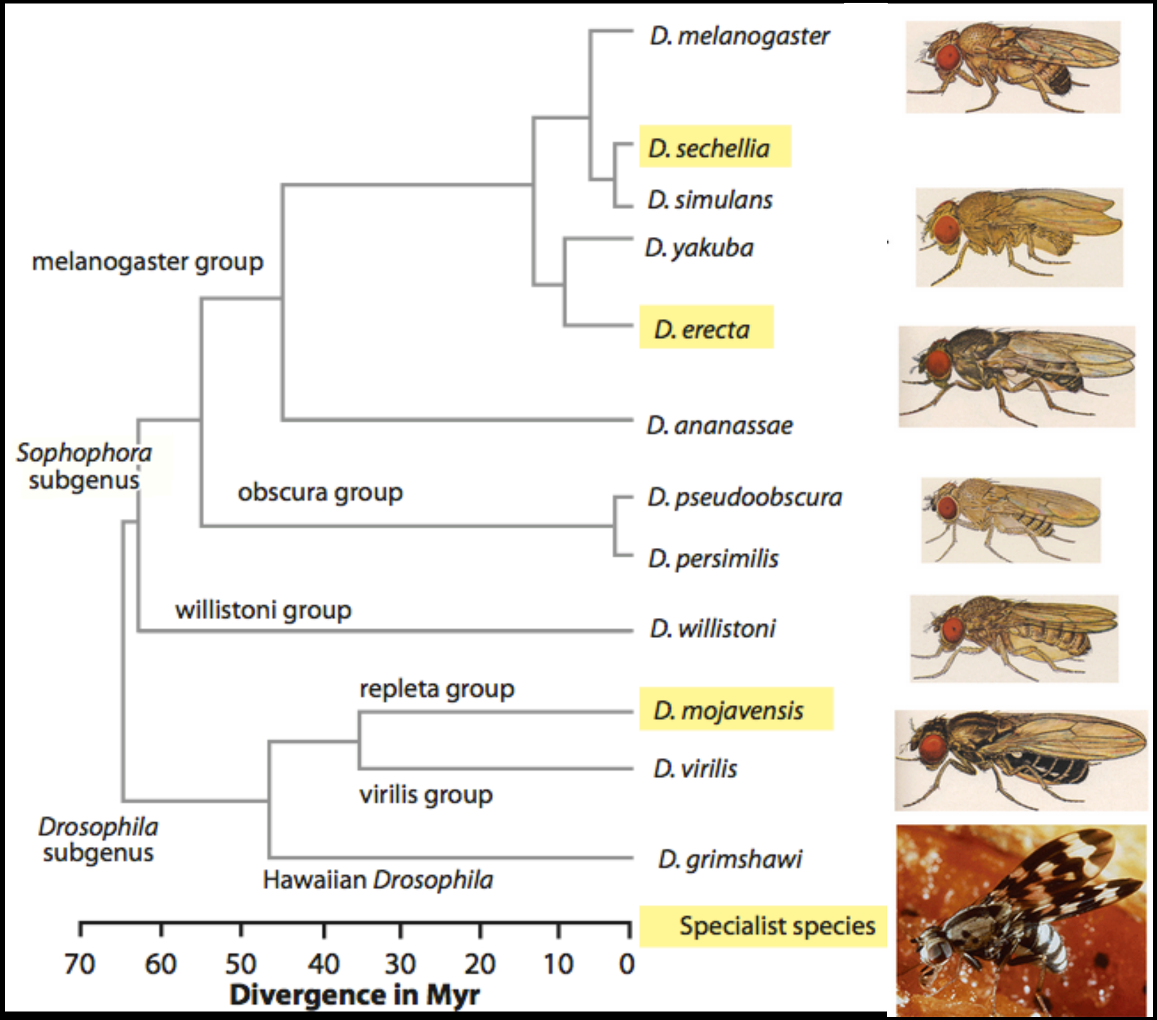
Priors directly influence posteriors



Millions of years

Obbard et al. 2012 Mol. Biol. Evol.

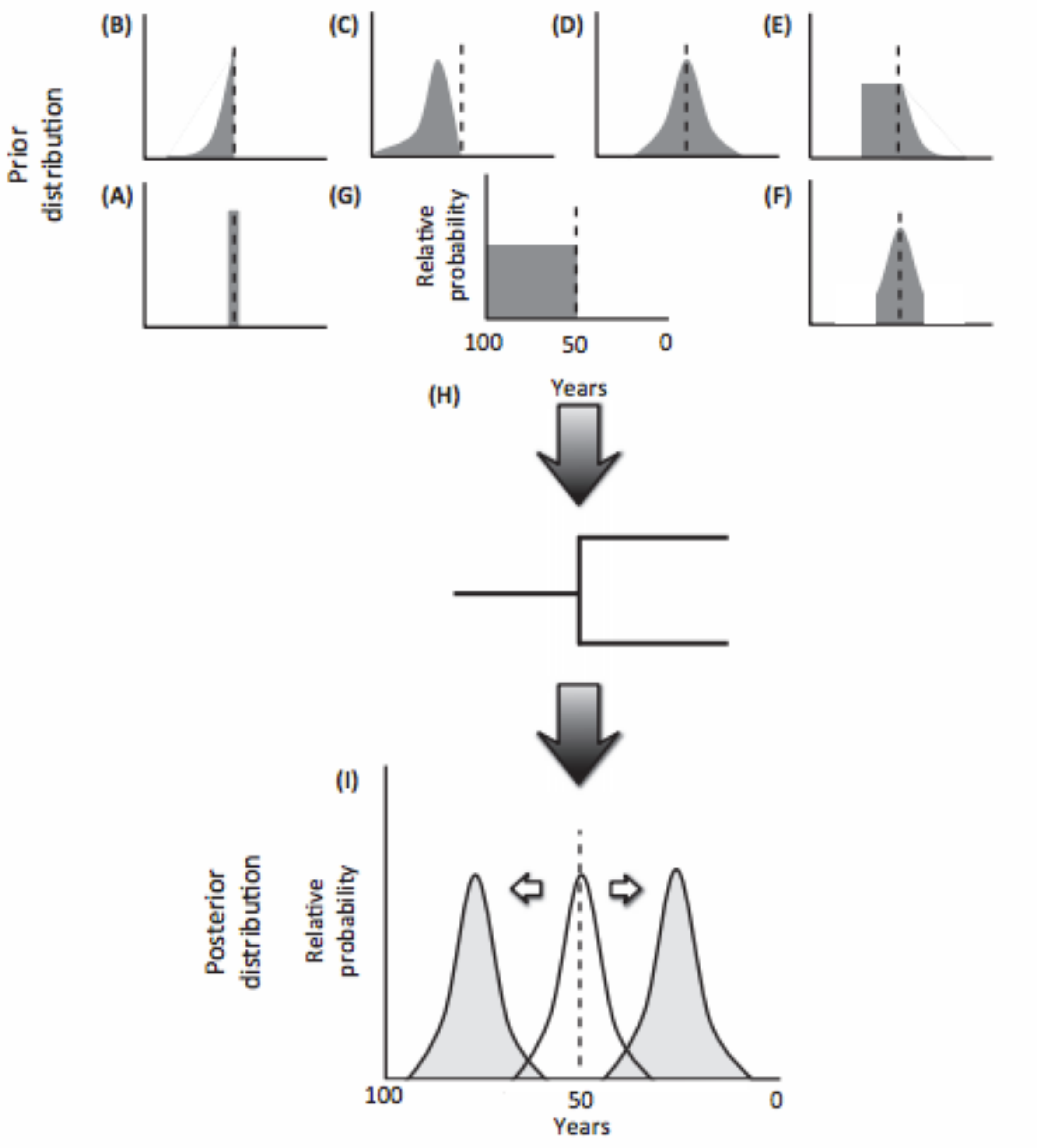
Thus, the age of this clade is fiction



Drosophila 12 Genomes Consortium 2007 Nature

Prior distributions matter

- Integrative science is challenging
- Discuss or collaborate with experts to evaluate your approach.



Wheat and Wahlberg 2013 Trends Ecology & Evolution

How do we gain dating confidence when we are in the dark?

- Fossils and DNA are likely to rarely agree
- How can we assess the temporal signal in the DNA in a robust manner?
  - Reducing prior biases and using lots of DNA data, while modeling likely violations of analysis models

Wheat and Wahlberg 2013
Trends Ecology & Evolution

Post-genomics challenge

“What we can measure is by definition uninteresting and what we are interested in is by definition unmeasureable”

- Lewontin 1974

“What we understand of the genome is by definition uninteresting and what we are interested in is by definition very damn difficult to sequence and assemble and annotate and analyze at genomic scale”

- Wheat 2015

For example:

- indels & inversions
- gene family dynamics
- evolutionary dynamics

What does a good P-value really tell you?

What type of selection?

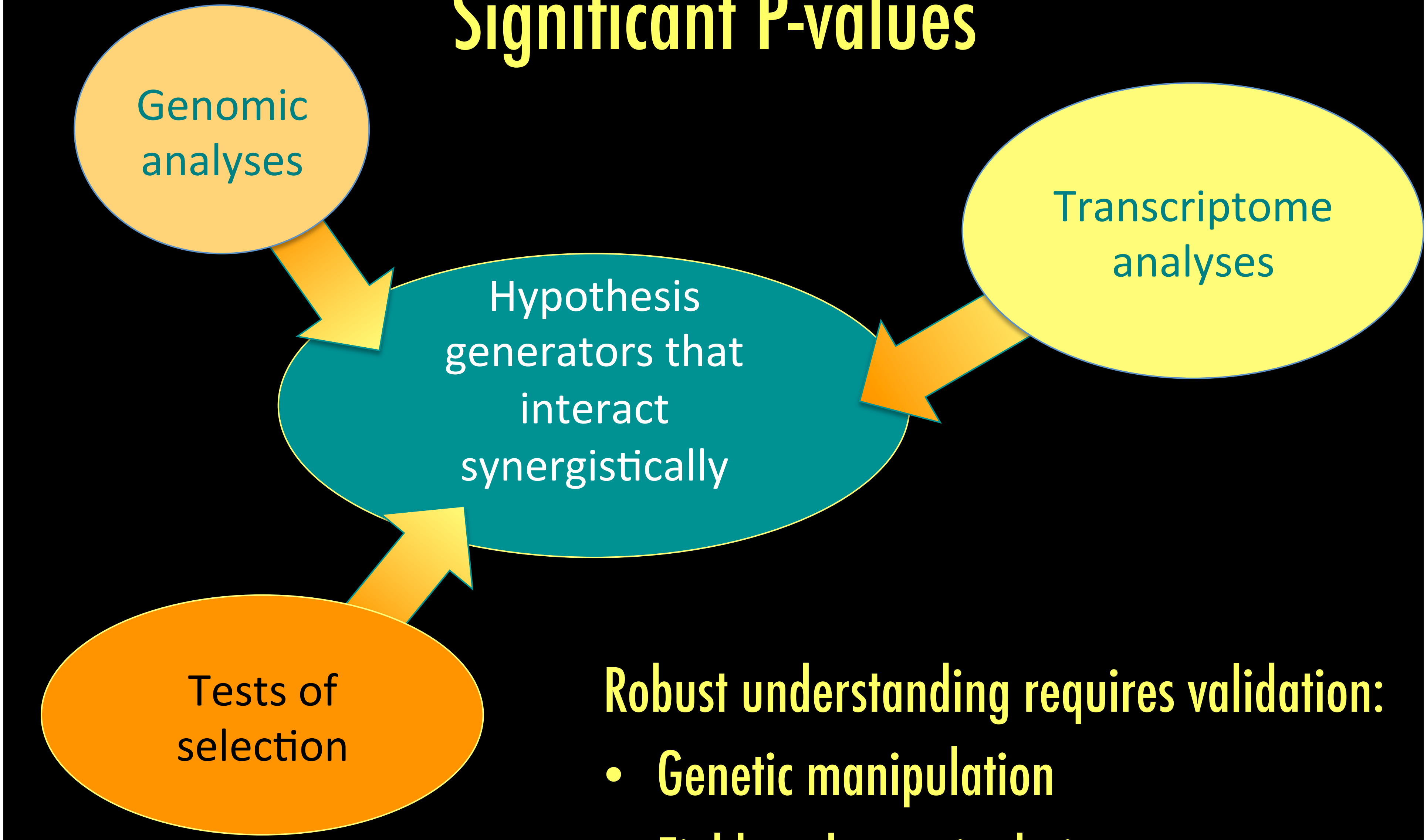
Are you chasing a good P-value?

Is method mismatched to mechanism?

When did selection happen?

What does a bad P-value really tell you?

Significant P-values



Genomic analyses

Transcriptome analyses

Hypothesis generators that interact synergistically

Tests of selection

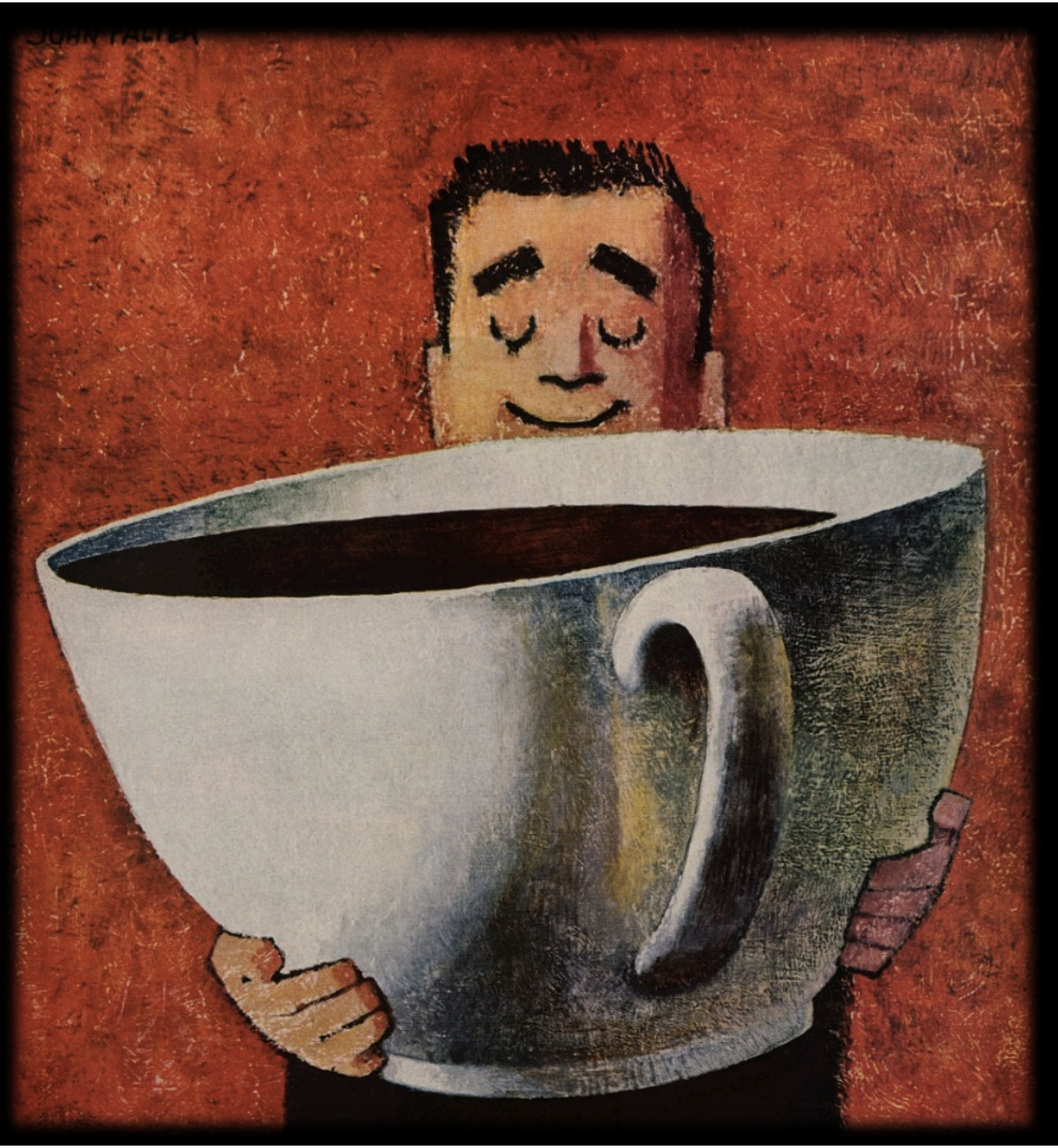
Robust understanding requires validation:

- Genetic manipulation
- Field study manipulations

Goal of this lecture

- Present a non-typical view of ecological genomics
  - So you have a more complete view of the field
- Make you uncomfortable
  - Provide a context for understanding your results
- Encourage you to rethink the reality presented by publication biases
  - Overcoming this bias is a continual challenge

sallyedelsteincollage.com



JOHN FALTER

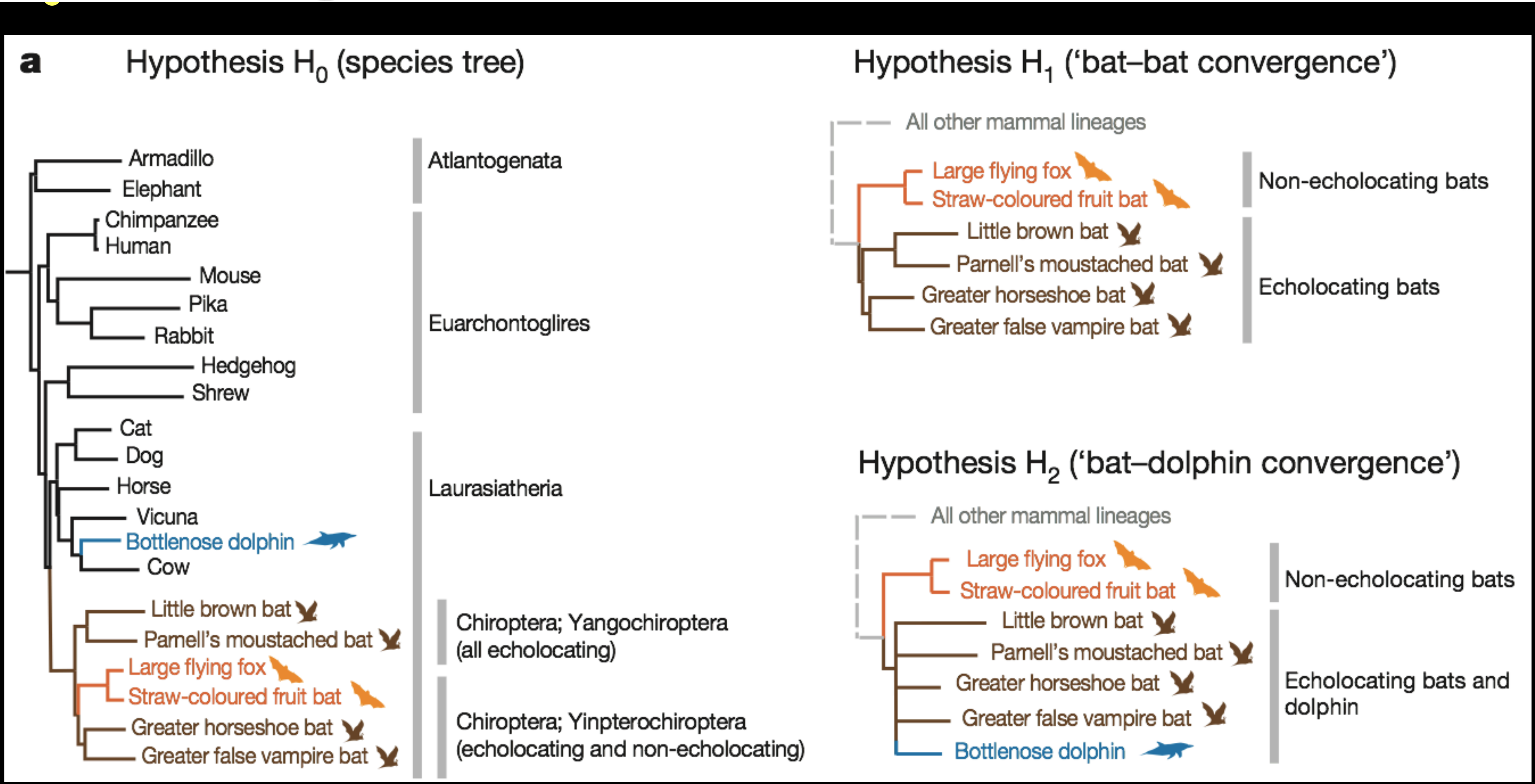
Outline

- Type I errors in studies
- How I try and avoid this
- RNA-Seq gone wrong ....

