Non-model species and RAD-sequencing

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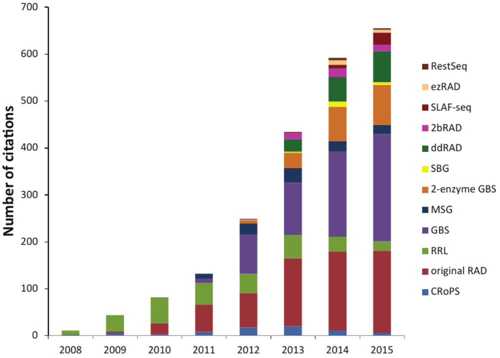
May 2017

RAD-Seq - young and successful NGS methods



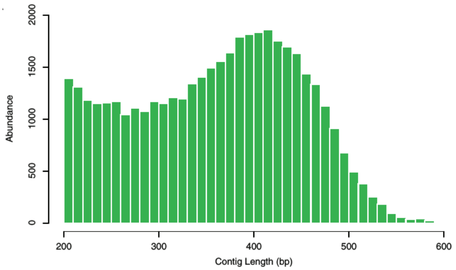
(Andrews et al., 2016)

Purpose of RAD-seq

- Genome-reduction method to fragments adjacent to restriction enzyme recognition sites.
- Increases depth of coverage per locus compared to whole genome sequencing
- High-throughput genotyping of populations (multiplexing using barcoding) at relatively low cost.
- Makes genome-scale population genetic studies possible for non-model species lacking a reference genome.

Reductive *de novo* genome sequencing and SNP identification

- RAD-Seq of the sunflower genome (Illumina)
  - 44.7M reads (PE:40bpx80bp)
- *De novo* assembly of ca. 15.2 Mb in >42,000 contigs
- Identified >94,000 putative SNPs across six lines



(Pegadaraju et al., 2013)

Population genomics and parallel adaptive differentiation in threespine sticklebacks

- Reference genome available
- >45,000 SNPs across 100 individuals ('genotyping by sequencing')
- Consistent signatures of selection between two oceanic and three freshwater populations
- Identified 31 candidate genes of evolutionary significance

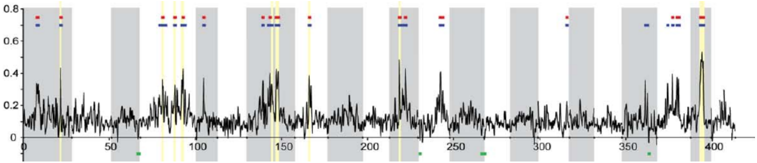
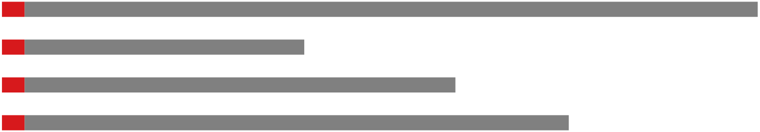


Figure: F_{ST} for SNPs in sliding windows across the genome between oceanic and freshwater populations

Original RAD-Seq protocol

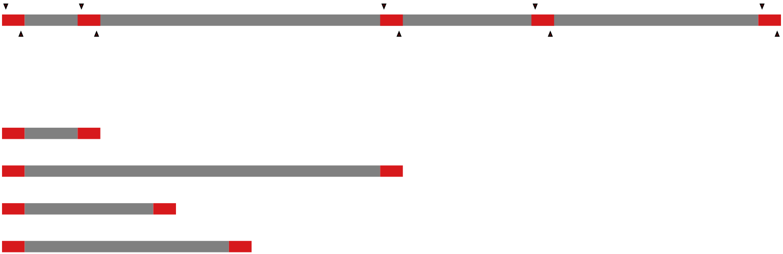
- Developed by (Baird et al., 2008; Miller et al., 2007).
- DNA fragments adjacent to restriction enzyme recognition sites



5’ GAATTC 3’
3’ CTTAAG 5’