LETTER 228 | NATURE | VOL 502 | 10 OCTOBER 2013

doi:10.1038/nature12511

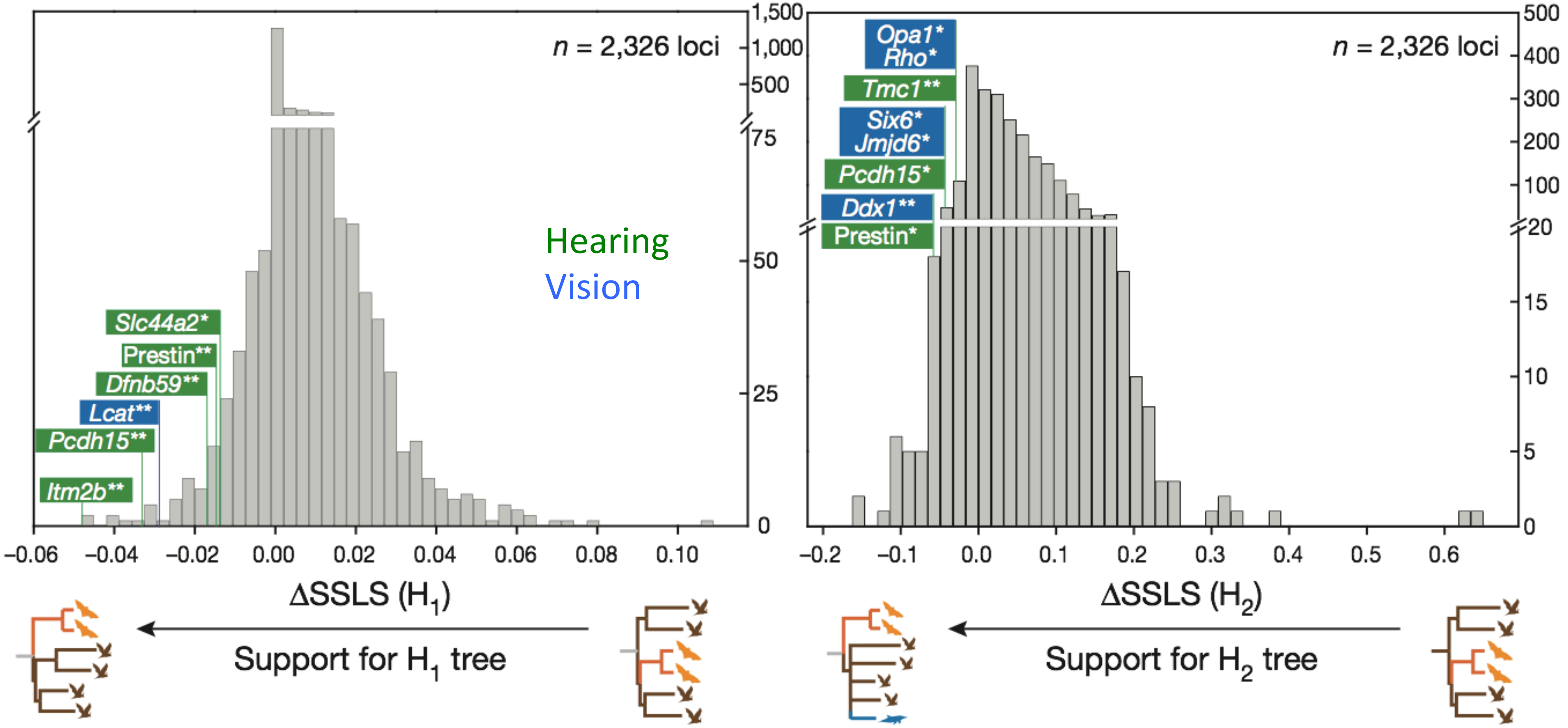
Genome-wide signatures of convergent evolution in echolocating mammals



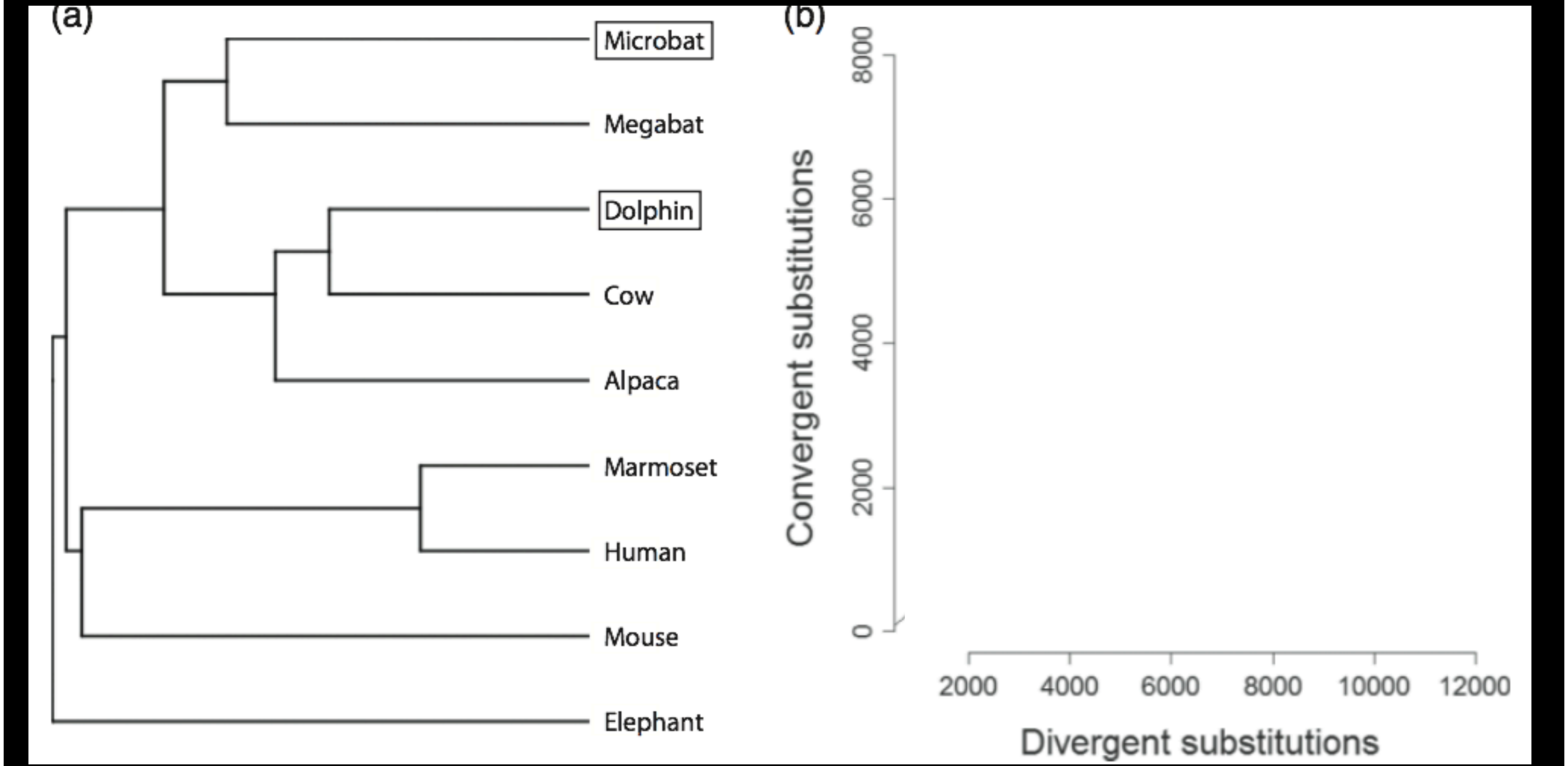
Parker et al. 2013. Nature 502:228–231.

"Strong and significant support for convergence among bats and the bottlenose dolphin was seen in numerous genes linked to hearing or deafness, consistent with an involvement in echolocation."

- 2326 orthologous genes
- site-wise log-likelihood support (SSLS)
  - Negative values support convergence H1,H2
    - 824 mean support for H1
    - 329 mean support for H2



Palmer failed to conduct orthogonal 'test' of findings or estimate proper 'null' expectation



Thomas and Hahn 2015. Mol Biol Evol 32:1232–1236.

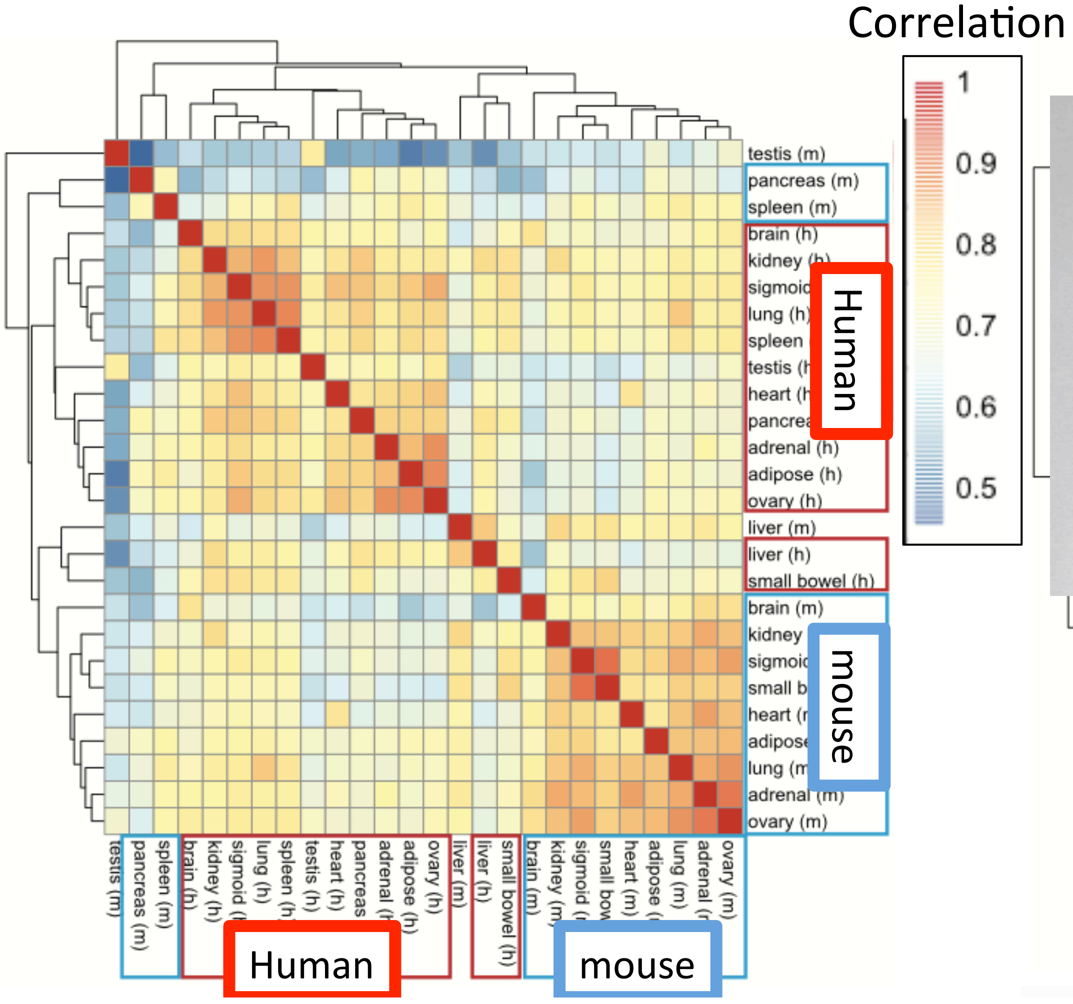
Synder mouse controversy

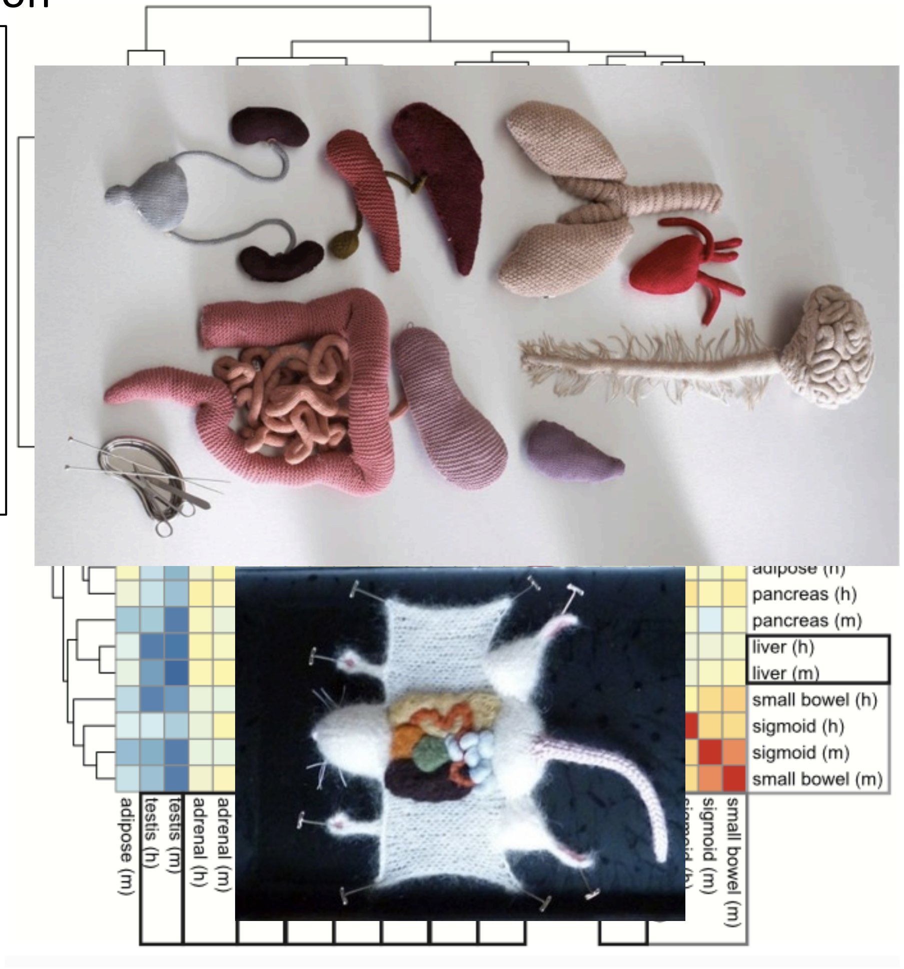
“the expression for many sets of genes was found to be more similar in different tissues within the same species than between species” Lin et al. 2014 PNAS

Human – Mouse TMRCA ~ 90 MYA

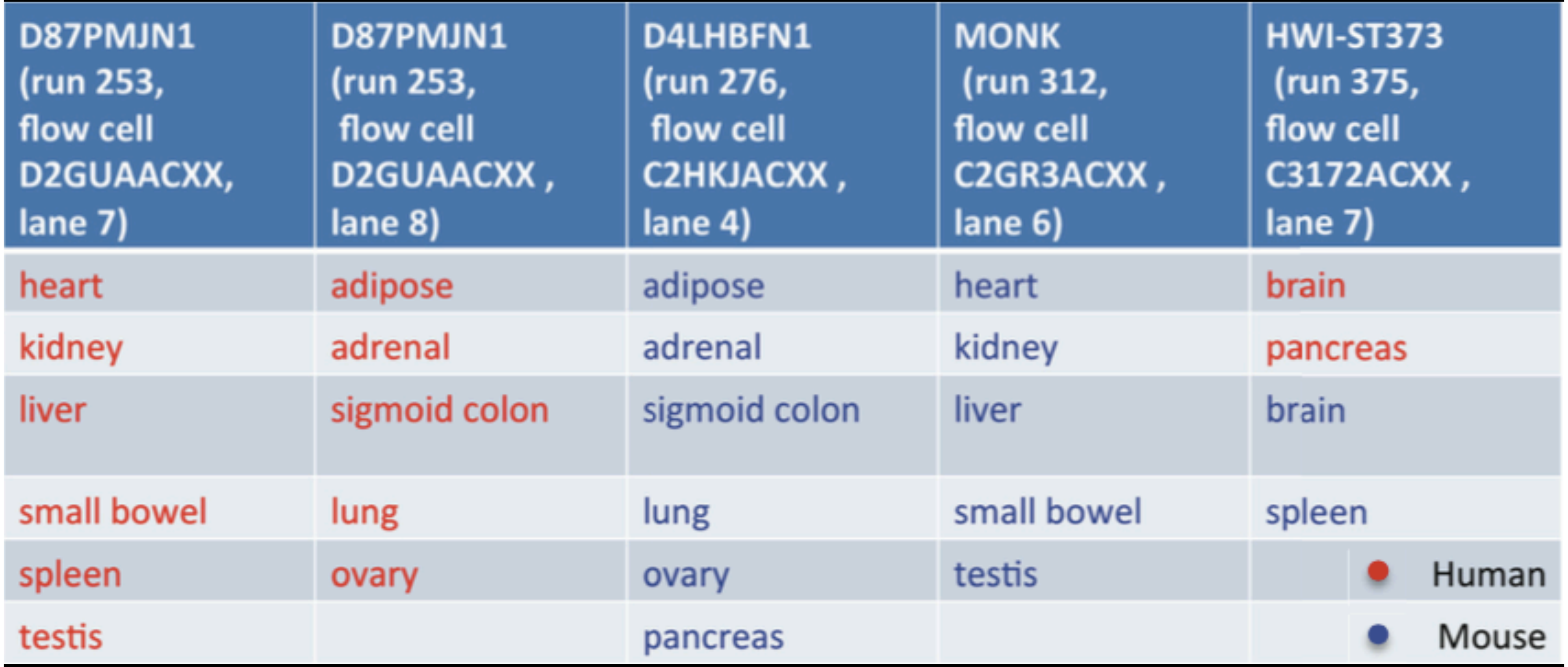
Brain – Kidney TMRCA?

“[after accounting] for the batch effect, ..human and mouse tend to cluster by tissue, not by species” Gilad and Mizrahi-Man 2015. F1000 Research





Batch effect: confounding sequencing grouping with biological grouping



D87PMJN1 (run 253, flow cell D2GUAACXX, lane 7)	D87PMJN1 (run 253, flow cell D2GUAACXX , lane 8)	D4LHBFN1 (run 276, flow cell C2HKJACXX , lane 4)	MONK (run 312, flow cell C2GR3ACXX , lane 6)	HWI-ST373 (run 375, flow cell C3172ACXX , lane 7)
heart	adipose	adipose	heart	brain
kidney	adrenal	adrenal	kidney	pancreas
liver	sigmoid colon	sigmoid colon	liver	brain
small bowel	lung	lung	small bowel	spleen
spleen	ovary	ovary	testis	🔴 Human
testis		pancreas		🔵 Mouse

Solution = Keep technical effects orthogonal to biological

- Mouse & Human in same lane, same tissues in same lane
    - Will your Core facility know to do this for you?

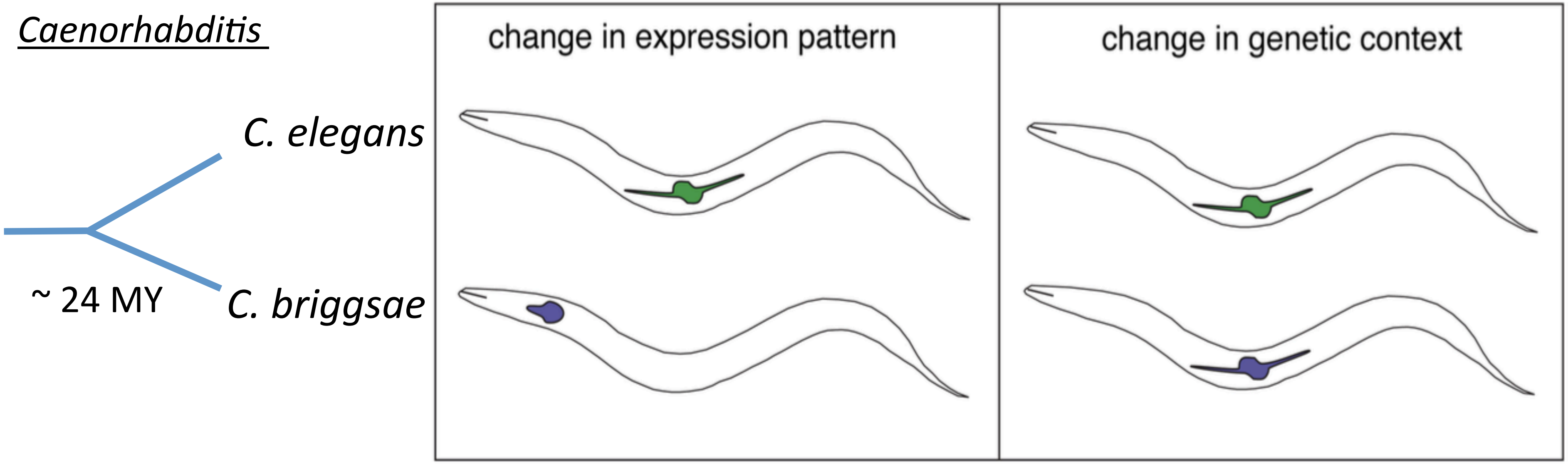
Evolutionary Inference = House of Cards?

The quality of our evolutionary inference

Is proportional to assumptions of orthology

Orthologous genes … can their phenotypic effects drift over evolutionary time?

- RNAi phenotypes assessed for1,300 genes in two nematodes
  - TMRA ~24 MYA
  - 7% had divergent phenotypic effects (in lab, etc.)
  - Likely higher in nature



Verster et al. 2014. PLoS Genet

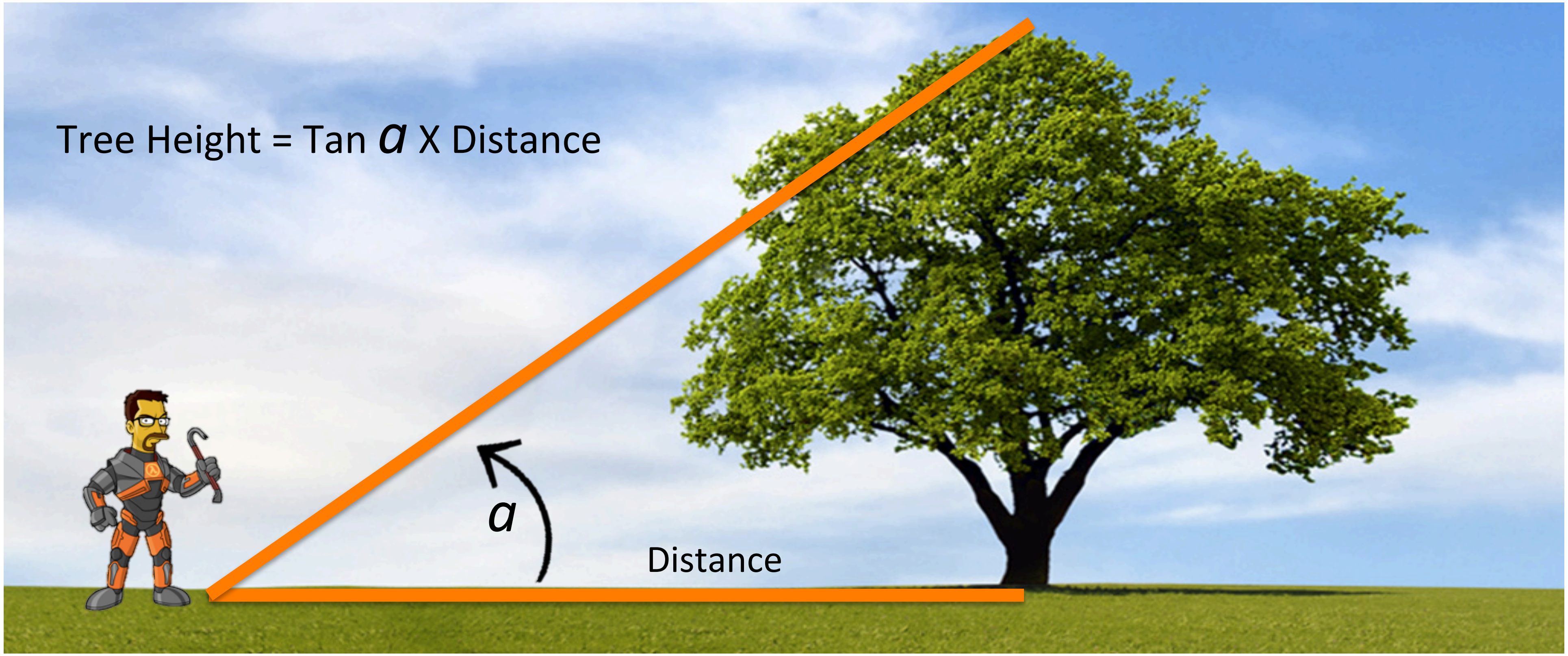
If I’m talking about all these errors …

How do I work to minimize making type I errors?

- I try and avoid over stating my work
- I ‘triangulate’

Triangulation for building evidence

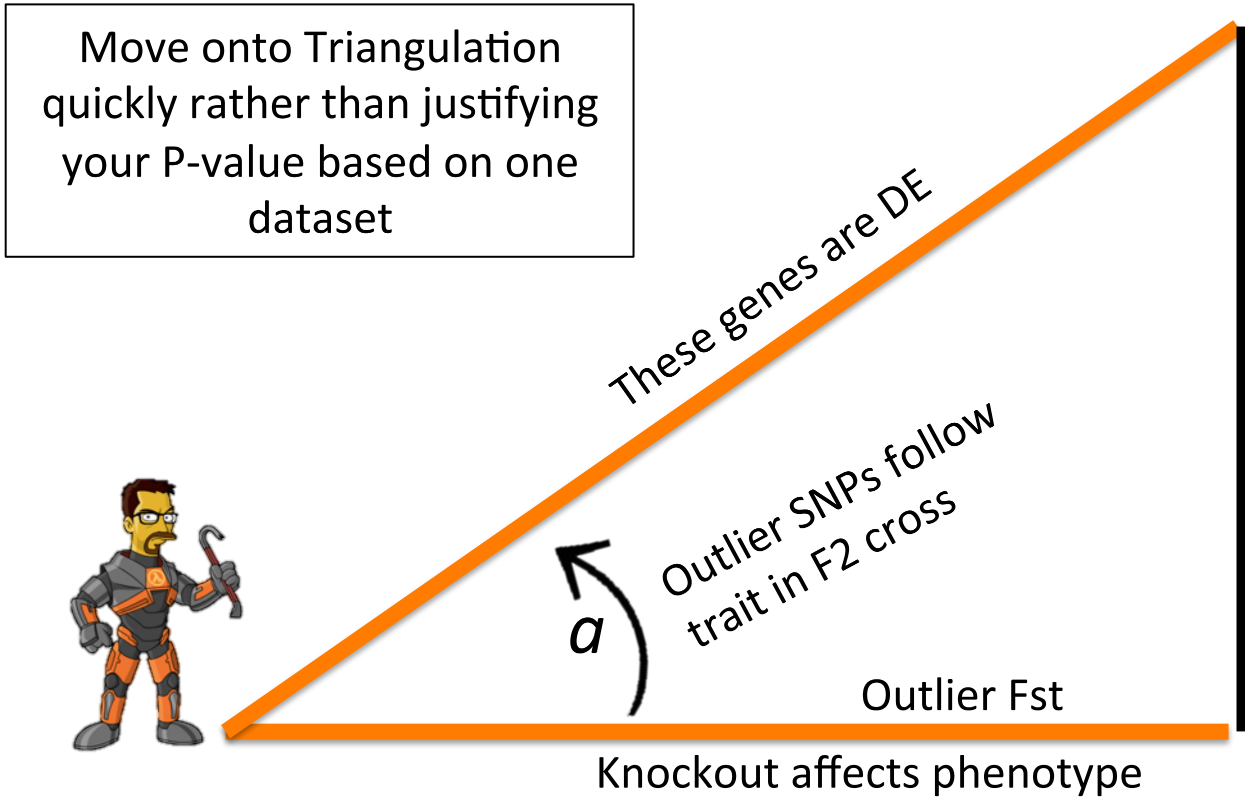
- Use more than one independent set of evidence
  - Derived from independent biological replicates
- Challenge is maintaining genomic scale
  - Genome wide SNP scan for outliers, QTL mapping, RNA-Seq, knockouts, manipulations, etc.



Triangulation for building evidence

- Use more than one independent set of evidence
  - Derived from independent biological replicates
- Challenge is maintaining genomic scale
  - Genome wide SNP scan for outliers, QTL mapping, RNA-Seq, knockouts, manipulations, etc.

Move onto Triangulation quickly rather than justifying your P-value based on one dataset


These genes are DE
Outlier SNPs follow trait in F2 cross
a
Outlier Fst
Knockout affects phenotype

Is it an adaptation?

What was ancestral state?

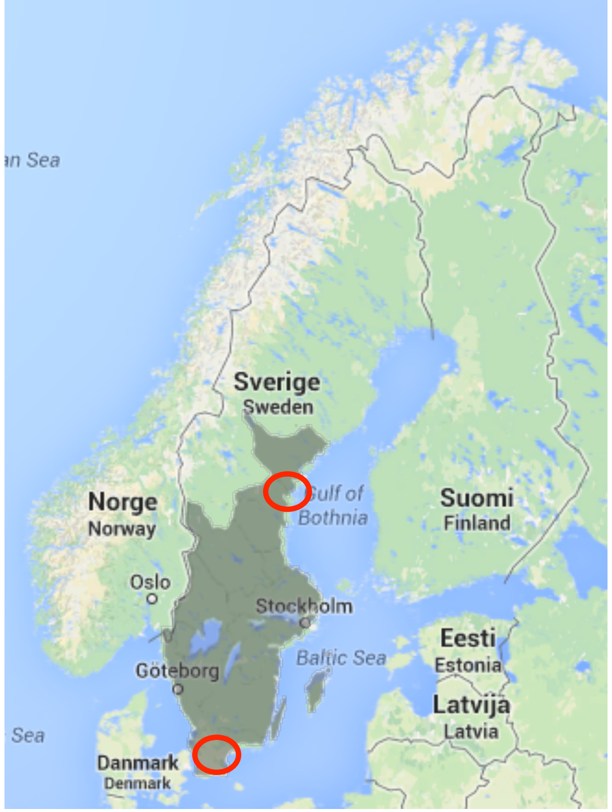
Is there any clinal variation?

Phenotype respond to chemical manipulation?

Response to selection experiment?

Genomic signal of Diapause adaptation

Speckled Wood
(*Pararge aegeria*)

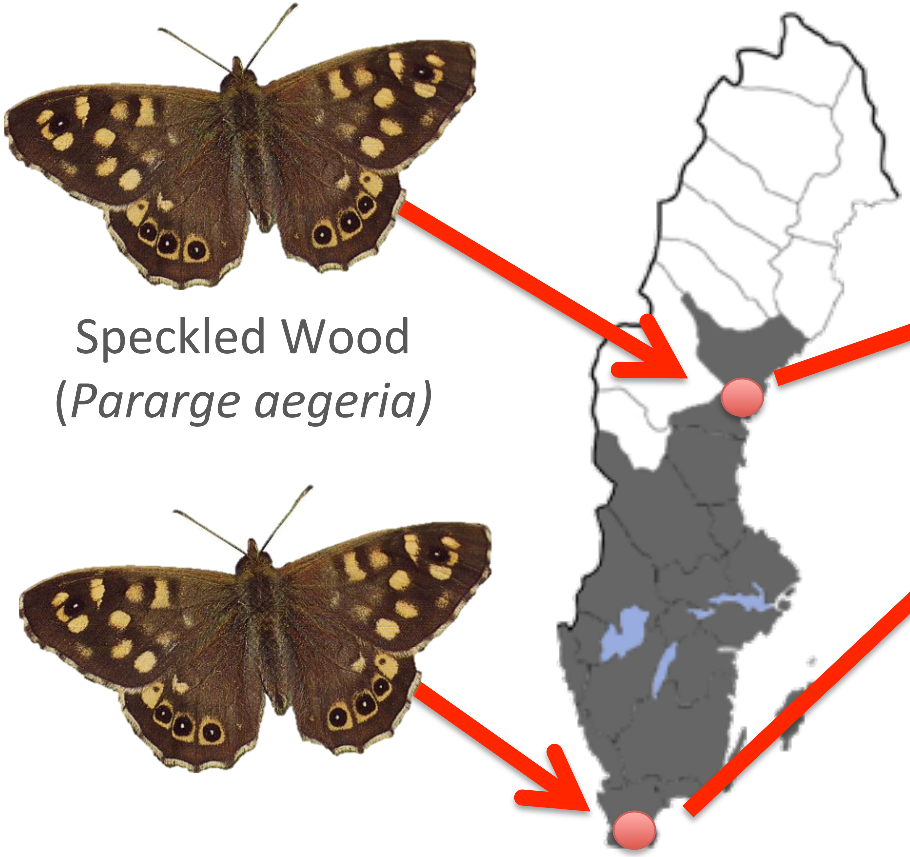


15 months ago, only :

- mtDNA and microsat loci
- Extensive ecological studies > 10 years

Stockholm University

Peter Pruisscher

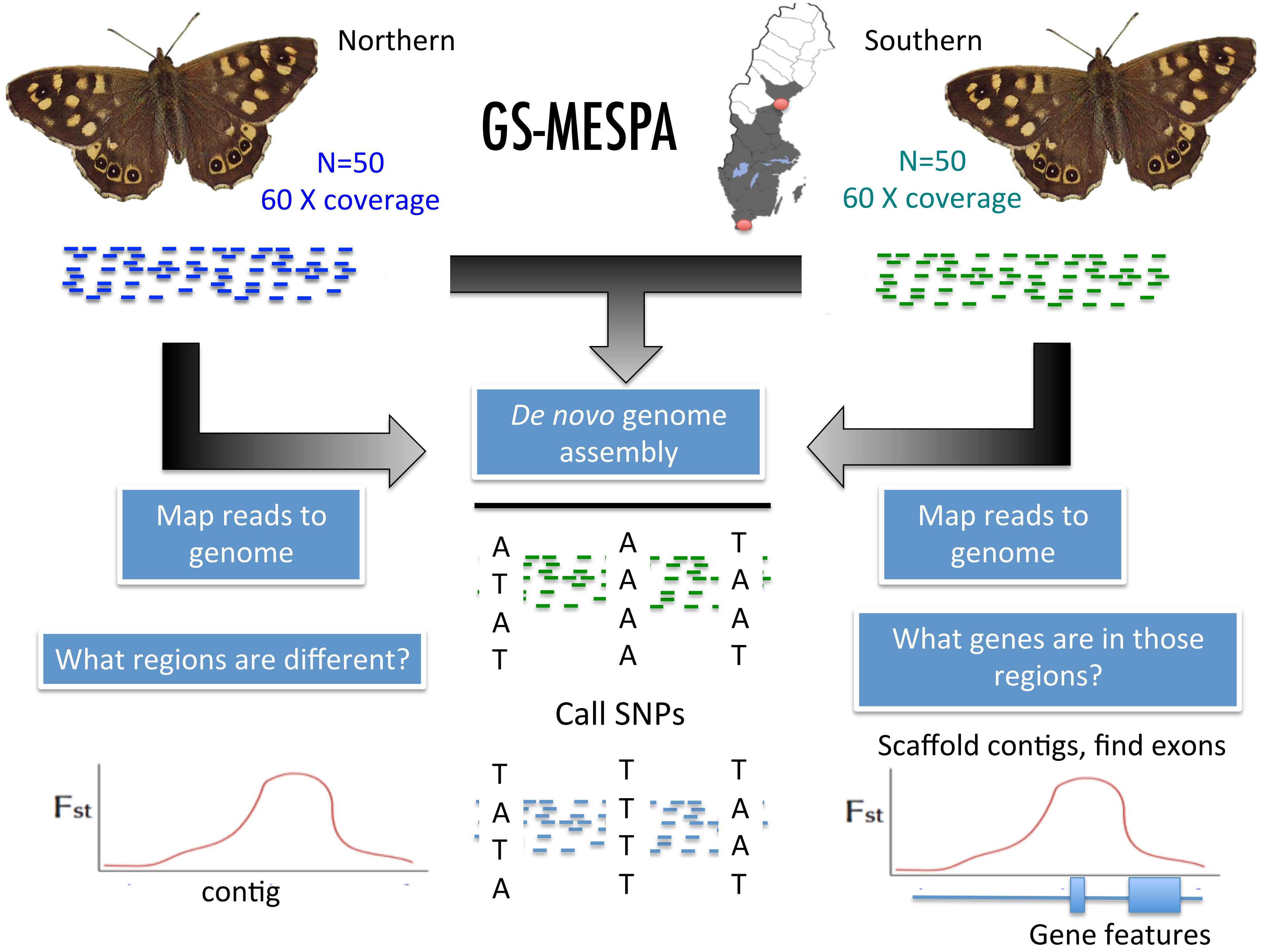


Speckled Wood
(*Pararge aegeria*)

Generations per year	% in diapause at 18 hours light
1	100 %
2	0 %

What is the genetic basis of adaptation to day length?

GS-MESPA



Northern

N=50
60 X coverage

Southern

N=50
60 X coverage

De novo genome assembly

Map reads to genome

Map reads to genome

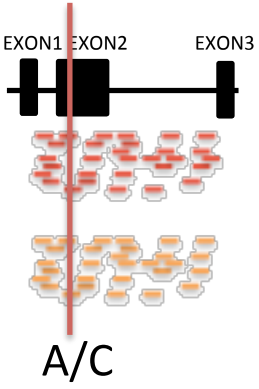
Call SNPs

What regions are different?

What genes are in those regions?

Scaffold contigs, find exons

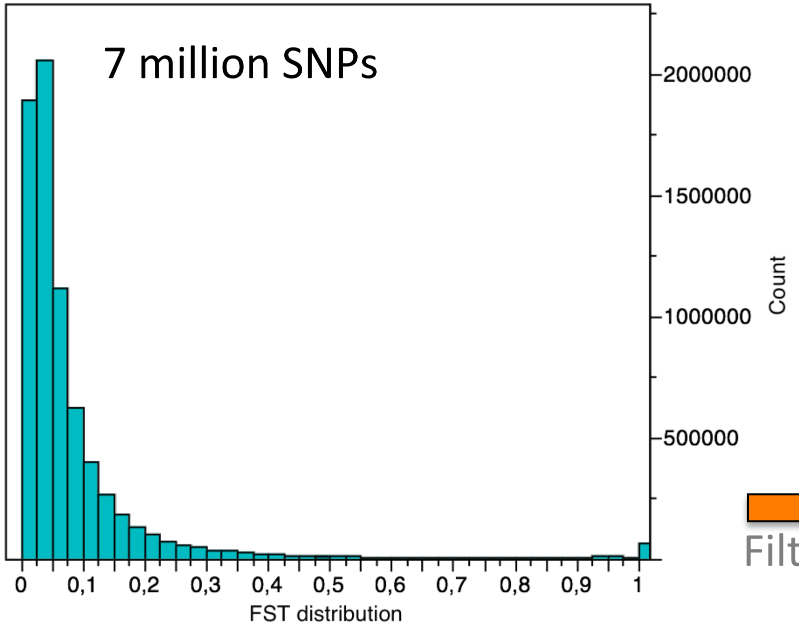
Fst outlier analysis for candidates



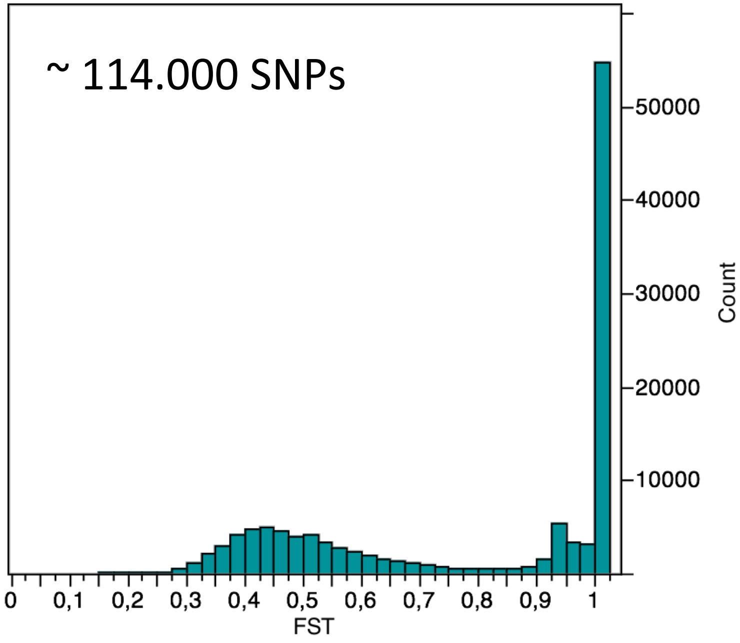
11,000 gene models & ~7 million SNPs

Quality Filtering

~ 114,000 SNPs of which 68,000 SNPs: FST >0.9

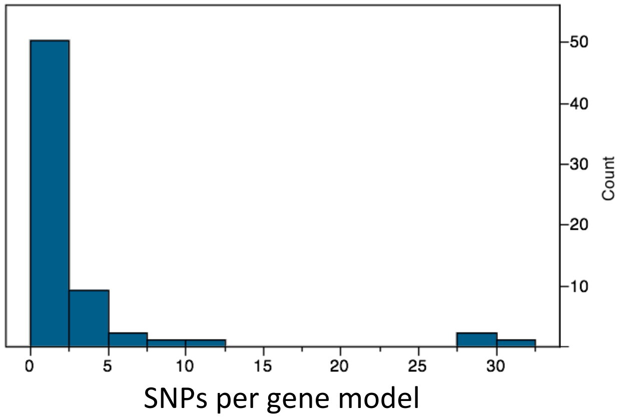


Filtering



Fixed variation in genes

1. Intergenic regions contain+/- 67,604 Fixed SNPs



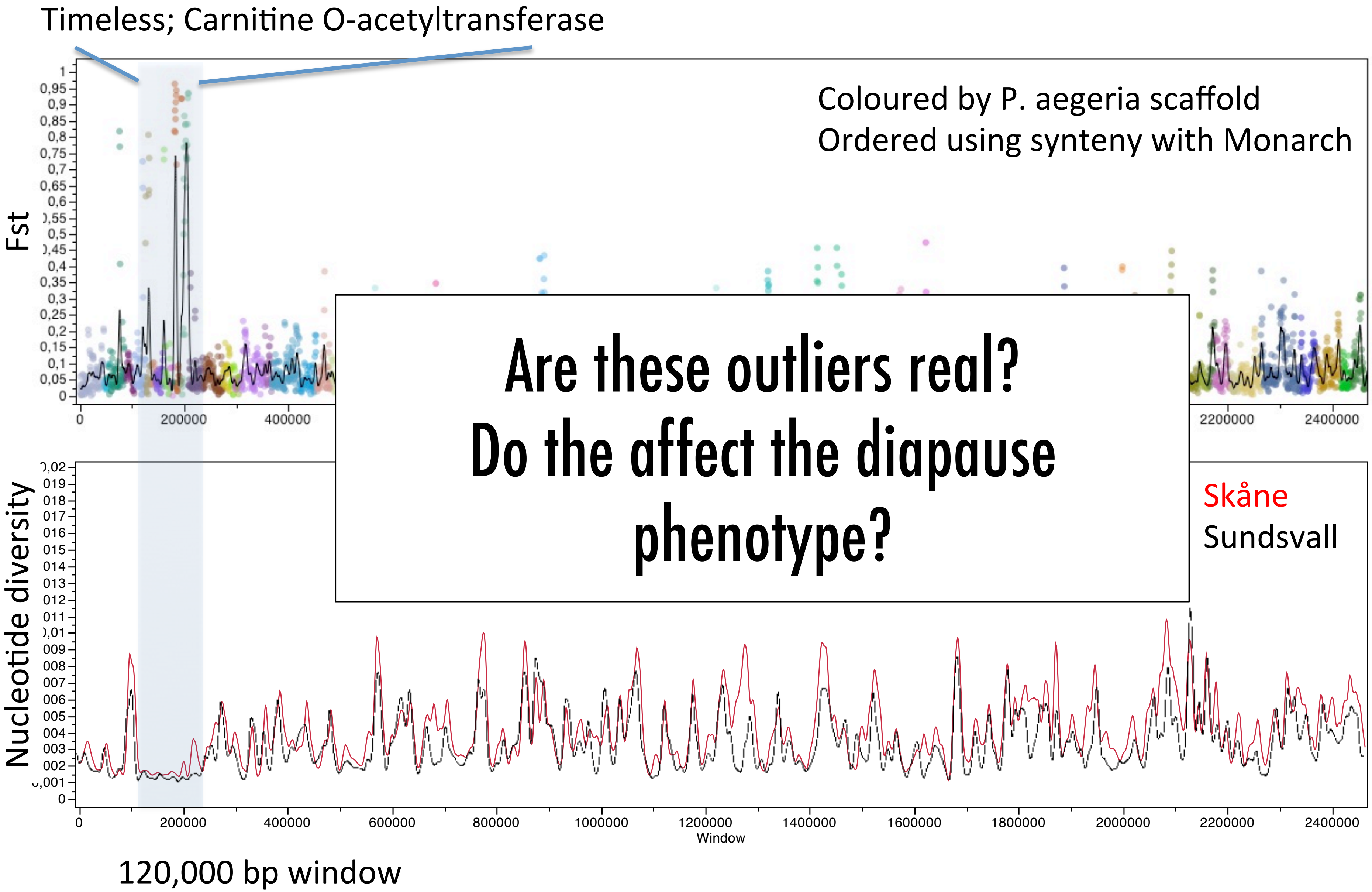
2. 67 gene models contain 209 fixed SNPs

3. Filter for SNPs in exons and introns

UniRef90_proteinnames	exon	gene	intergenic	Total	D.plex scaffold	Bmori_chr
Timeless	**2**	0	0	2	DPSC300014	chr4
Carnitine O-acetyltransferase	**3**	25	1	29	DPSC300014	chr4
Trypsin-like protein	**2**	14	14	30	DPSC300041	chr5
Vasa-like protein	**1**	2	0	3	DPSC300379	chr19
Period	**2**	2	1	5	DPSC30005	chr1

Is there a foot-print of selection around these SNPs?

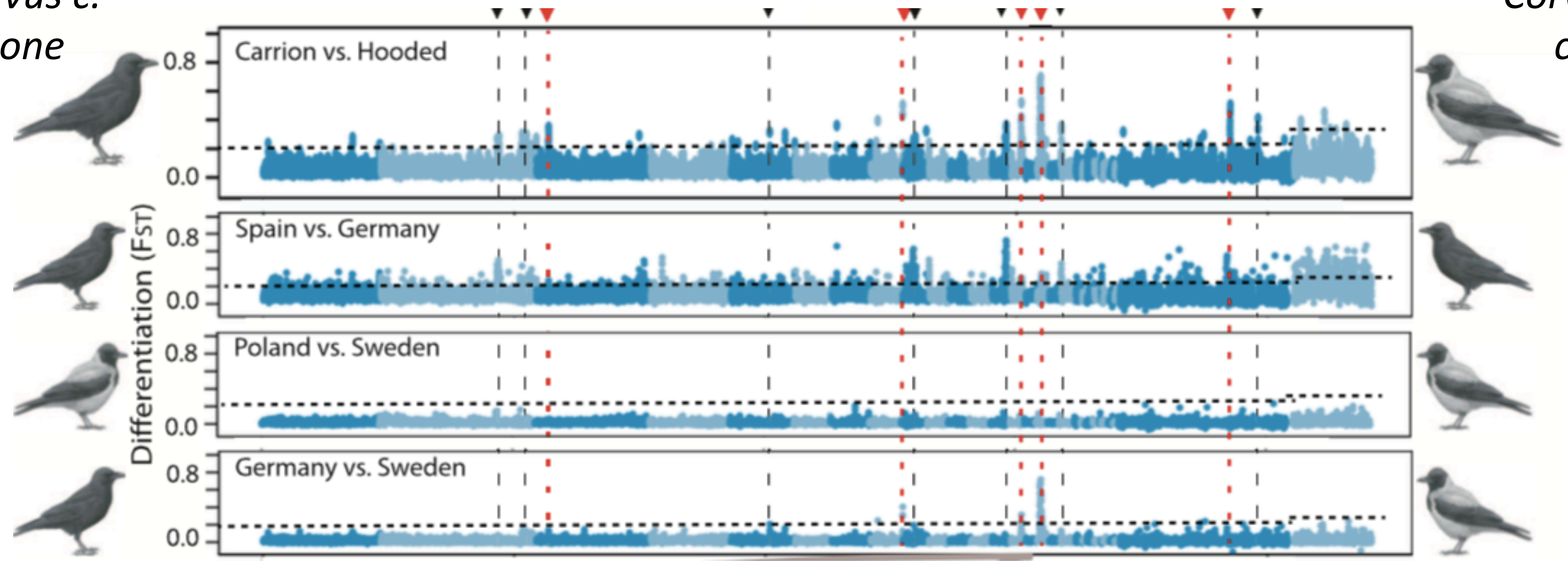
Region around timeless



Are these outliers real?
Do the affect the diapause phenotype?