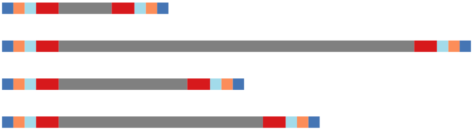
EcoRI recognition site

Step 1: cut DNA



- Note: Bias in GC content of restriction site samples the genome non-randomly

Step 2: ligate P1 adapter

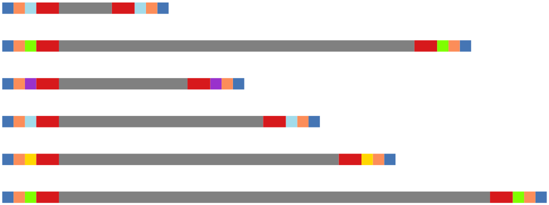


Amplification primer site

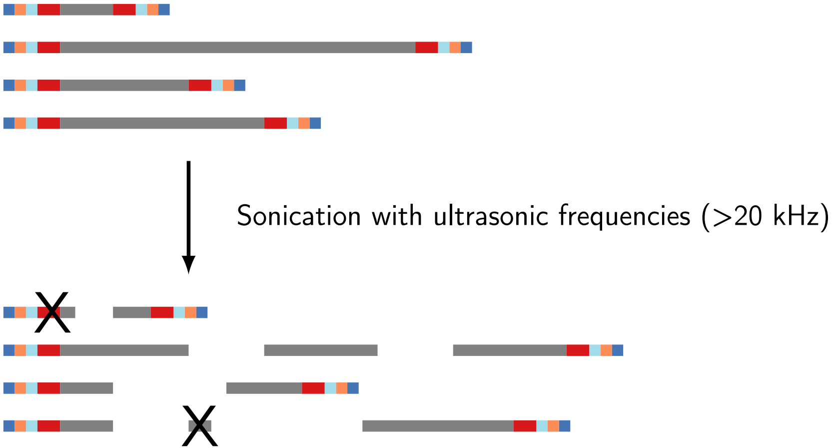
Sequencing primer site (Illumina-specific)

Barcode

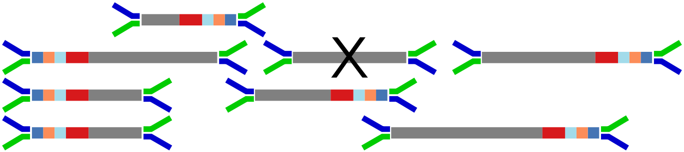
Barcoding allows to pool samples



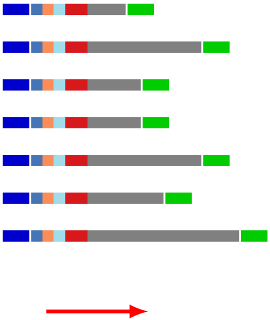
Step 3: Shearing and size selection



Step 4: Ligation of P2 adapter with 'Y' structure



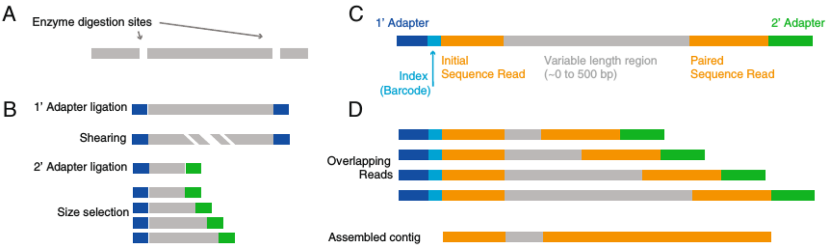
Step 5: Sequence amplified reads on Illumina



Sequence 100 or so bp on Illumina

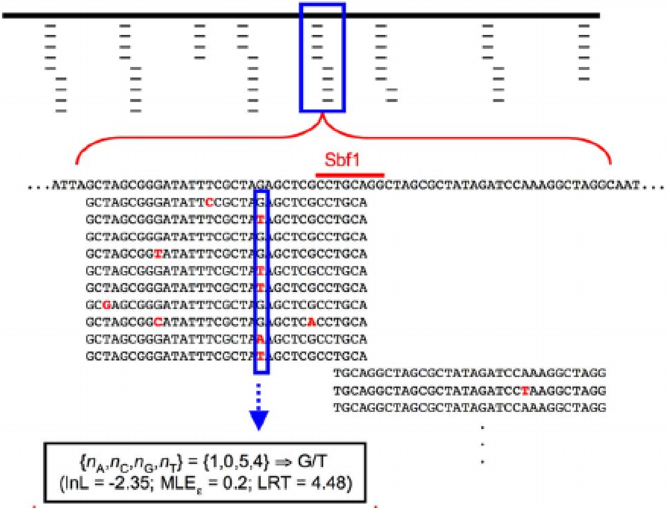
Random shearing of 3'ends helps to detect PCR duplicates