Corvus c.
corone

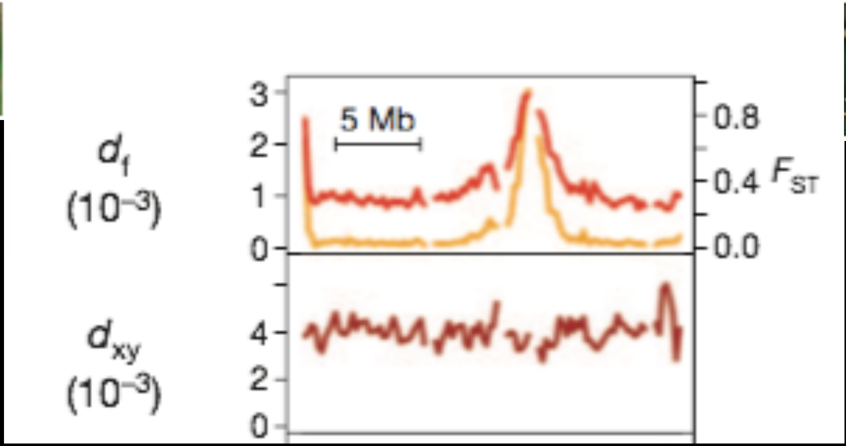
Corvus c.
cornix



[redacted]

Islands of speciation or background selection?

D_{xy}:
An absolute measure of differentiation, increase due to mutations

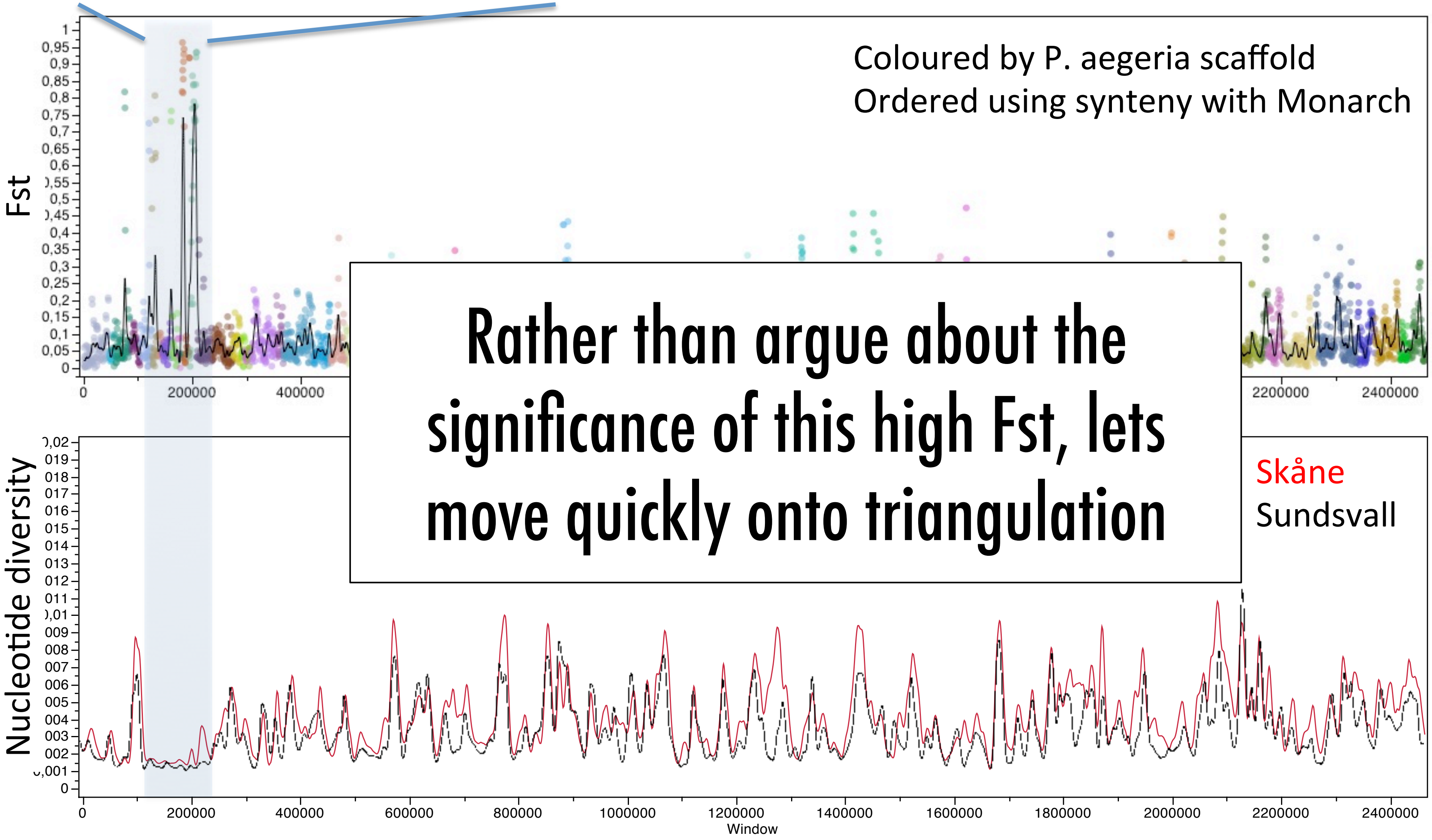


Fst:
A relative measure of differentiation, increases due to freq. change

The absence of high Dxy in regions of high Fst suggest a role of background selection driving these patterns rather than genomic ‘islands’ driving speciation.

Cruickshank and Hahn. 2014. Molecular Ecology.

Region around timeless



Timeless; Carnitine O-acetyltransferase

Coloured by P. aegeria scaffold
Ordered using synteny with Monarch

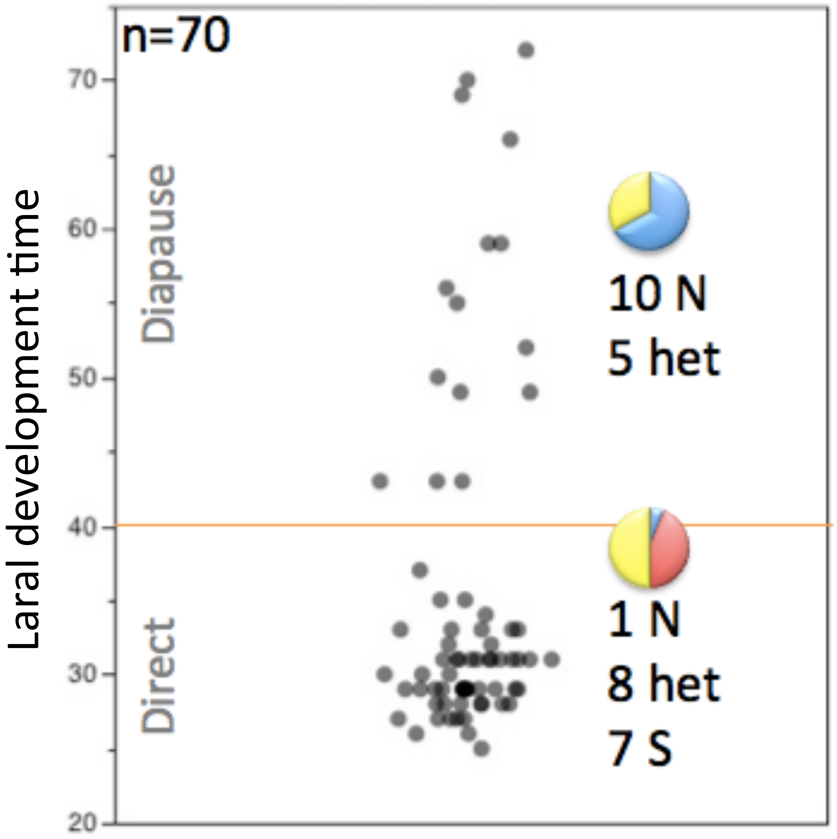
Rather than argue about the significance of this high Fst, lets move quickly onto triangulation

Skåne
Sundsvall

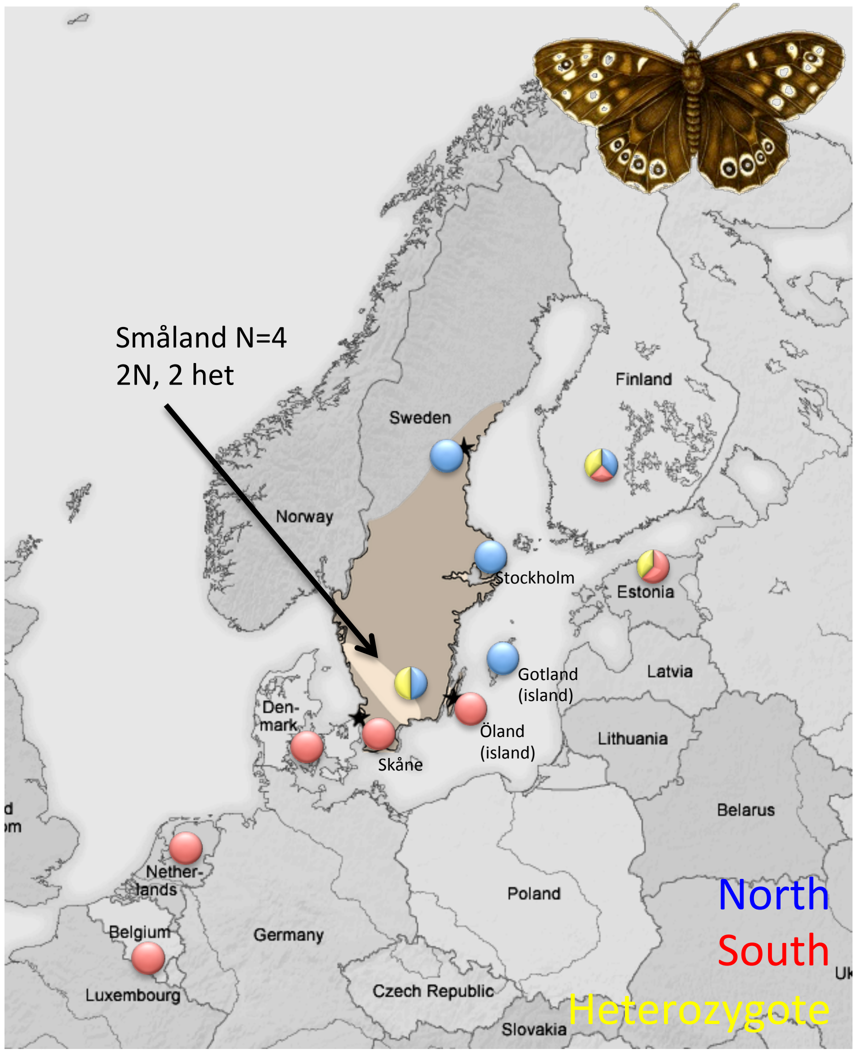
120,000 bp window

Triangulating Timeless

SNP genotyping in F2 cross



Clinal anlaysis


Småland N=4
2N, 2 het

1001 ways for your pipeline to break

An overview of genomic pipeline challenges

Christopher West Wheat

STOCKHOLMS UNIVERSITET

Informatics and Biology

- We need to make sure we put the ‘bio’ into the bioinformatics
  - Do results pass 1st principals tests
  - Always double check data from your core facility or service company
  - Use independent analyses as ‘controls’ on accuracy
    - What are your + and – controls?
    - Do independent methods converge?

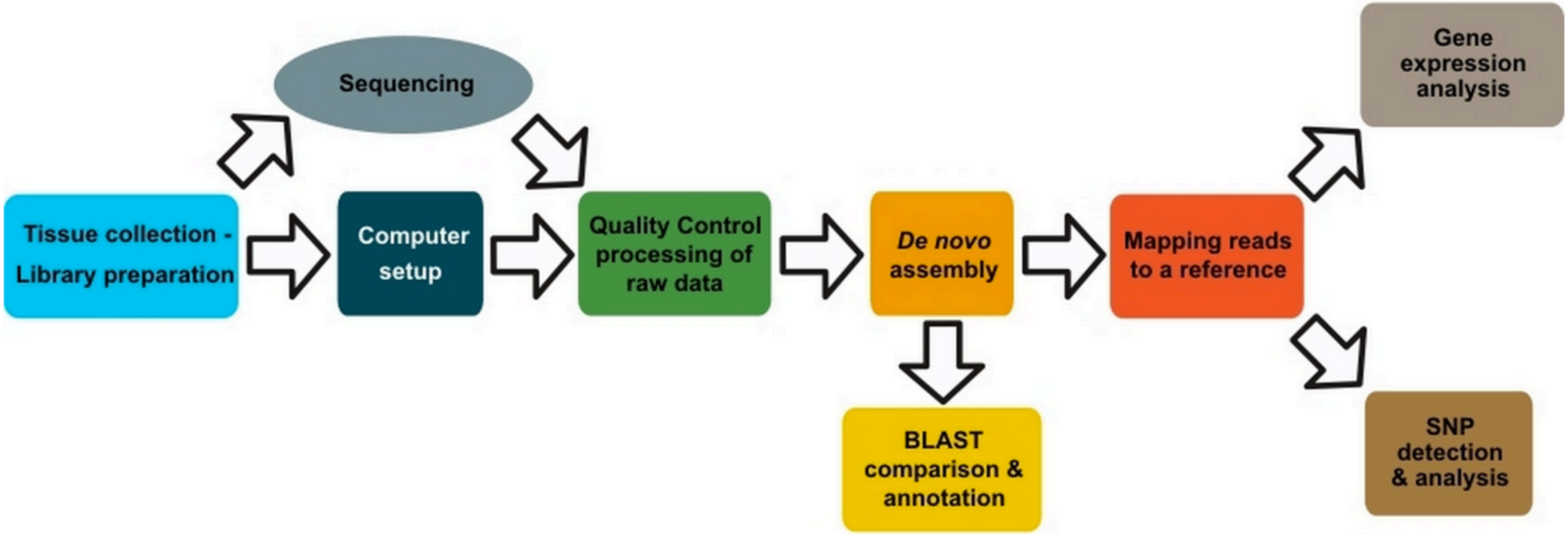
- Need to re-assess our common metrics for potential bias in the genomic age
  - Bootstraps on genomic scale data
  - P-values, outlier analyses, demographic null models

Outline

- Transcriptome analyses in non-model species
  - –Walk through pipeline and highlight issues of concern
  - –What is validation?

- Insights from candidate genes
  - –Can Second Gen methods get us there?

Pipeline Overview



Tissue collection - Library preparation

Sequencing

Computer setup

Quality Control processing of raw data

De novo assembly

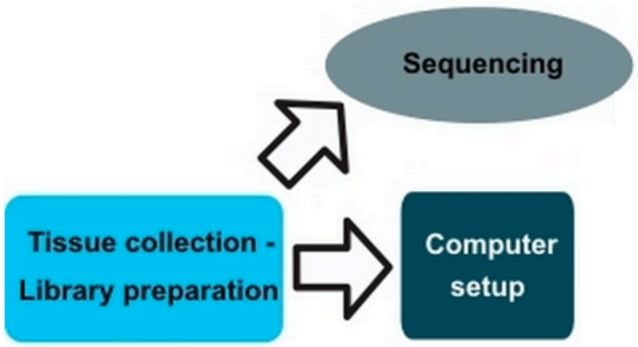
BLAST comparison & annotation

Mapping reads to a reference

Gene expression analysis

SNP detection & analysis

Pipeline Overview



How can I study my data using open source?

How much RAM do I need?

Are 16 cores enough?

Can I use my laptop?

What software & how do I get it?

Why Linux?

How much HD space is needed?

Computer Infrastructure

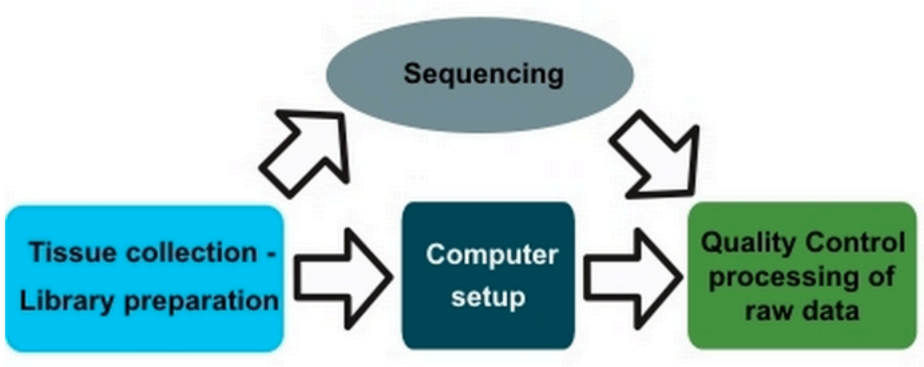
RNAseq dataset:

4 conditions X 2 tissues X 3 families X 3 replicates = 72 X $10^{\wedge 6}$ reads

	File Sizes (Gb)	CPUs	RAM (Gb)	Time
Raw files *gz	(1.5			~3 hours / file
Raw files expanded				
TA assembly				weeks
Mapping (BAM)				ours / file
Annotation	10			~6 – 12 days
Analysis	< 20 Mb	4	4	~< 1 hour
Visualization	BAM files	≥ 4	≥ 8	

Get ready for your data by downloading similar sized dataset from the Short Read Archive. Do not wait till it arrives

Pipeline Overview

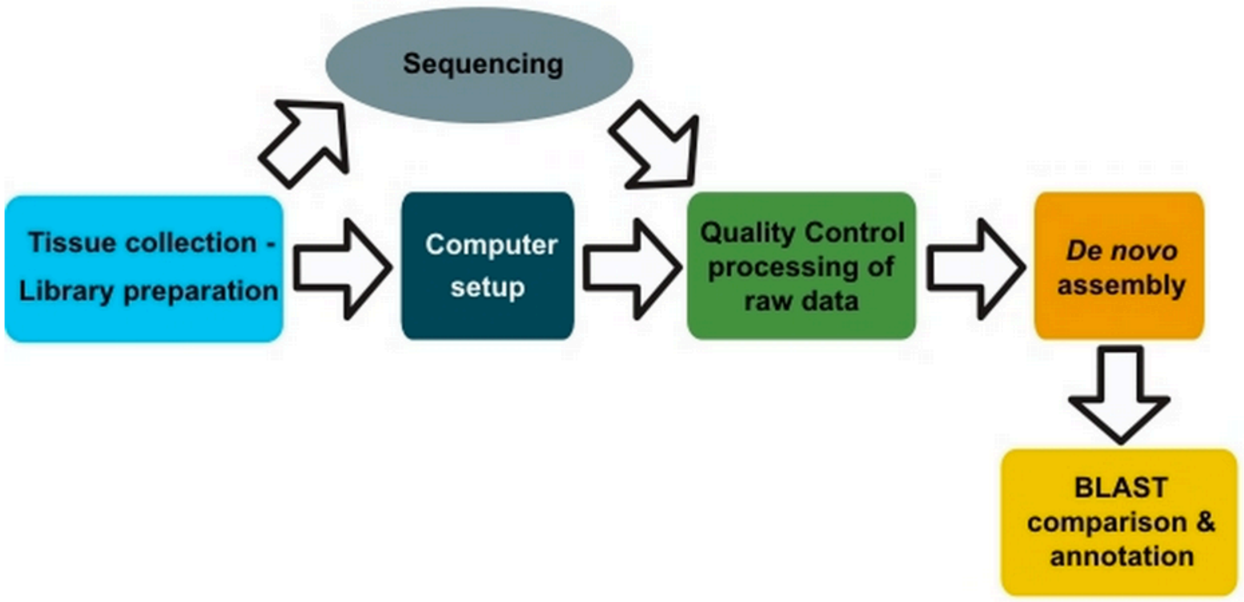

Sequencing
Tissue collection - Library preparation
Computer setup
Quality Control processing of raw data

Core facilities and non-model species

Statements from core facilities that are not true:

- Here is your data
- You can't do RNA-Seq without a genome
- We'll have your data back in < 1 month

Pipeline Overview


Sequencing
Tissue collection - Library preparation
Computer setup
Quality Control processing of raw data
De novo assembly
BLAST comparison & annotation

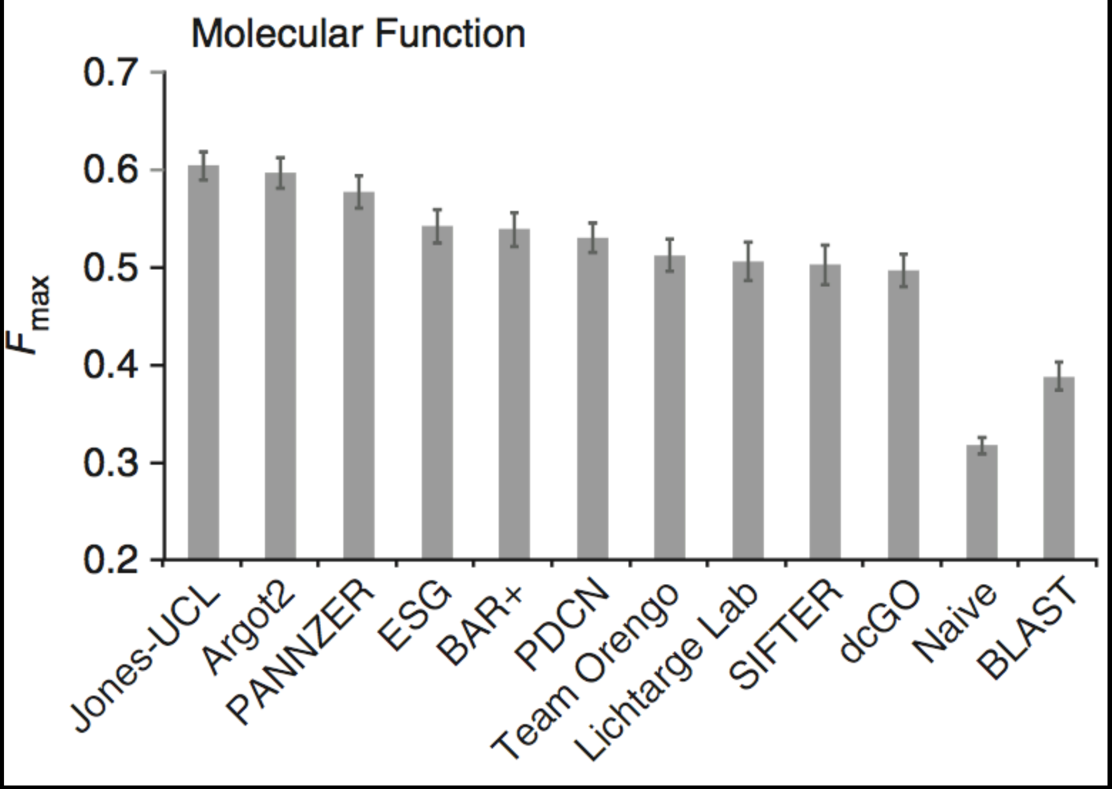
Gene Ontology: order in the chaos

- Addresses the need for consistent descriptions of gene products in different databases in a species-independent manner

- GO project has developed three structured controlled vocabularies (ontologies) that describe gene products in terms of their associated
  - biological processes
  - cellular components
  - molecular functions

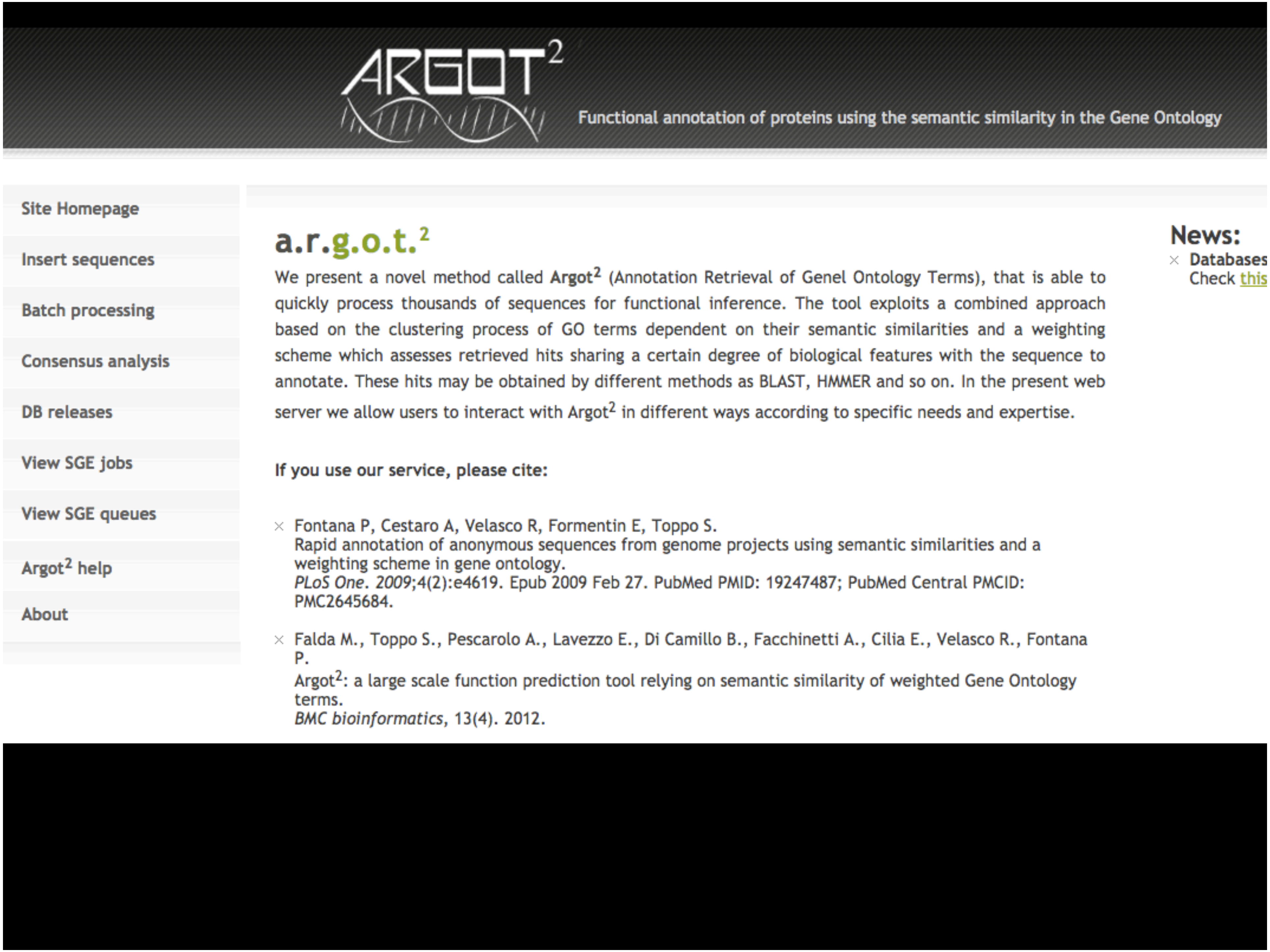
http://www.geneontology.org/

Comparisons among annotation tools



Radivojac et al.: **A large-scale evaluation of computational protein function prediction**. *Nat Meth* 2013, **10**:221–227.

Falda et al. **Argot2: a large scale function prediction tool relying on semantic similarity of weighted Gene Ontology terms**. *BMC Bioinformatics* 2012, **13**:S14.



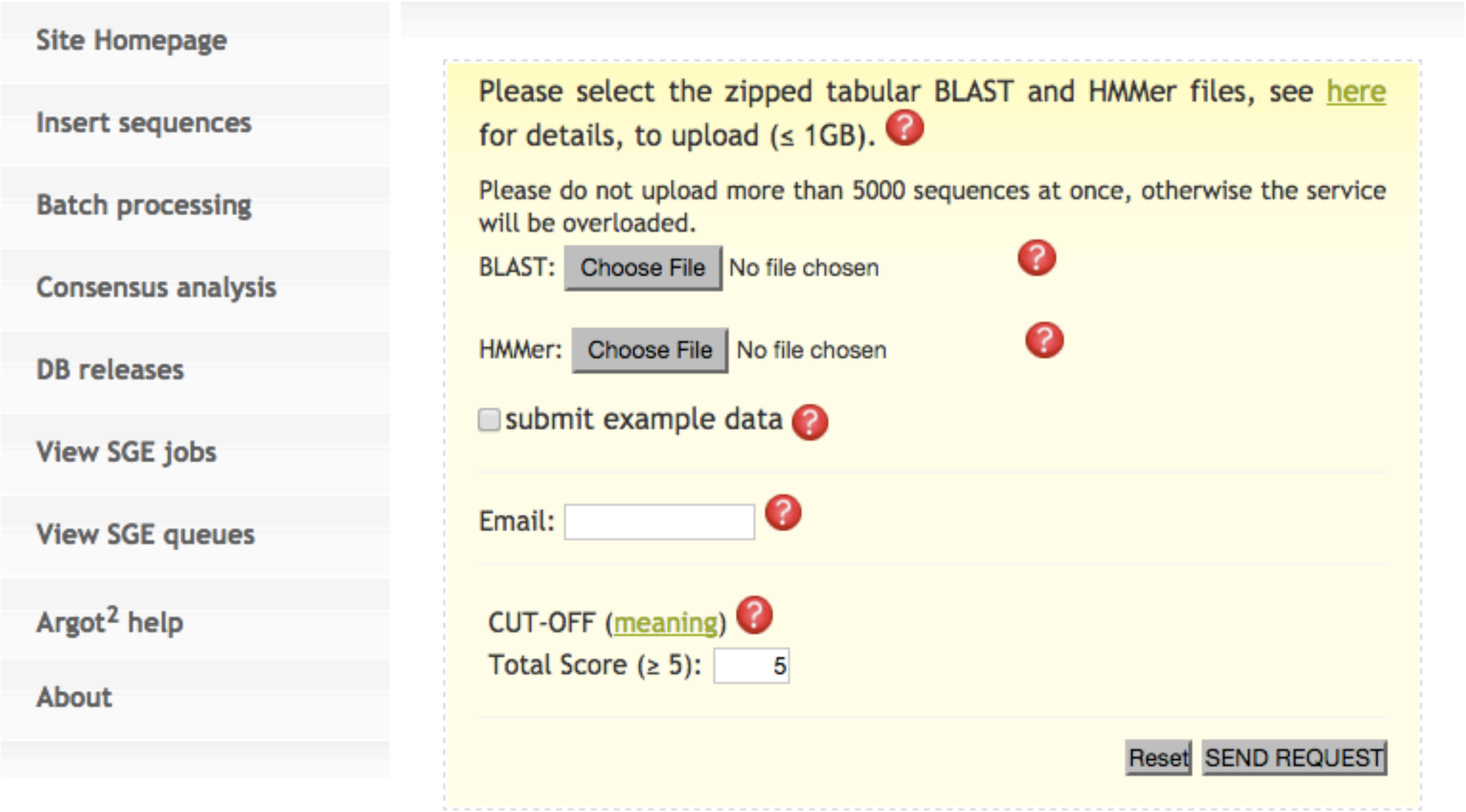
a.r.g.o.t.^{2}

We present a novel method called **Argot**^{2} (Annotation Retrieval of Genel Ontology Terms), that is able to quickly process thousands of sequences for functional inference. The tool exploits a combined approach based on the clustering process of GO terms dependent on their semantic similarities and a weighting scheme which assesses retrieved hits sharing a certain degree of biological features with the sequence to annotate. These hits may be obtained by different methods as BLAST, HMMER and so on. In the present web server we allow users to interact with Argot^2 in different ways according to specific needs and expertise.

If you use our service, please cite:

- Fontana P, Cestaro A, Velasco R, Formentin E, Toppo S.
  Rapid annotation of anonymous sequences from genome projects using semantic similarities and a weighting scheme in gene ontology.
  PLoS One. 2009;4(2):e4619. Epub 2009 Feb 27. PubMed PMID: 19247487; PubMed Central PMCID: PMC2645684.
- Falda M., Toppo S., Pescarolo A., Lavezzo E., Di Camillo B., Facchinetti A., Cilia E., Velasco R., Fontana P.
  Argot^2: a large scale function prediction tool relying on semantic similarity of weighted Gene Ontology terms.
  BMC bioinformatics, 13(4). 2012.

Batch processing for GO terms



Please select the zipped tabular BLAST and HMMer files, see here for details, to upload (≤ 1GB). ?

Please do not upload more than 5000 sequences at once, otherwise the service will be overloaded.

BLAST: Choose File No file chosen ?

HMMer: Choose File No file chosen ?

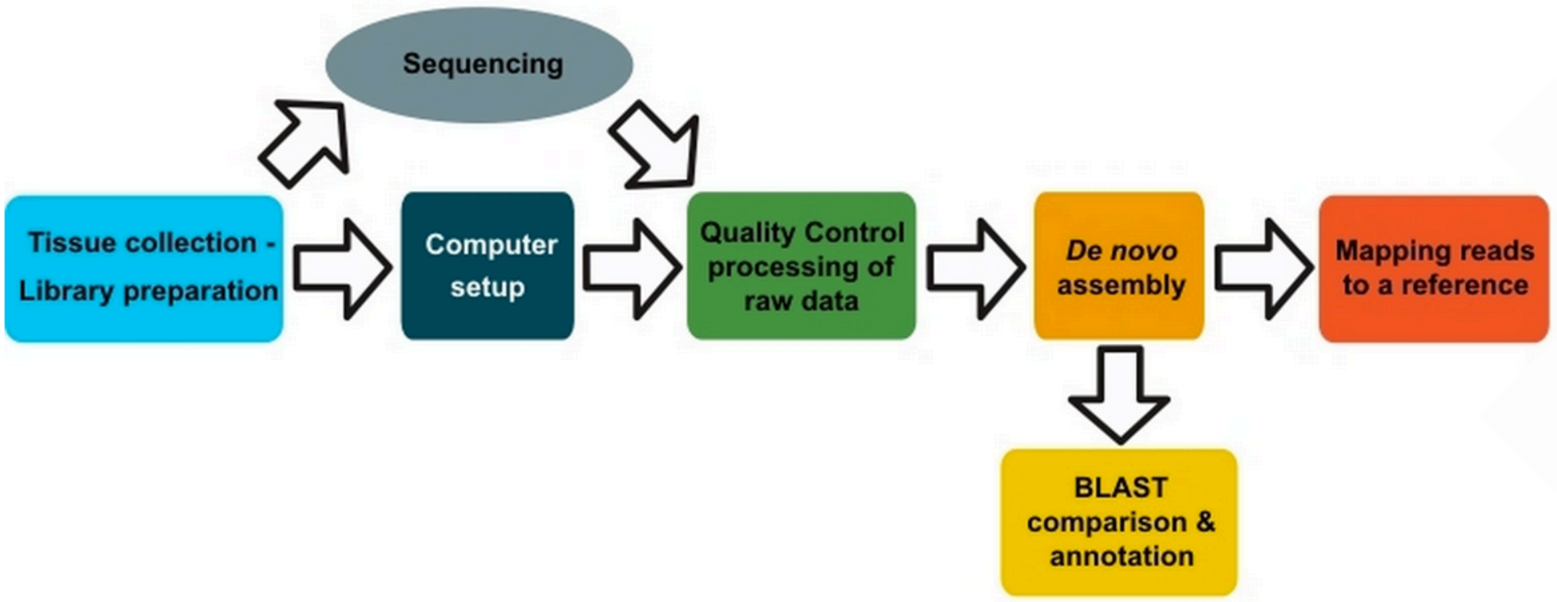
□ submit example data ?

Email: ?

CUT-OFF (meaning) ?

Total Score (≥ 5): 5

Pipeline Overview


Sequencing
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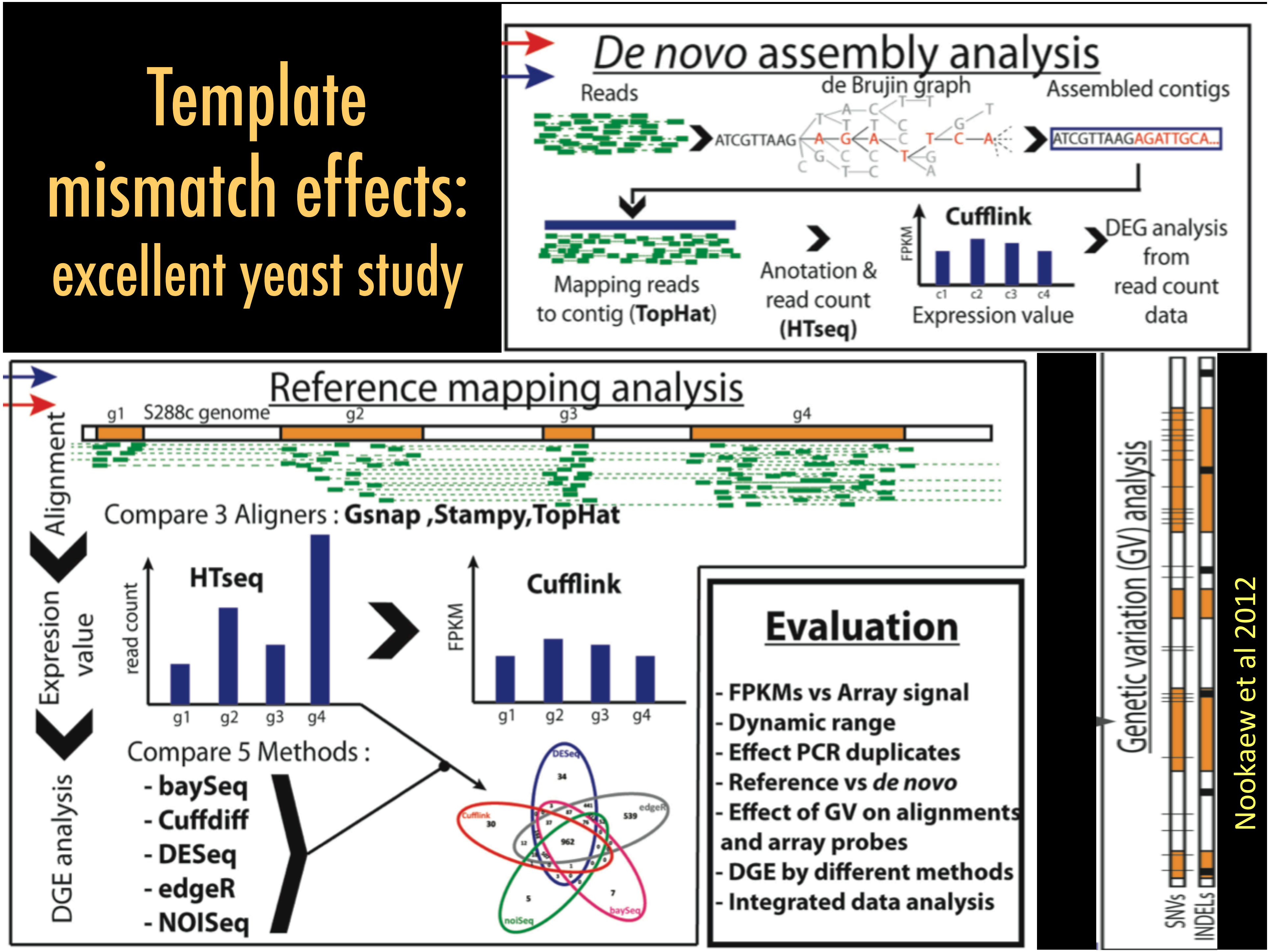
De novo assembly
Mapping reads to a reference
BLAST comparison & annotation

Template mismatch effects: excellent yeast study

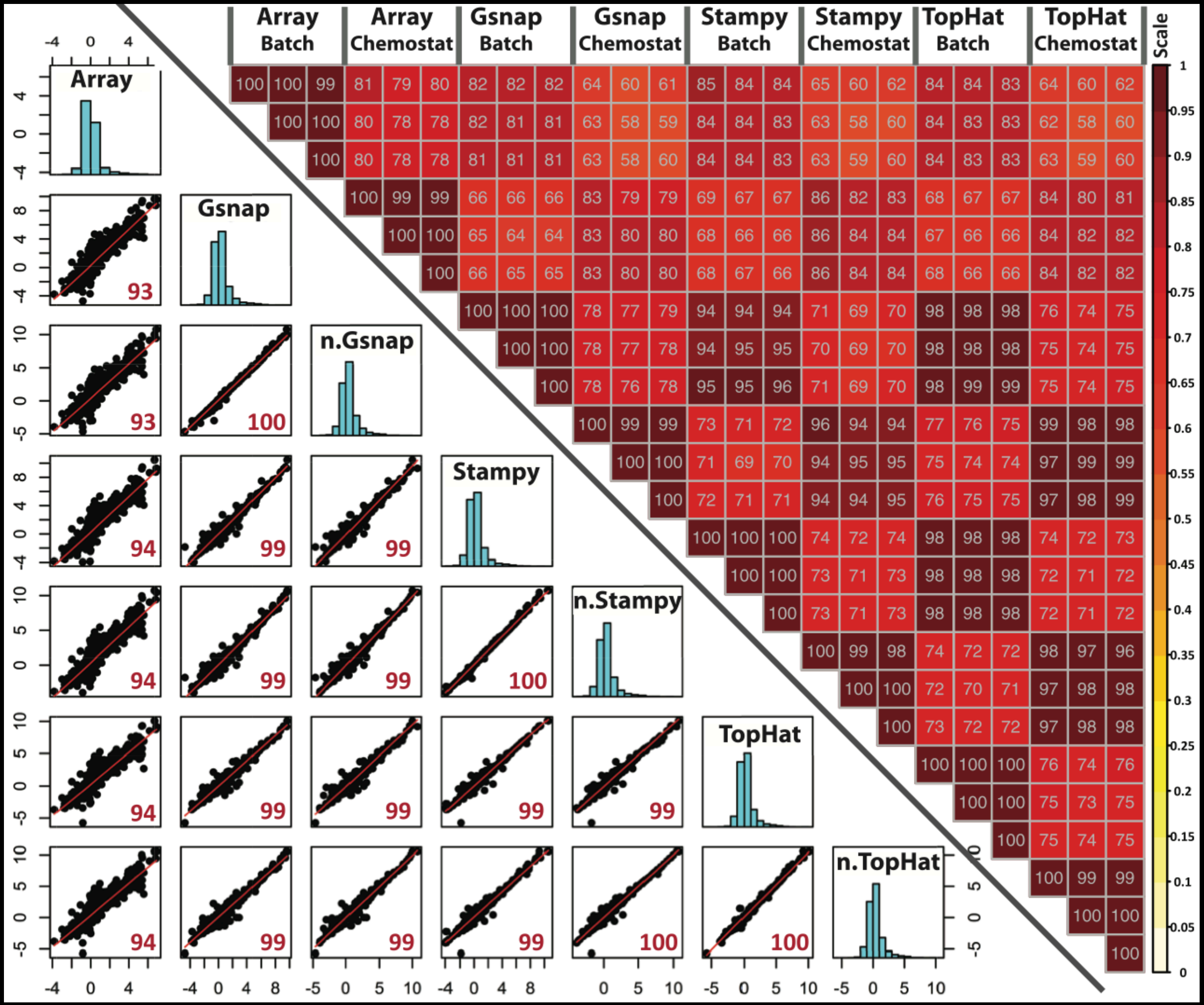


Evaluation

- FPKMs vs Array signal
- Dynamic range
- Effect PCR duplicates
- Reference vs *de novo*
- Effect of GV on alignments and array probes
- DGE by different methods
- Integrated data analysis

Nookaew et al 2012

Does alignment software matter?



Array Batch			Array Chemostat			Gsnap Batch			Gsnap Chemostat			Stampy Batch			Stampy Chemostat			TopHat Batch			TopHat Chemostat		
100	100	99	81	79	80	82	82	82	64	60	61	85	84	84	65	60	62	84	84	83	64	60	62
	100	100	80	78	78	82	81	81	63	58	59	84	84	83	63	58	60	84	83	83	62	58	60
		100	80	78	78	81	81	81	63	58	60	84	84	83	63	59	60	84	83	83	63	59	60
			100	99	99	66	66	66	83	79	79	69	67	67	86	82	83	68	67	67	84	80	81
				100	100	65	64	64	83	80	80	68	66	66	86	84	84	67	66	66	84	82	82
					100	66	65	65	83	80	80	68	67	66	86	84	84	68	66	66	84	82	82
						100	100	100	78	77	79	94	94	94	71	69	70	98	98	98	76	74	75
							100	100	78	77	78	94	95	95	70	69	70	98	98	98	75	74	75
								100	78	76	78	95	95	96	71	69	70	98	99	99	75	74	75
									100	99	99	73	71	72	96	94	94	77	76	75	99	98	98
										100	100	71	69	70	94	95	95	75	74	74	97	99	99
											100	72	71	71	94	94	95	76	75	75	97	98	99
												100	100	100	74	72	74	98	98	98	74	72	73
													100	100	73	71	73	98	98	98	72	71	72
														100	73	71	73	98	98	98	72	71	72
															100	99	98	74	72	72	98	97	96
																100	100	72	70	71	97	98	98
																	100	73	72	72	97	98	98
																		100	100	100	76	74	76
																			100	100	75	73	75
																				100	75	74	75
																					100	99	99
																						100	100
																							100

Nookaew et al. **A comprehensive comparison of RNA-Seq-based transcriptome analysis from reads to differential gene expression and cross-comparison with microarrays: a case study in Saccharomyces cerevisiae**. *Nucleic Acids Research* 2012, **40**:10084–10097.