Paired-end sequencing of RAD-tags allows for *de novo* genome sequencing



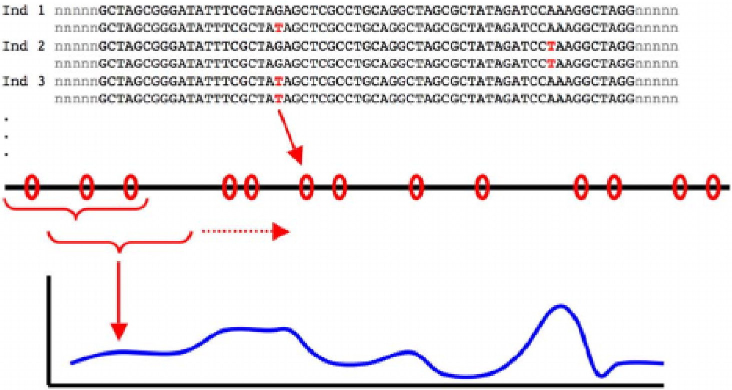
(Pegadaraju et al., 2013)

Calling SNPs from RAD-tags



(Hohenlohe et al., 2010)

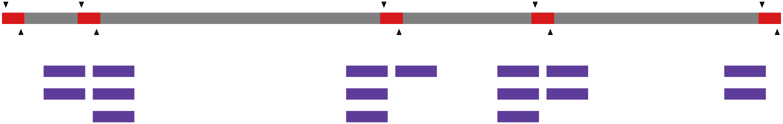
Summary statistics (e.g. population differentiation) along sliding windows



(Hohenlohe et al., 2010)

Double-digest RAD-seq (Peterson et al., 2012)

Single digest RAD-Seq



Double digest RAD-seq

Sequencing of fragments:

- within a specific size range
- flanked by two different cutting sites

EcoRI recognition site

SbfI recognition site

ddRAD compared to single-digest RAD sequencing

1. Rapid and 'cheap' protocol (8 hrs hands-on): Doesn't require difficult and high cost of shearing and enzymatic end-repair.

ddRAD compared to single-digest RAD sequencing

2. Lower number of loci but increased coverage and, thus, higher chance to target the same loci in different individuals.

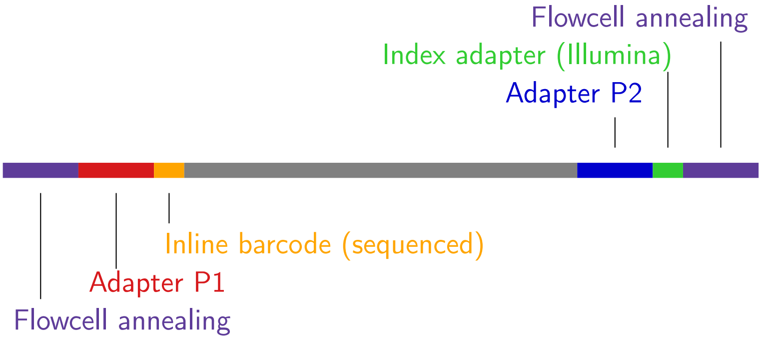
ddRAD compared to single-digest RAD sequencing

3. Coverage expected to be equal among individuals and highest for fragment lengths targeted by size selection.

ddRAD compared to single-digest RAD sequencing

4. Combinatorial indexing allows to multiplex more individuals (up to 12 barcodes were affordable for single-digest RAD-Seq).