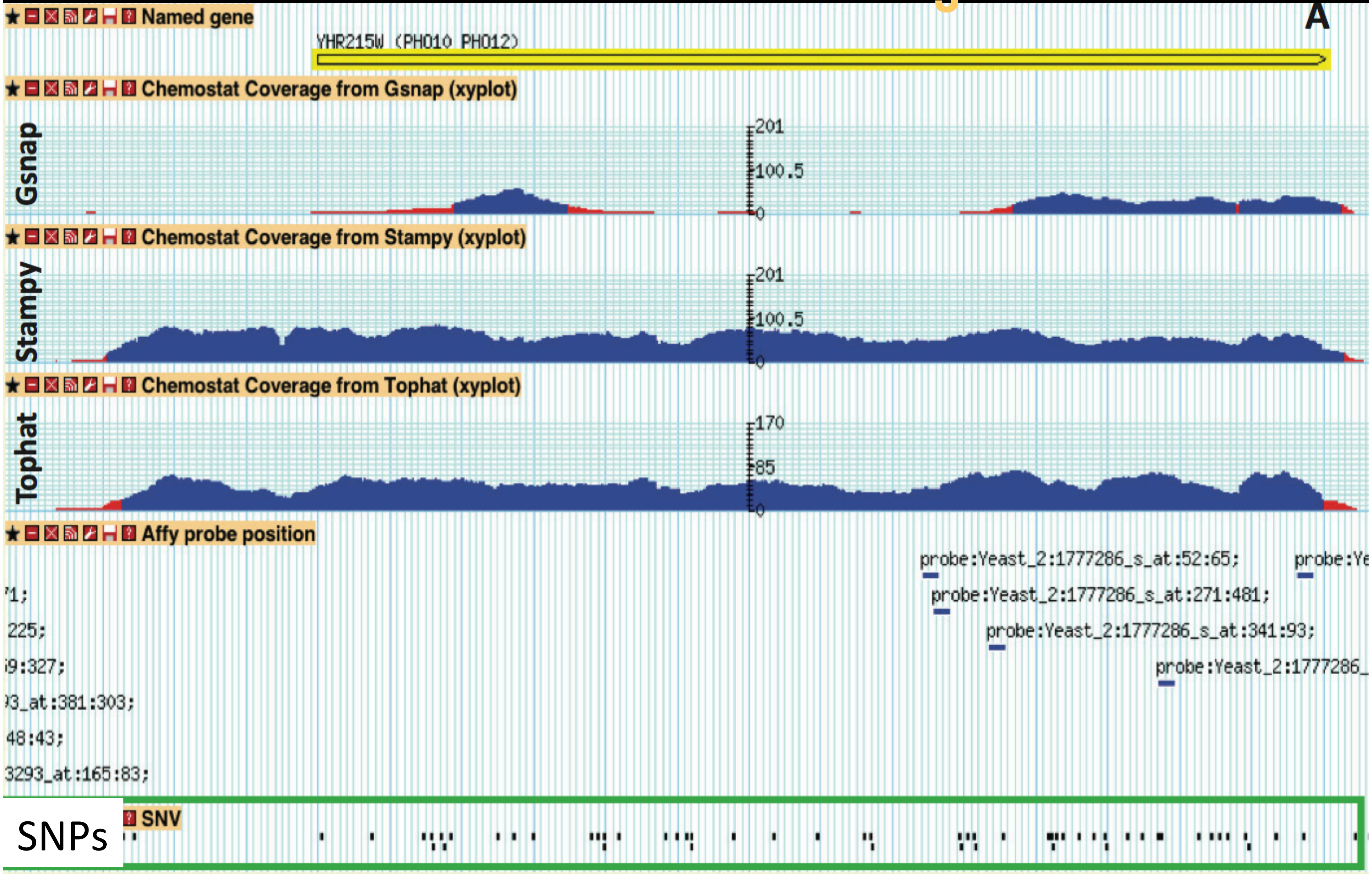
Mappers don’t appear to matter

Wrong

- Genomic scale data can hide widespread biases that unless you specifically look, are hard to find

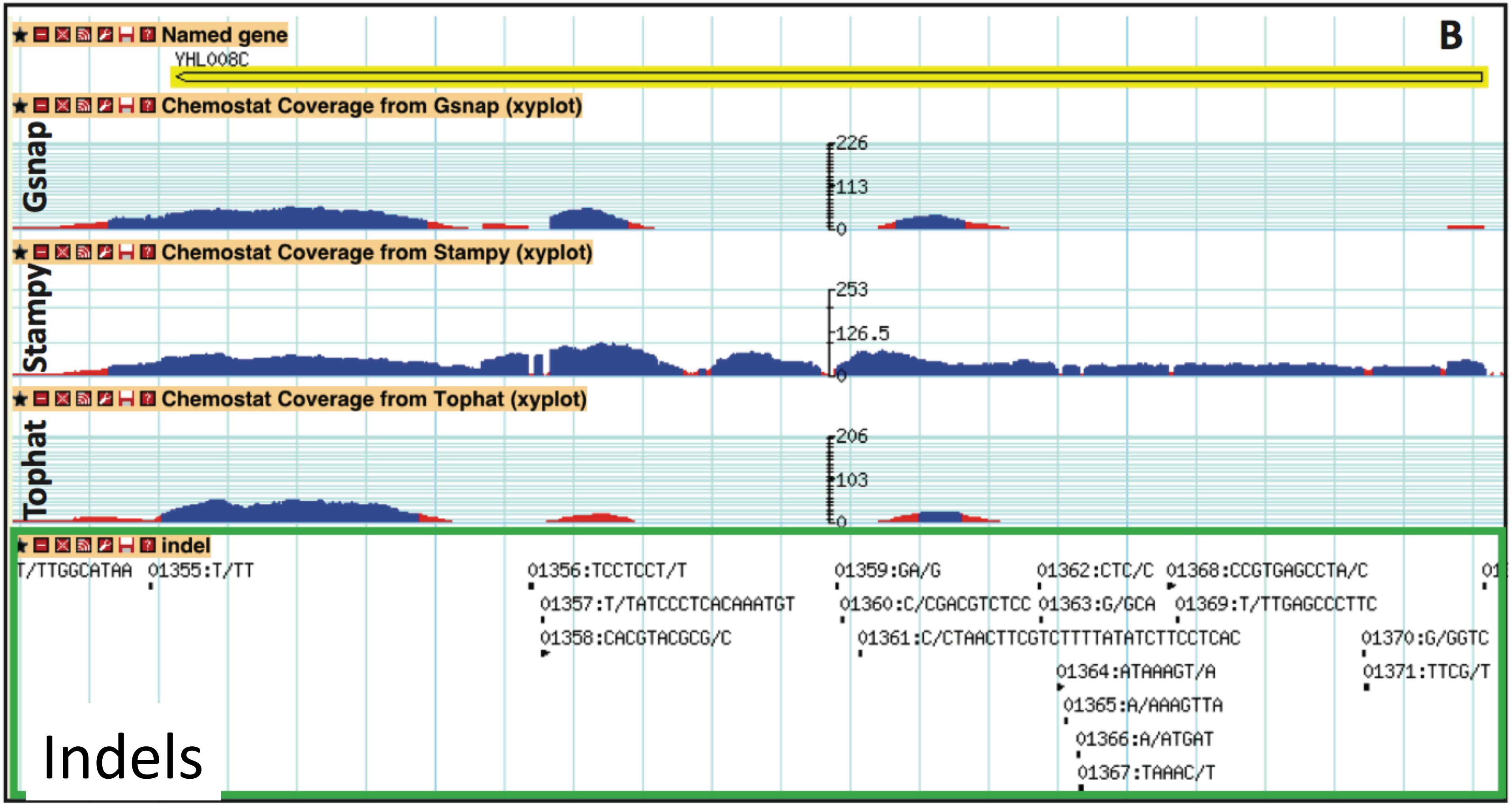
- Mapping programs differ in their settings and design
  - DNA to DNA vs. RNA to DNA
  - Are usually compared using species without much genetic variation
  - Indels, splicing, SNPs all affect mapper performance

SNP effects can be large


A
Named gene
YHR215W (PHO10 PHO12)
Chemostat Coverage from Gsnap (xyplot)
Gsnap
201
100.5
0
Chemostat Coverage from Stampy (xyplot)
Stampy
201
100.5
0
Chemostat Coverage from Tophat (xyplot)
Tophat
170
85
0
Affy probe position
probe:Yeast_2:1777286_s_at:52:65;
probe:Ye
probe:Yeast_2:1777286_s_at:271:481;
probe:Yeast_2:1777286_s_at:341:93;
probe:Yeast_2:1777286_
'1;
225;
i9:327;
)3_at:381:303;
48:43;
3293_at:165:83;
SNV
SNPs

Nookaew et al. **A comprehensive comparison of RNA-Seq-based transcriptome analysis from reads to differential gene expression and cross-comparison with microarrays: a case study in Saccharomyces cerevisiae**. *Nucleic Acids Research* 2012, **40**:10084–10097.

Insertions & deletions (indels) have large effects



Indels

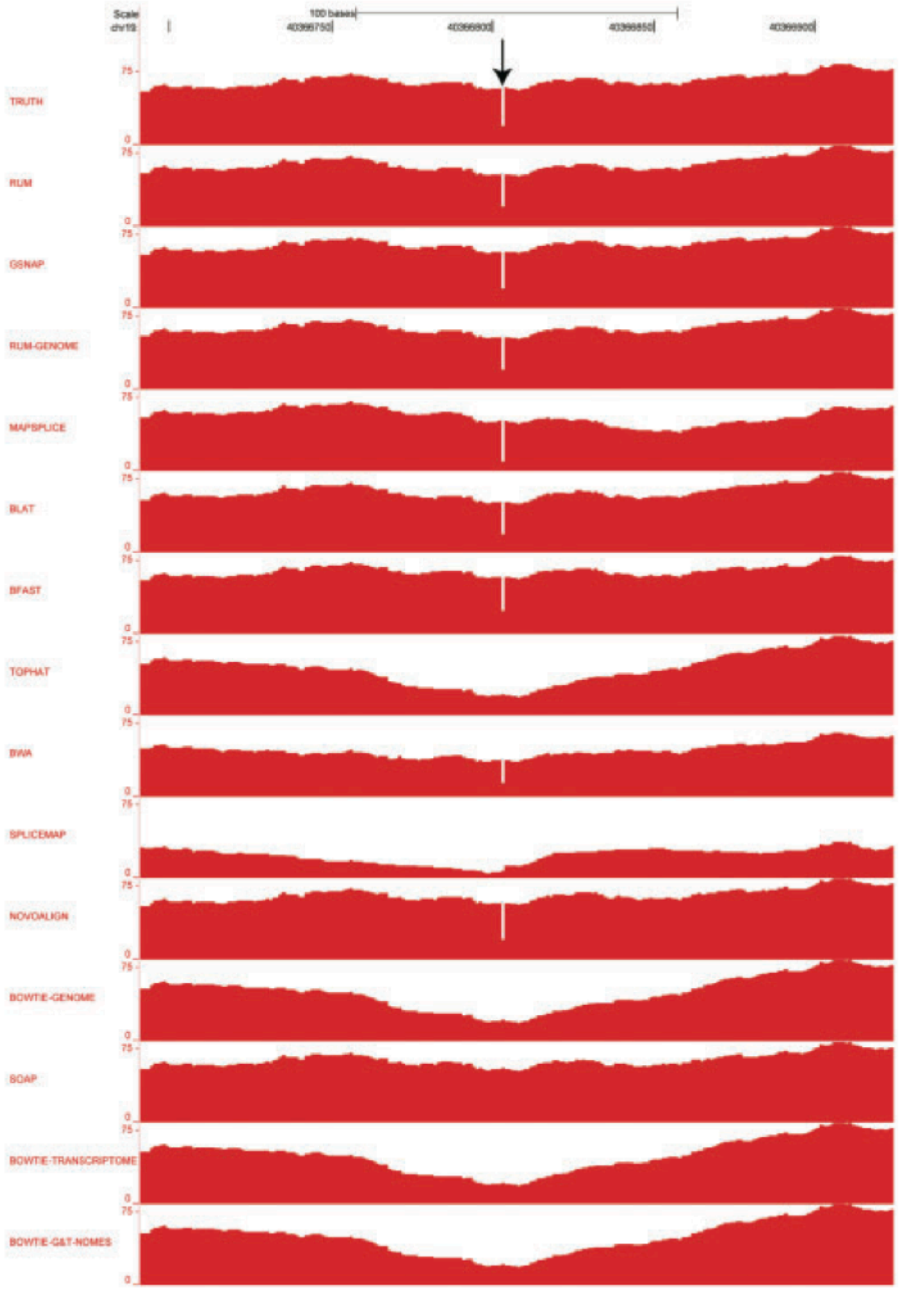
Nookaew et al. **A comprehensive comparison of RNA-Seq-based transcriptome analysis from reads to differential gene expression and cross-comparison with microarrays: a case study in Saccharomyces cerevisiae**. *Nucleic Acids Research* 2012, **40**:10084–10097.

15 mapping results

Dramatic differences in ability to handle a 2 bp insertion in reference compared to reads

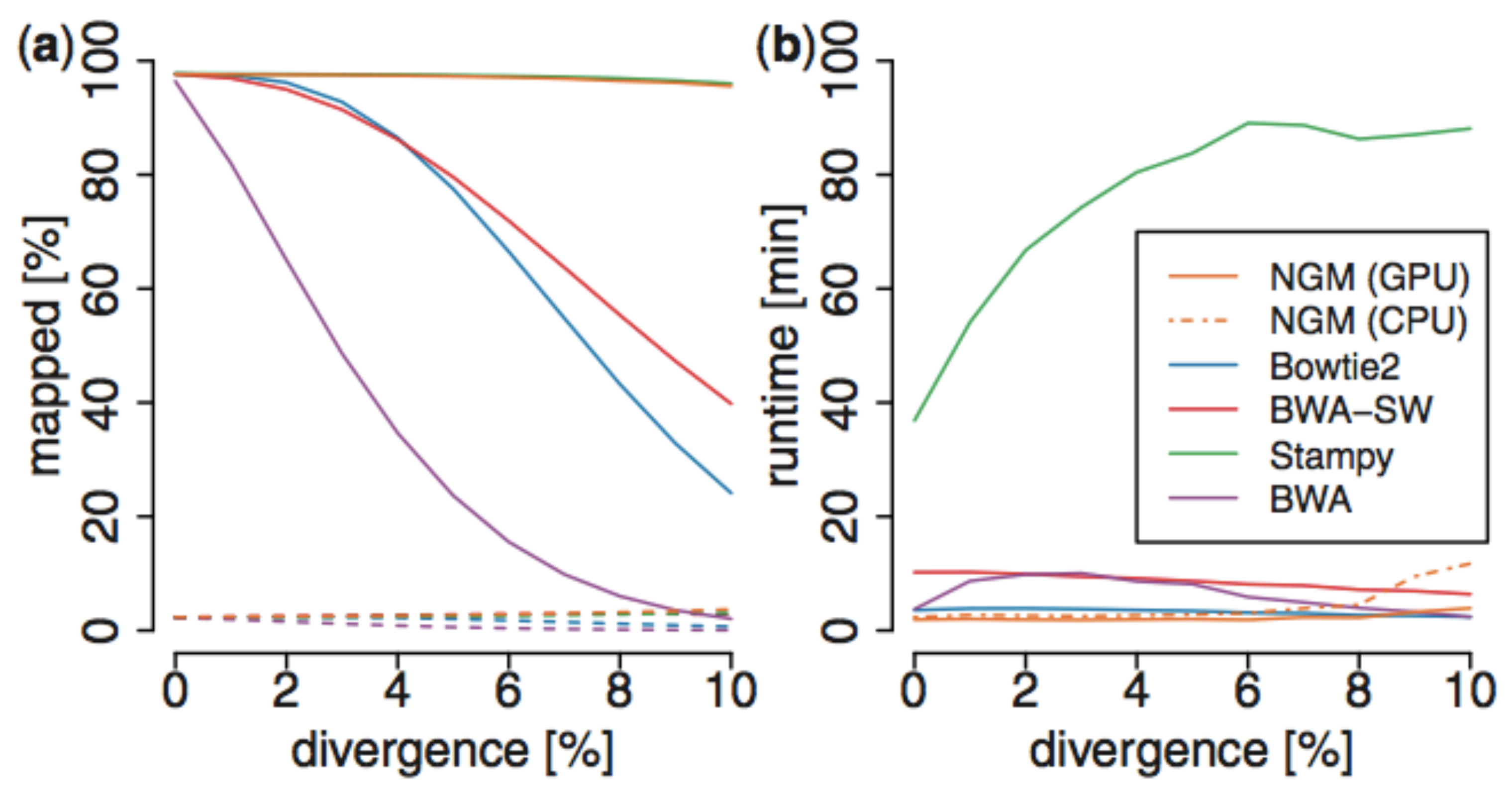
TopHat, SpliceMap, Bowtie and Soap

- do not identify indels
- they fail to accurately align reads to these regions



Grant GR, Farkas MH, Pizarro A, Lahens N, Schug J, Brunk B, Stoeckert CJ, Hogenesch JB, Pierce EA: **Comparative Analysis of RNA-Seq Alignment Algorithms and the RNA-Seq Unified Mapper (RUM)**. *Bioinformatics* 2011, doi:10.1093/bioinformatics/btr427.

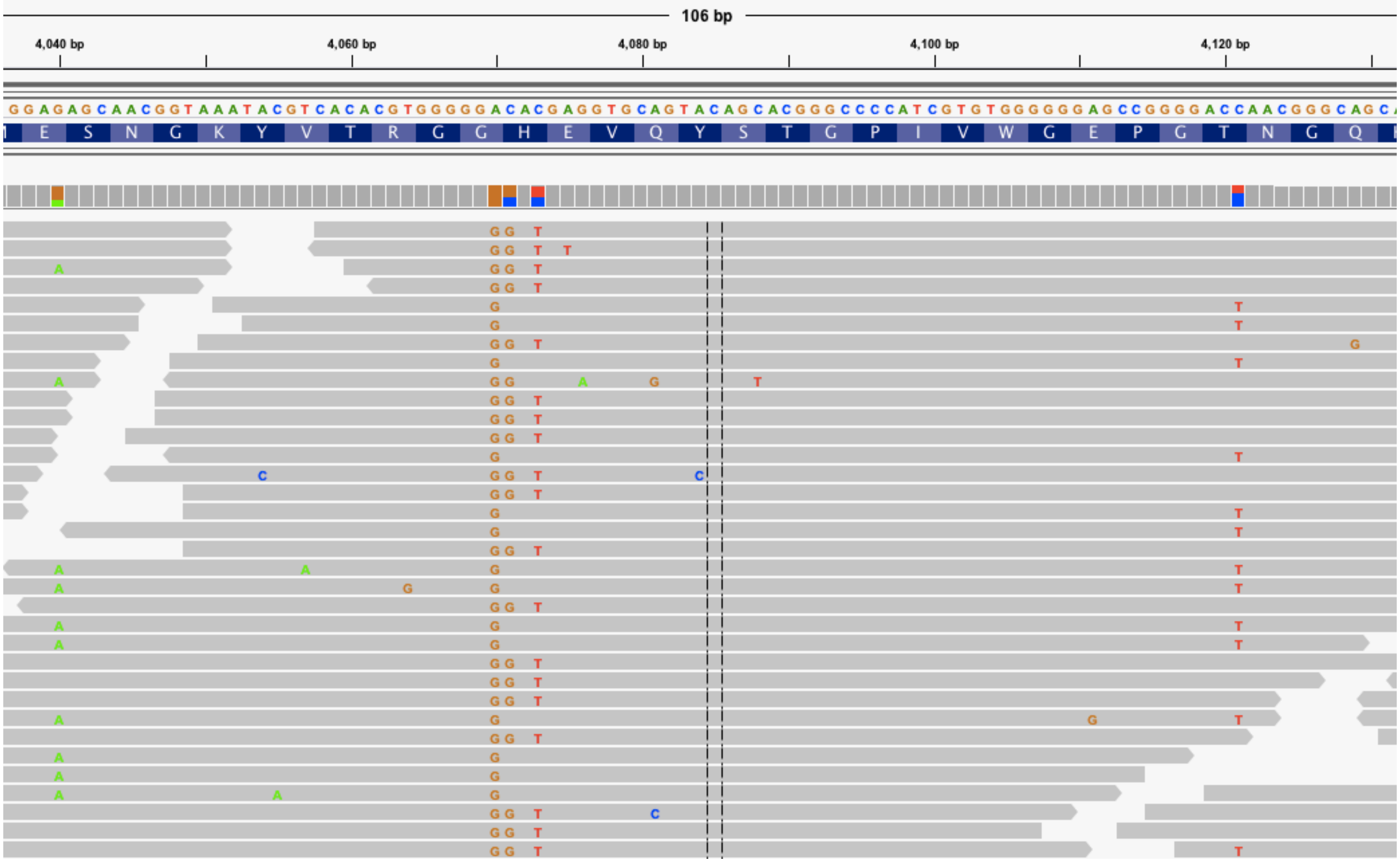
Allelic bias in read mapping



- Essentially identical to allele specific PCR bias … but on a scale you can't detect unless you care to look
- Do your genes of interest have more than 3 SNPs / 100 bp?

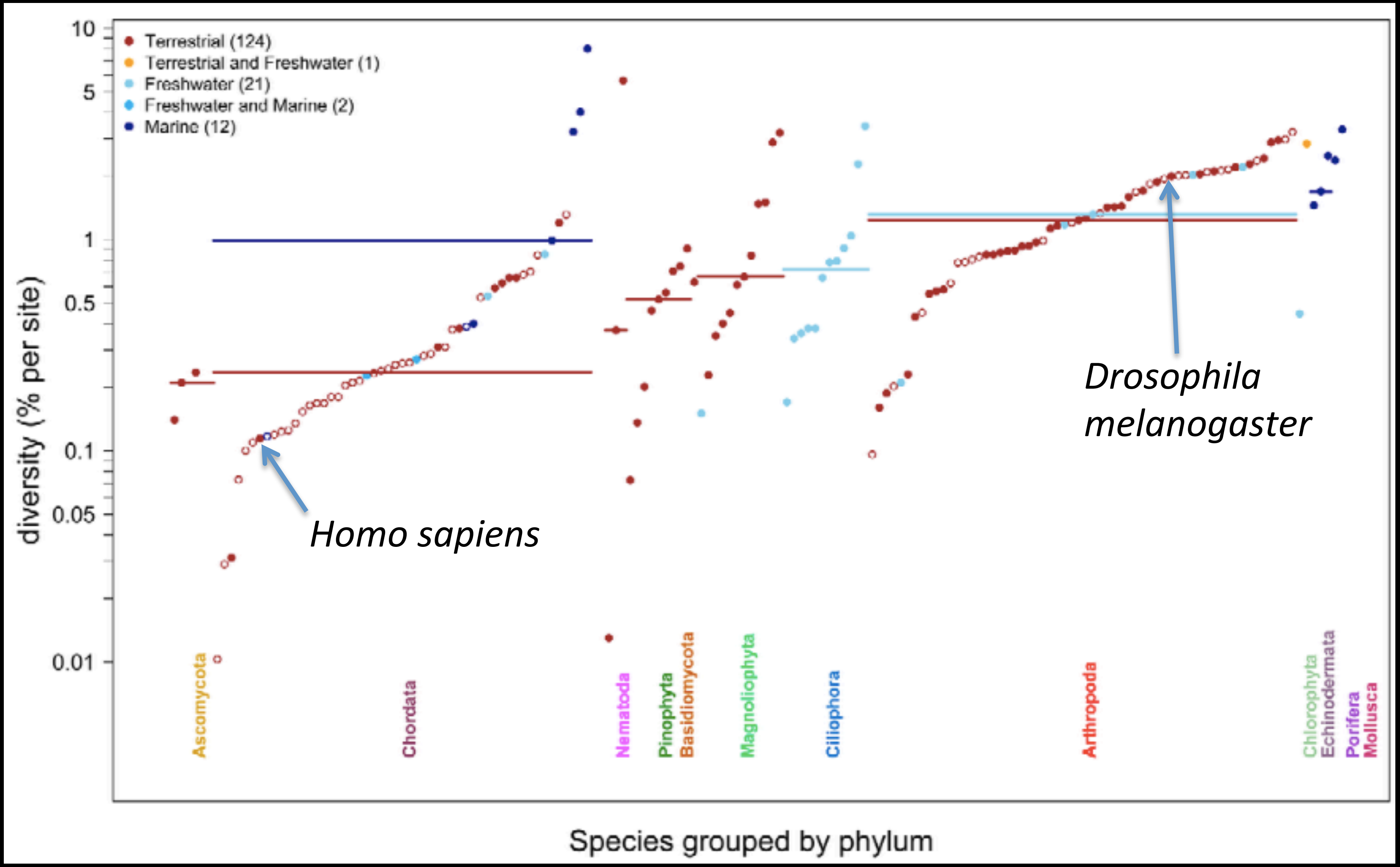
Sedlazeck et al. 2013 *Bioinformatics*

100 bp window with 4 – 5 SNPs differing from reference


106 bp
4,040 bp
4,060 bp
4,080 bp
4,100 bp
4,120 bp
GGAGAGCAACGGTAAATACGTCACACGTGGGGGACACGAGGTGCAGTACAGCACGGGCCCCATCGTGTGGGGGGAGCCGGGGGACCAACGGGCAGC
E S N G K Y V T R G G H E V Q Y S T G P I V W G E P G T N G Q

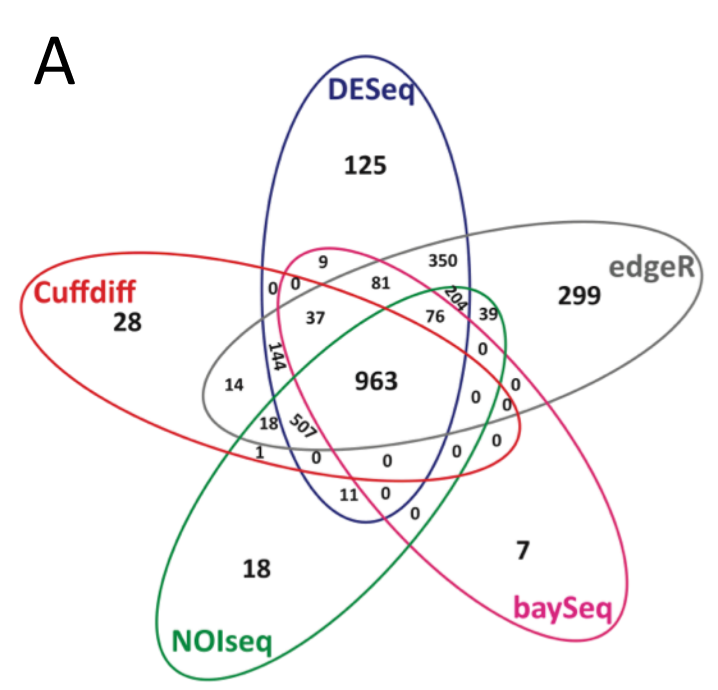
Mapping reads in outbred species

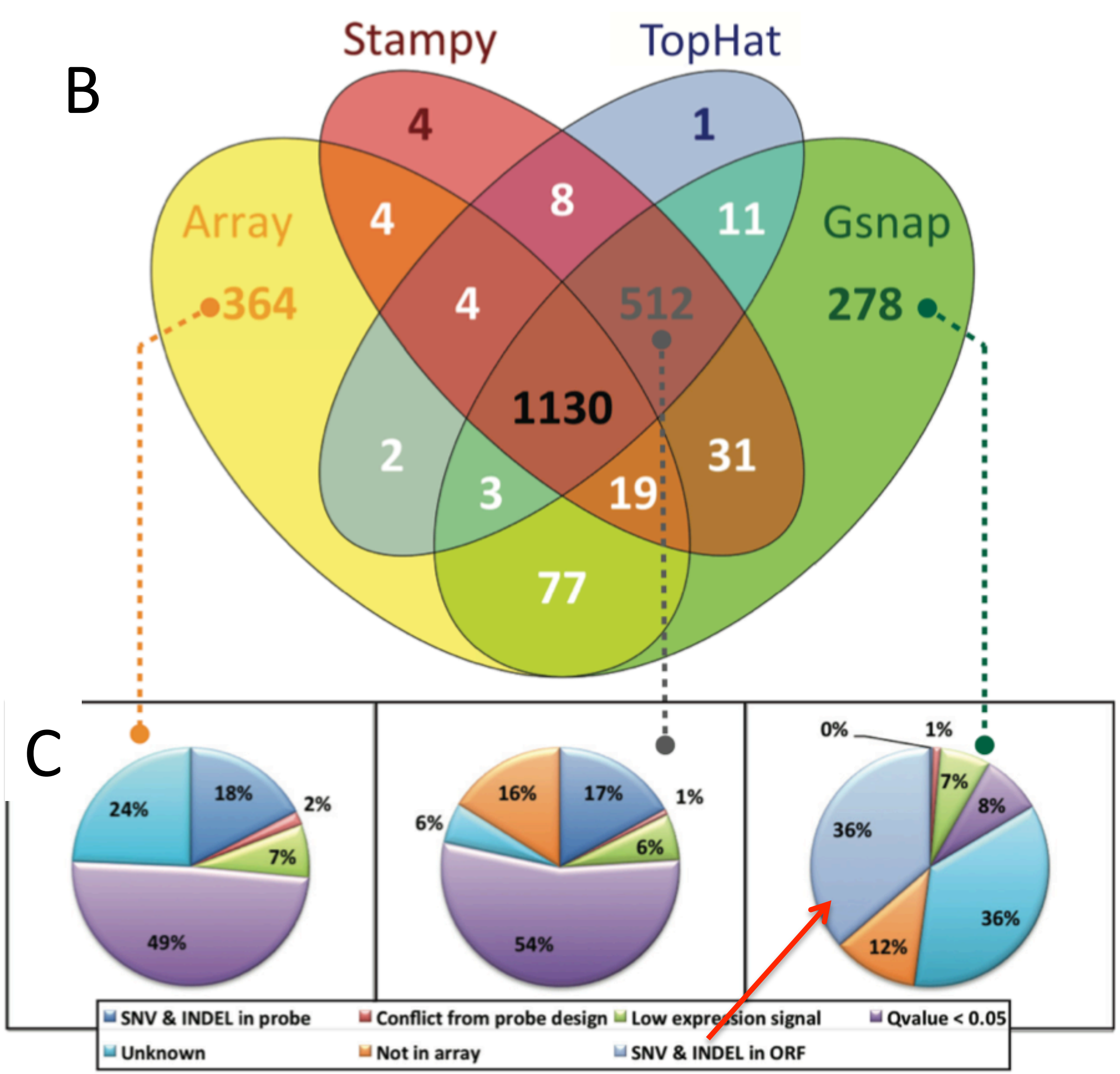
Average genome polymorphism levels (ignores indels)



Leffler *et al.* 2012 *Plos Biol*

Sig. expression differences by method





A: Stampy mapping

B: Cuffdiff analysis

C: Likely error source

RNA-Seq

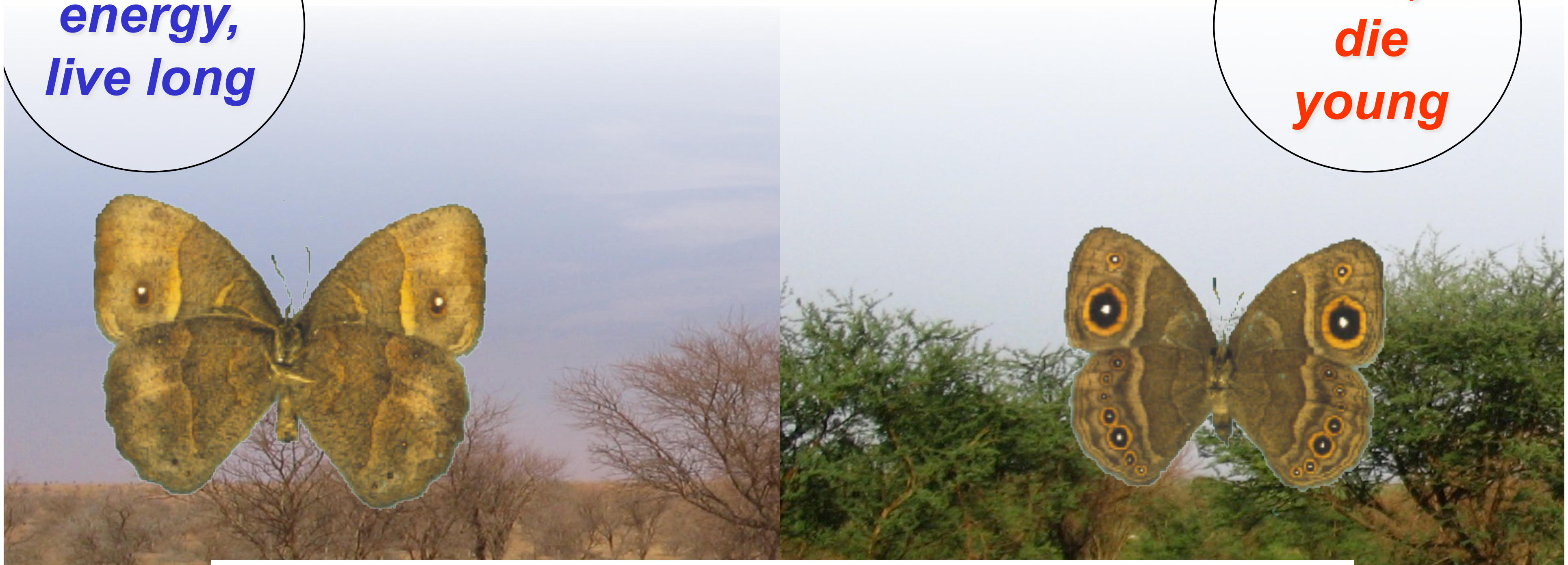
Real world example

2 factor analysis with family effects

Bicyclus anynana

Save energy, live long

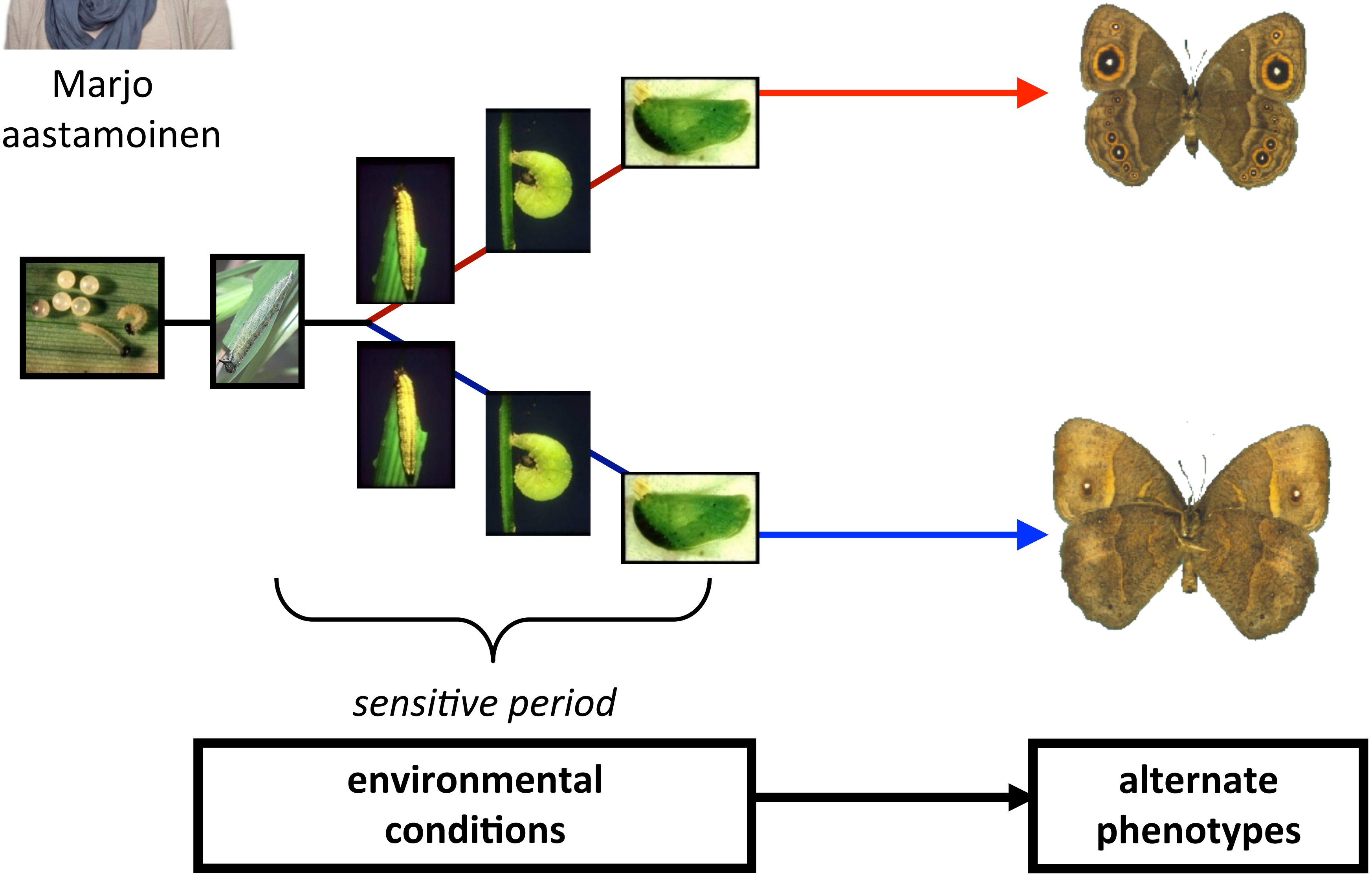
Live fast, die young



long	lifespan	short
delayed	reproduction	fast
inactive	behaviour	active
high	fat reserves	low
cryptic	wing pattern	conspicuous

Bicyclus anynana

Marjo Saastamoinen

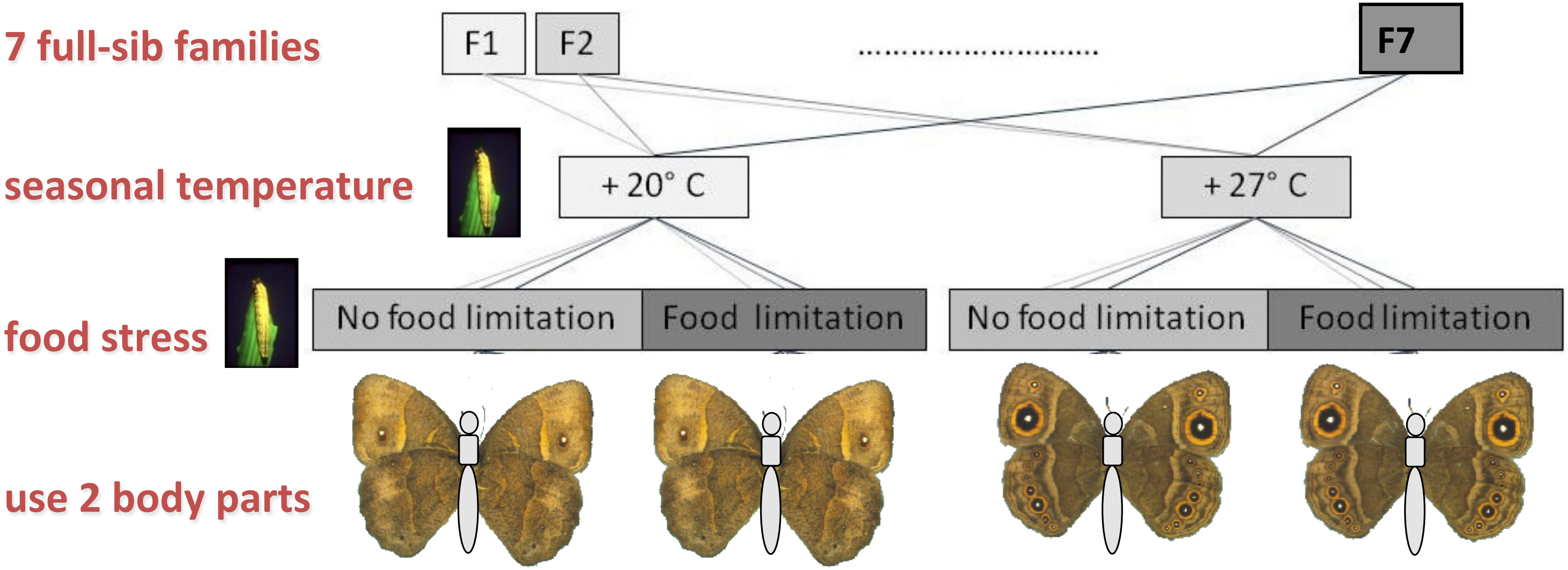


sensitive period

environmental conditions

alternate phenotypes

Experimental design



7 full-sib families

F1 F2 .... F7

seasonal temperature

+ 20° C

+ 27° C

food stress

No food limitation

Food limitation

No food limitation

Food limitation

use 2 body parts

- 2 seasonal x 2 food stress x 2 body parts = **8 conditions**
- 7 families with n = 2 - 3 per condition → **144 RNA libraries**
- 10 million reads / library

华大基因 BGI

Vicencio Oostra

body part	# libraries	# clean reads (per library)	# nucleotides (per library)	GC content
abdomen	72	15,261,019	3,052,203,767	45%
thorax	72	15,633,416	3,126,683,150	46%
total	144	2,224,399,290	444,879,858,000	45%

Trinity

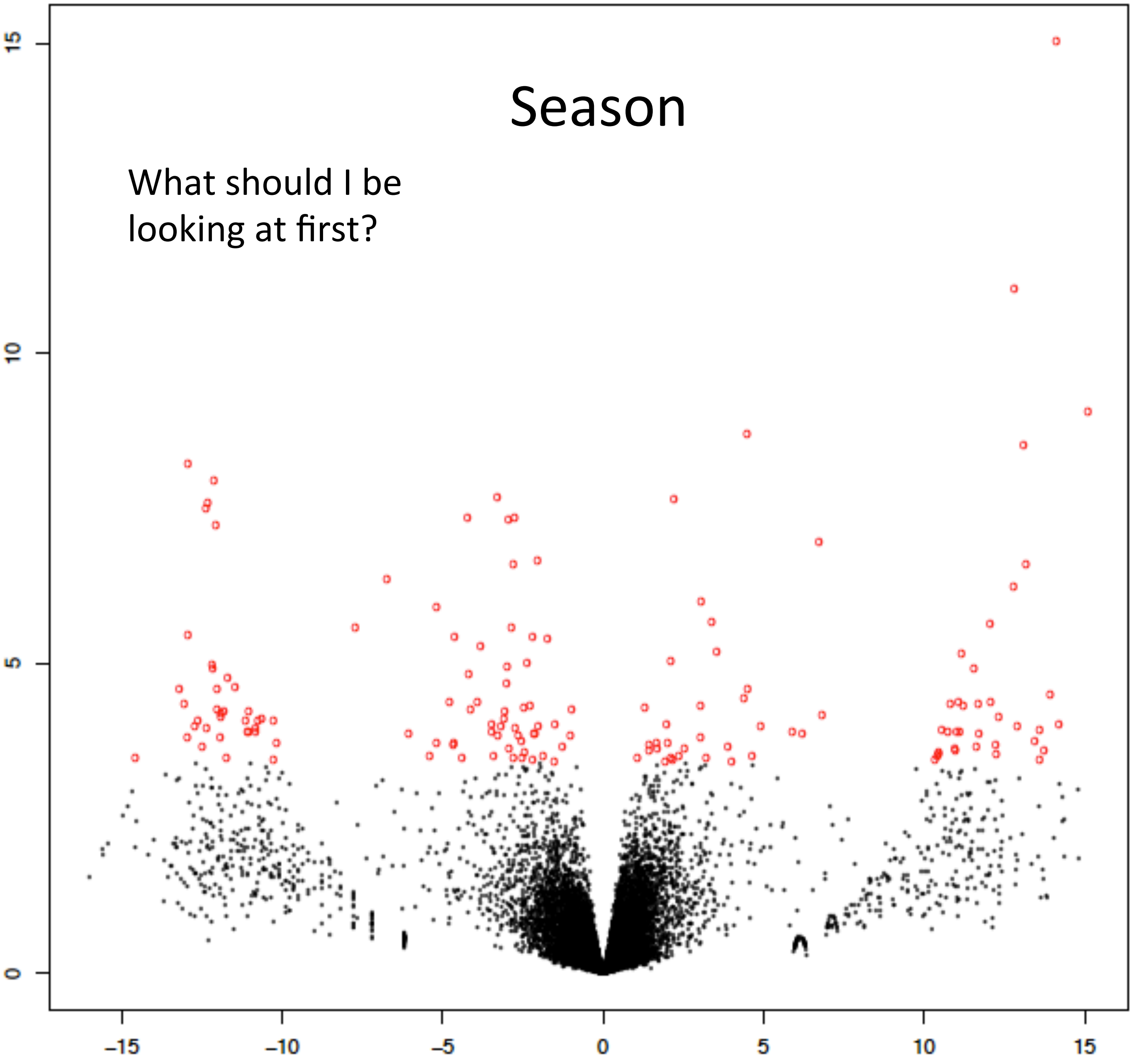
14 samples: one from each family, thorax and abdomen

69,075 contigs

edgeR

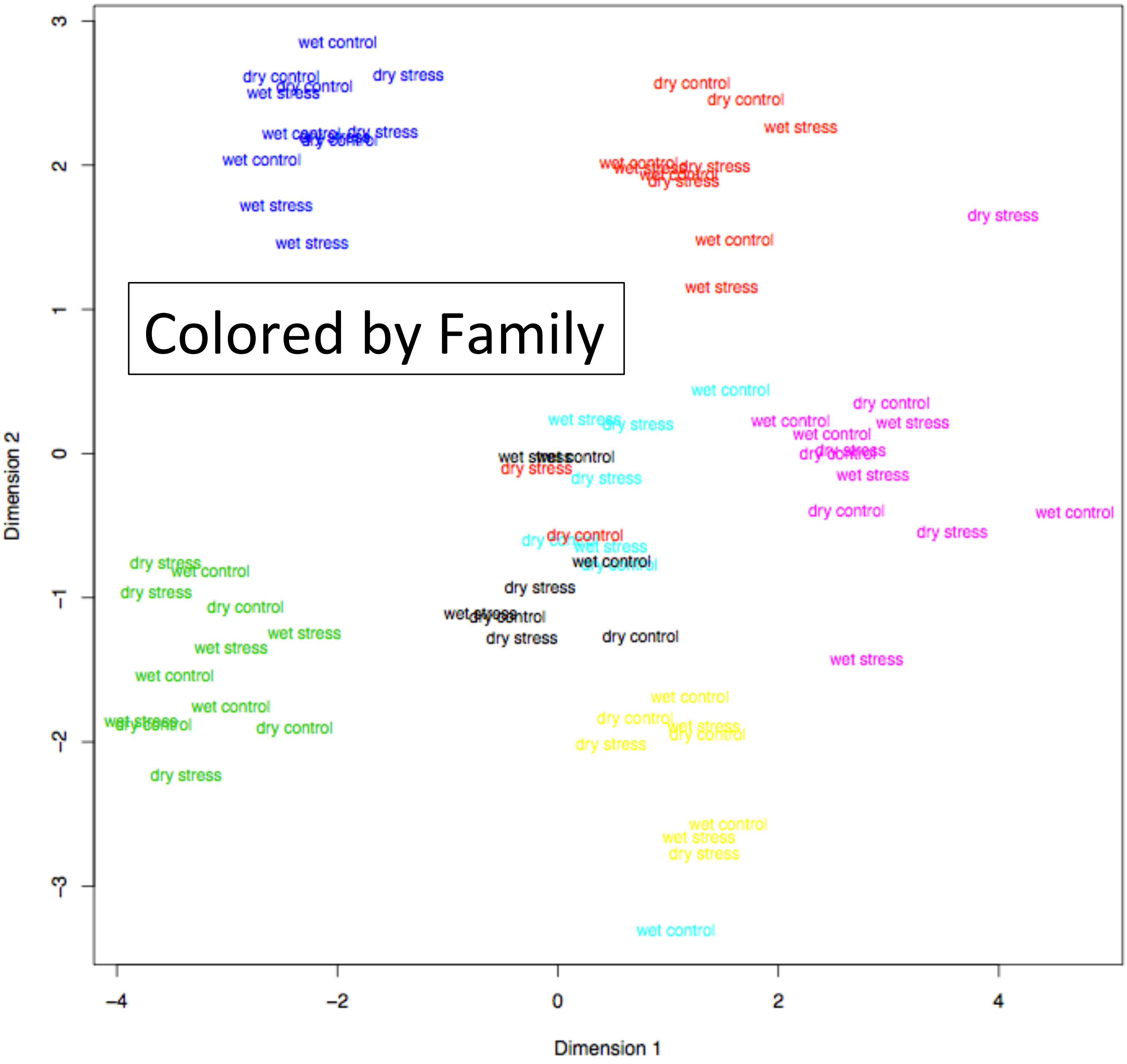
Bioconductor
OPEN SOURCE SOFTWARE FOR BIOINFORMATICS

```
# reads ~   season + stress + family +
            season*stress + season*family + stress*family
            season*stress*family
```

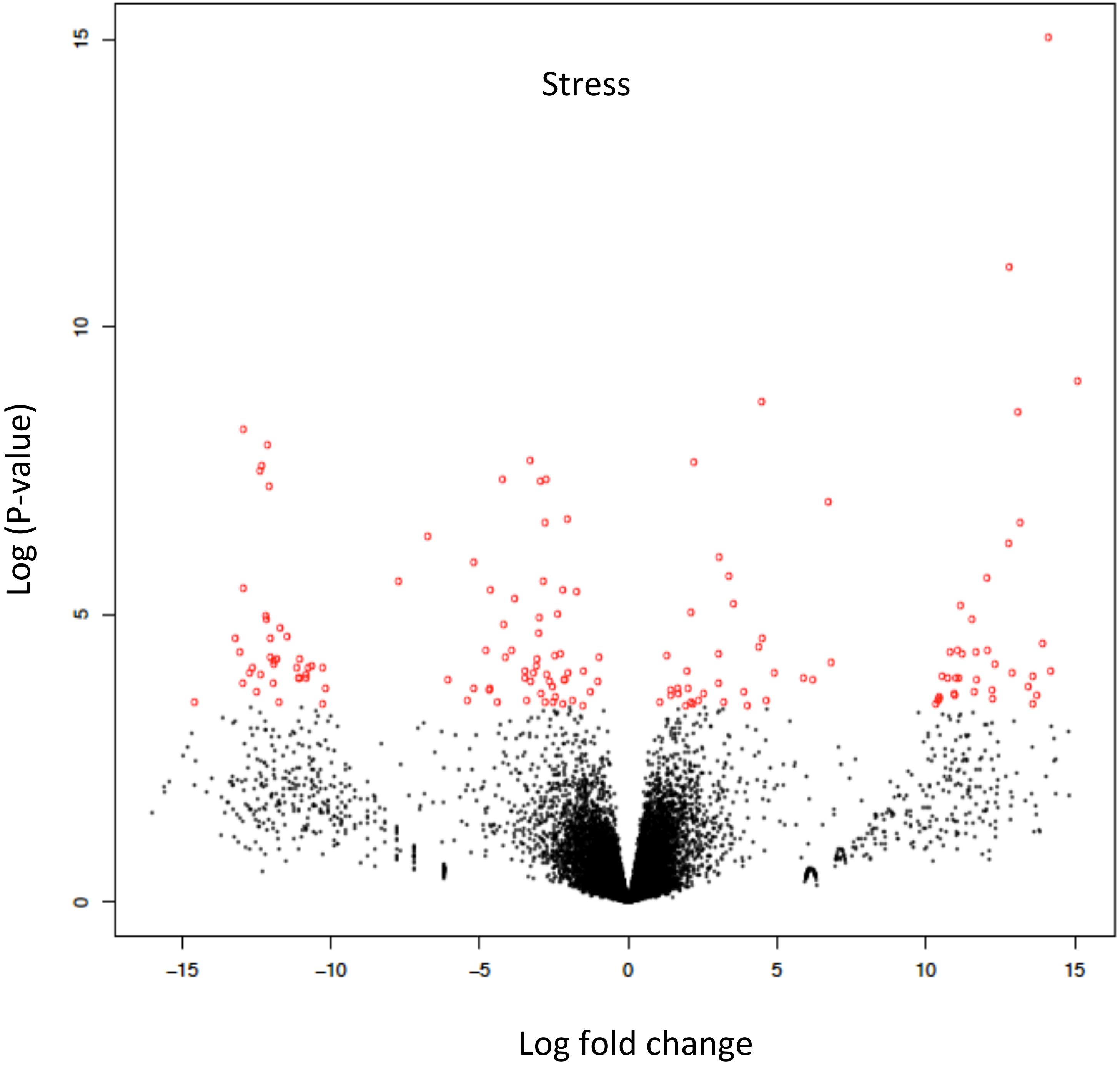


Season

What should I be
looking at first?

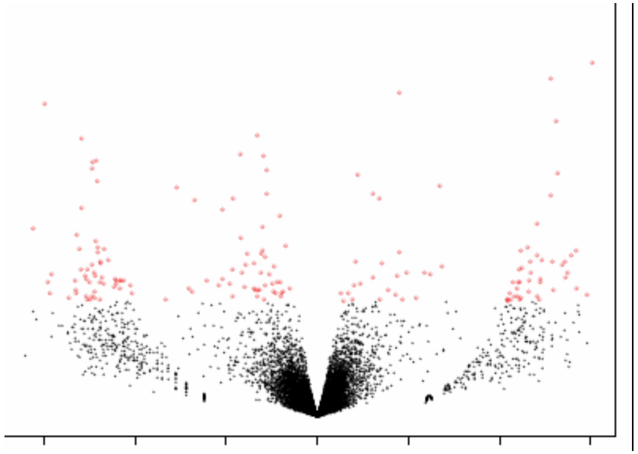


Colored by Family

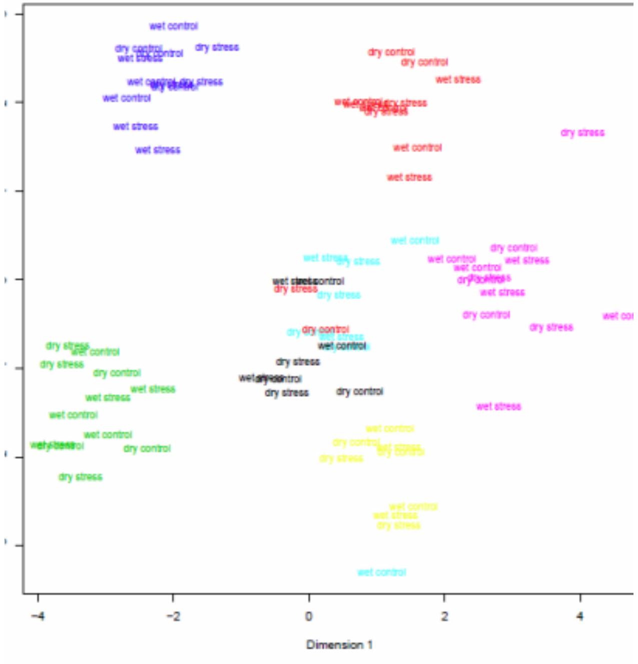

Stress
Log (P-value)
15
10
5
0
−15
−10
−5
0
5
10
15
Log fold change

Log (P-value)
Log fold change

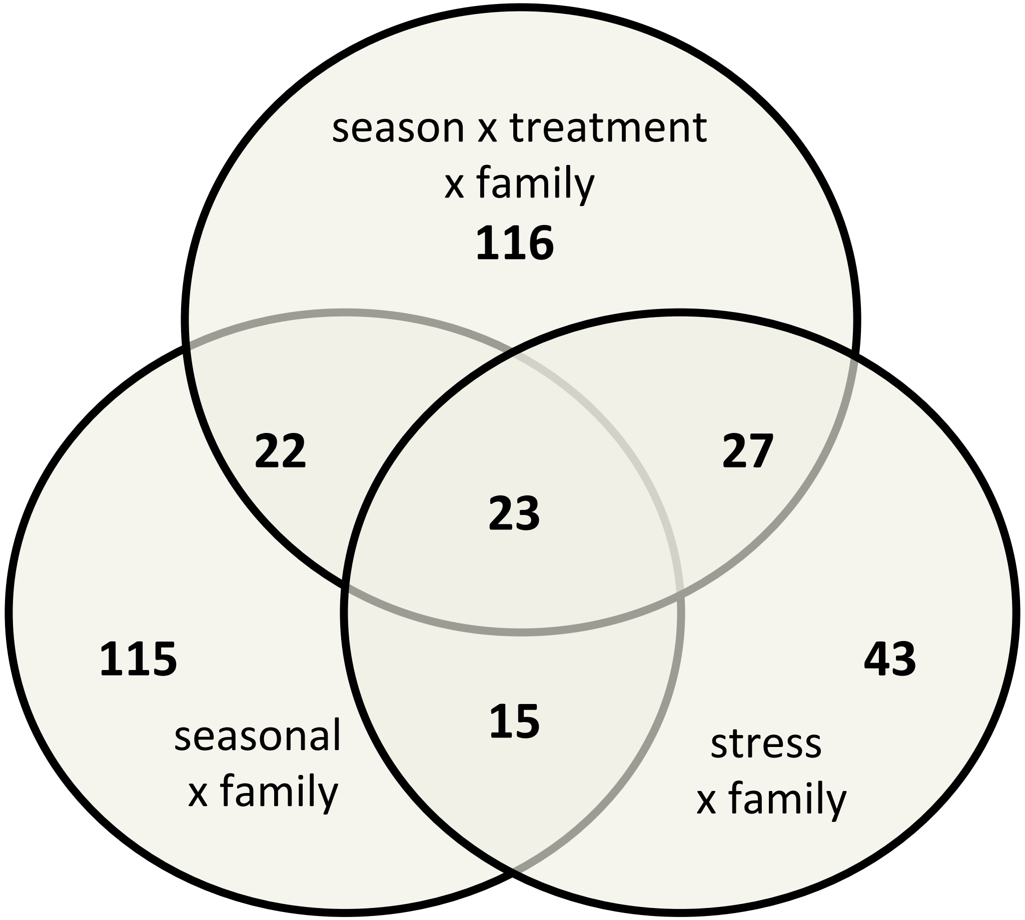
Effect of filtering the mapping to Trinity contigs



71 zero-read samples allowed



GLM results



- Plastic responses:
  - – Effects without any interaction with Family

- Genetic response:
  - ○ Effects that have an interaction with family
  - ○ Potential targets of natural selection

```
reads ~    season + stress + family + season*stress  +
           season*family + stress*family + season*stress*family
```

100 My

Bombyx mori
Whole genome sequence,
predicted gene set

320 My

Drosophila melanogaster
Extensive genomic &
functional resources

D. melanogaster lacks an orthologous reproductive physiology

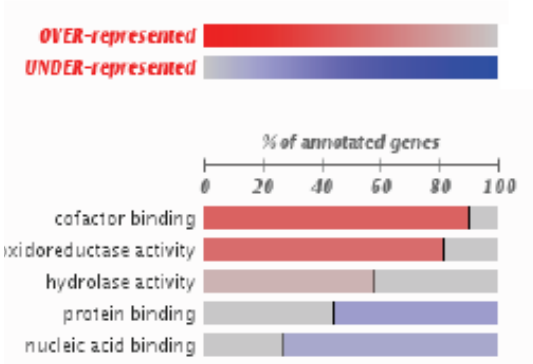
Blastx

Blastp

Assembly 2.0	Bmori06 PepEd90	Flybase gene ID
Contig_57178	BGIBMGA002704	CG33126
Contig_6821	BGIBMGA003247	CG6519
Contig_1004	BGIBMGA003248	CG6519
Contig_20226	BGIBMGA003248	CG6519
Contig_27720	BGIBMGA003248	CG6519
Contig_5260	BGIBMGA003249	CG6519
Contig_27110	BGIBMGA004806	CG33126
Contig_27390	BGIBMGA004806	CG33126
Contig_26901	BGIBMGA004865	CG33126
Contig_4713	BGIBMGA004866	CG33126
Contig_20081	BGIBMGA005329	CG3149
Contig_9982	BGIBMGA006733	CG6783
Contig_15387	BGIBMGA008859	CG4178
Contig_25362	BGIBMGA008859	CG4178
Contig_36071	BGIBMGA008859	CG4178

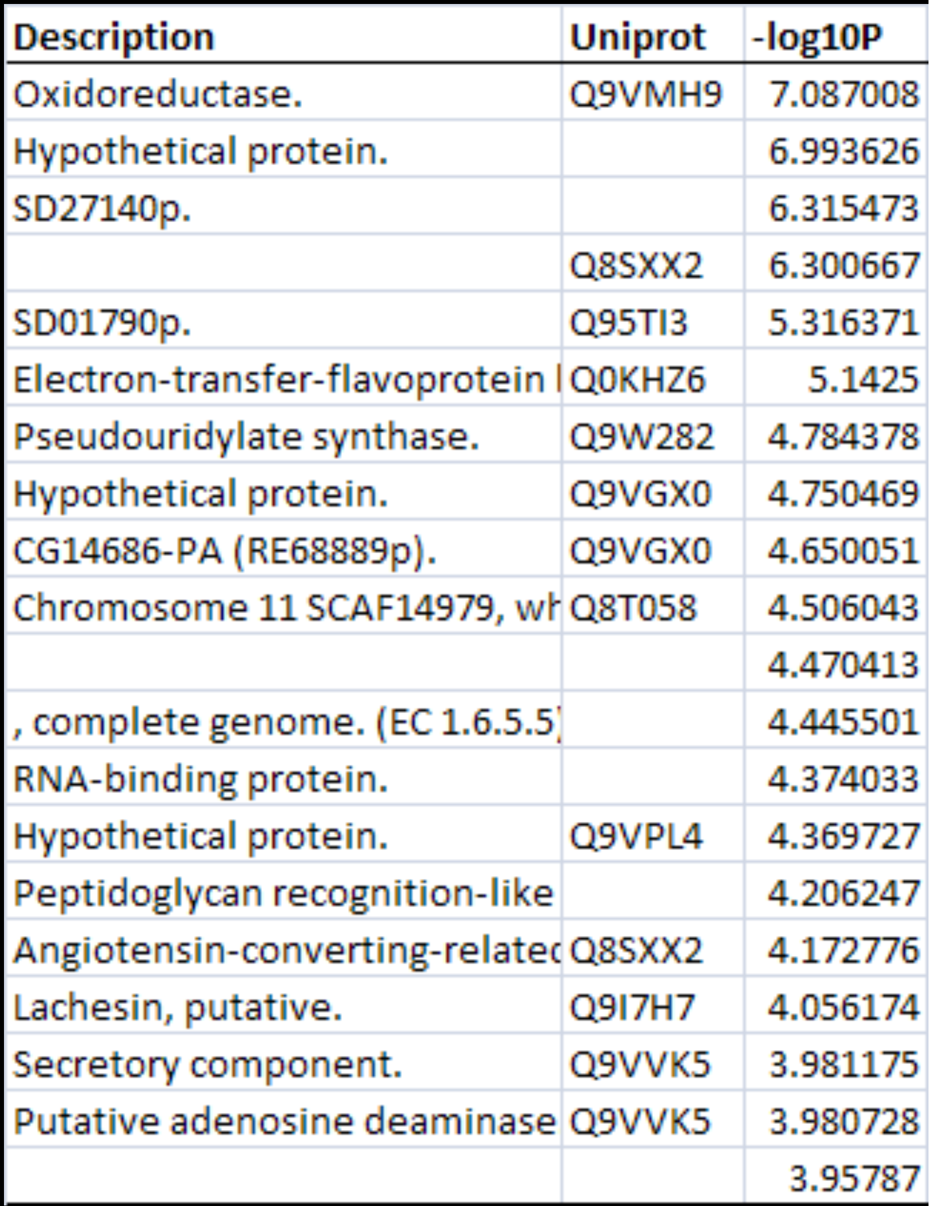
Gene Set Enrichment analysis using Gene Ontology database

Fatiscan Analysis



Most studies are annotation limited

- What is the biological meaning of the top P-value genes?
- Low P-value or expression genes are certainly important
- Gene set enrichments are key to insights
  - Thus, annotation is very important



Description	Uniprot	-log10P
Oxidoreductase.	Q9VMH9	7.087008
Hypothetical protein.		6.993626
SD27140p.		6.315473
	Q8SXX2	6.300667
SD01790p.	Q95TI3	5.316371
Electron-transfer-flavoprotein I	Q0KHZ6	5.1425
Pseudouridylate synthase.	Q9W282	4.784378
Hypothetical protein.	Q9VGX0	4.750469
CG14686-PA (RE68889p).	Q9VGX0	4.650051
Chromosome 11 SCAF14979, wh	Q8T058	4.506043
		4.470413
, complete genome. (EC 1.6.5.5)		4.445501
RNA-binding protein.		4.374033
Hypothetical protein.	Q9VPL4	4.369727
Peptidoglycan recognition-like		4.206247
Angiotensin-converting-relatec	Q8SXX2	4.172776
Lachesin, putative.	Q9I7H7	4.056174
Secretory component.	Q9VVK5	3.981175
Putative adenosine deaminase	Q9VVK5	3.980728
		3.95787

7 of 20 (35%) no Uniprot ID

Sources of error

Transcriptome assembly can be huge source of bias:

- Fragmentation creates multiple contigs of same gene
- SNPs and alternative splicing generates more contigs
- 1 locus = frag. X SNPs X alt. splicing = many contigs

We can observe effects in expression analyses:

- – Family effect mapping bias
- – Pseudo-inflation in Gene Set Enrichment Analyses

Put the BIO in your informatics!!

Use independent analyses as 'controls' on accuracy

– What are your + and – controls?

	Analysis # 1	Analysis # 2	Analysis # 3
Mapper	TopHat2	STAR	?
Normalization	none	TMM	TMM
Analysis	PCA	RSEM	EDGER

Should independent methods converge?

Interrogate your results

- “you need to be in charge of the analysis” – B. Cresko

- This will give you confidence
  - Bring freedom to your findings (no waterboarding)

- Graph your results – visualize the patterns
  - PCA or MDS plot
  - P-value distributions

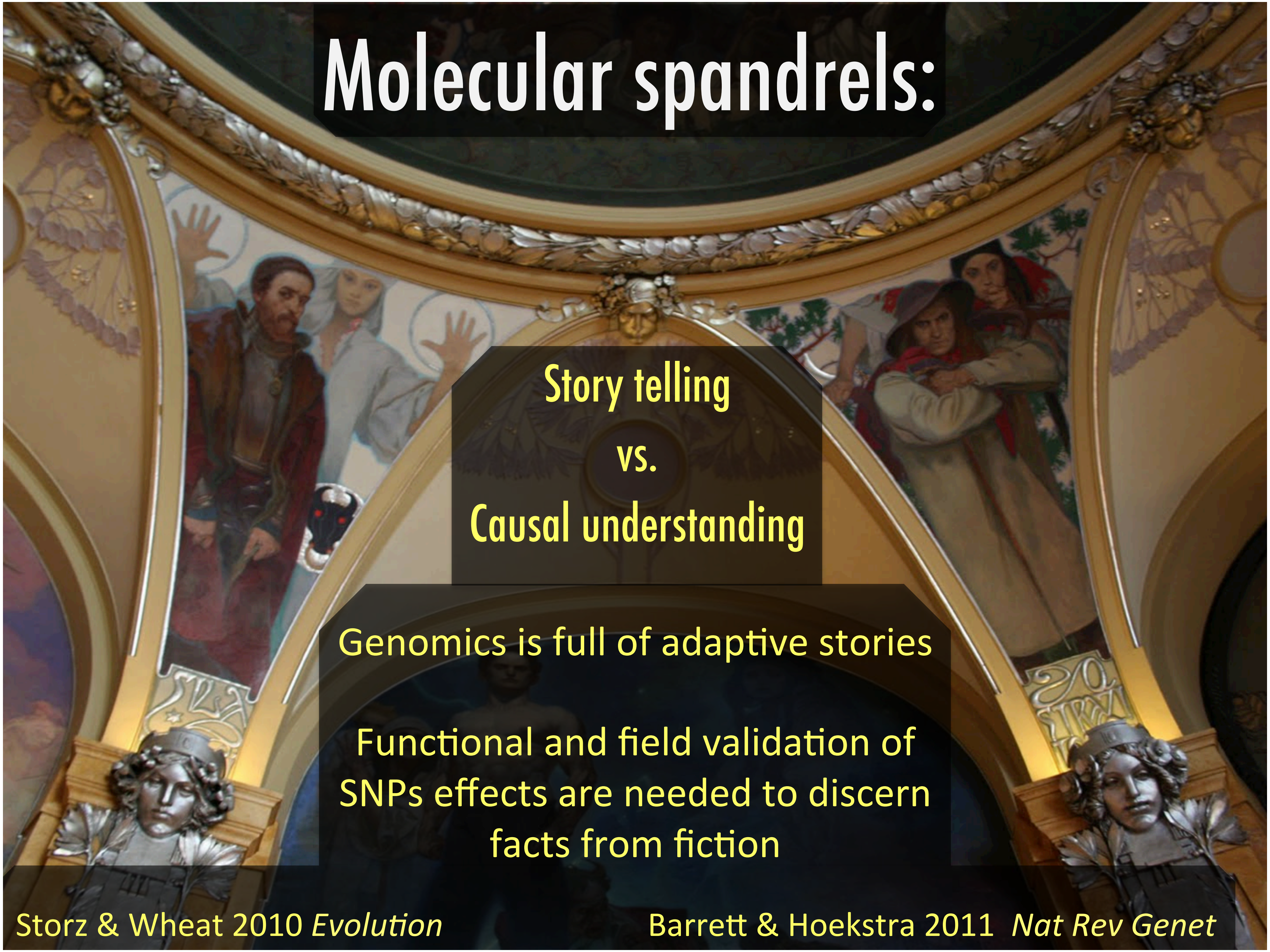
- Assess gene copy number in gene set enrichment analyses (GSEA)
  - Do these levels fit to 1st principals expectations?
  - Do you have extra copies due to your Transcriptome assembly?

A major challenge for Ecological Genomics

- What causes natural selection in the wild?
  - How does genetic variation at one region of the genome interact with its environment (genomic, abiotic, and biotic)

- DNA alone can’t tell us about selection dynamics in the wild
  - Molecular tests are very weak and uninformative about selection dynamics

- Research community is demanding actual demonstration of natural selection when making claims of adaptive role
  - Triangulate!!!!



Molecular spandrels:

Story telling

vs.

Causal understanding

Genomics is full of adaptive stories

Functional and field validation of SNPs effects are needed to discern facts from fiction

Storz & Wheat 2010 *Evolution*

Barrett & Hoekstra 2011 *Nat Rev Genet*

Ongoing work

- Currently trying to write commentary on biases in field
- Please send along other examples I might have missed
  - Feedback / critique is greatly appreciated

Karl Gotthard

Pararge aegeria

Peter Pruisscher

Ram Neethiraj

Stockholm University

Academy of Finland

VETENSKAPSRÅDET
THE SWEDISH RESEARCH COUNCIL

Knut och Alice Wallenbergs Stiftelse