Combinatorial indexing allows for high multiplexing levels in ddRAD-Seq



48 x 12 = 576 (multiplexing level)

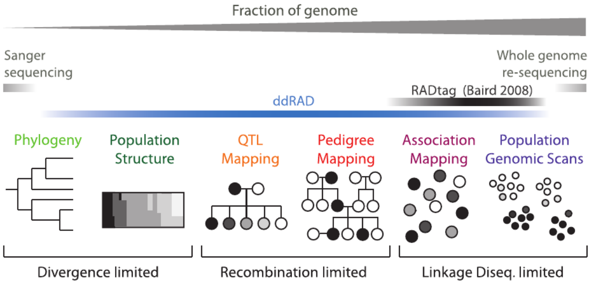
added first, with ligation of adapters, allows to pool samples

added second, with PCR primer, allows to combine multiple pools

Great adjustability of the number of markers makes ddRAD suitable for a broader range of approaches than RAD-Seq

Number of markers adjusted by:

- Cutting frequency of restriction enzymes
- Size selection



(Peterson et al., 2012)

How to predict the number of fragments

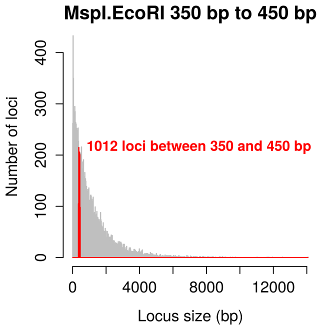
Based on our own study on Guppy

- Targeted coverage: 20x per individual
- Pooling: 60 individuals
- Sequencing output: 24M reads (12M fragments, minimum for Illumina MiSeq v2 paired-end kits)
- Fragments per individual: $12M/60 = 200{,}000$
- Target: 10,000 fragments (to reach a 20x coverage)

What combination of restriction enzymes to use to obtain the appropriate cutting frequency?

In silico genome digestion

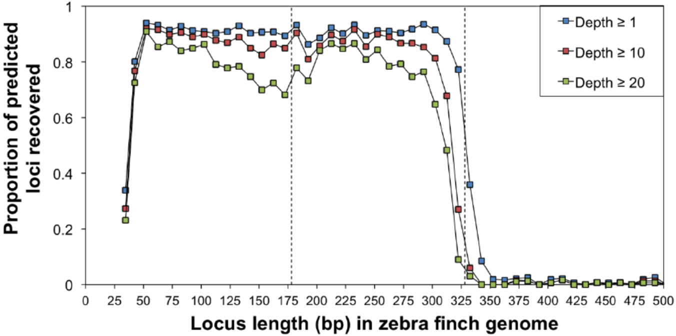
Simulate restriction enzyme digestion with the R package simRAD (Lepais and Weir, 2014)



Based on 10% of the entire genome size

Without reference genome: try, sequence and re-adjust

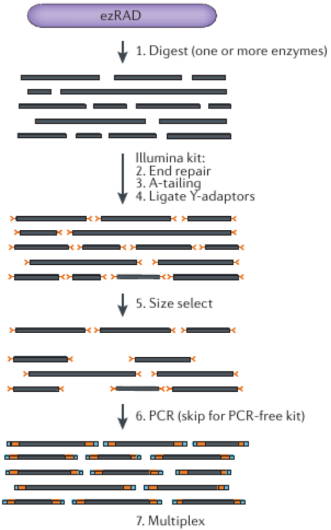
Recovery of *in silico* predicted loci



(DaCosta and Sorenson, 2014)

Targeted: 178-328bp, but short restriction fragments (38–178 bp) were carried through the agarose gel size selection step

ezRAD (Toonen et al., 2013)



(Andrews et al., 2016)

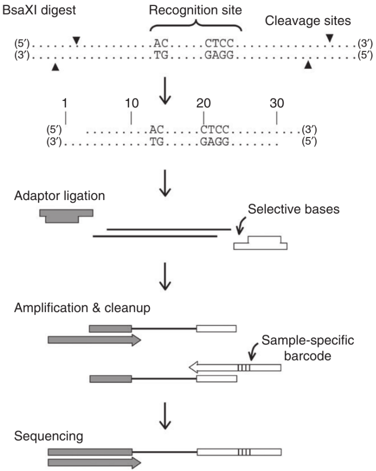
ezRAD (Toonen et al., 2013)

Advantage

- non-PCR kits can avoid PCR duplication and bypass any PCR bias.

2bRAD (Wang et al., 2012)

- Type IIb restriction endonuclease to excise 36-bp fragments.
- Number of loci customized by base-selective adapters.



2bRAD (Wang et al., 2012)

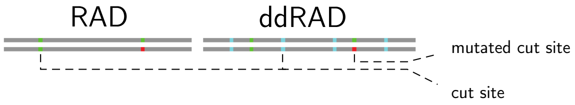
Advantages

- Extremely simple and cost-effective: no purification or size selection.
- No biases due to fragment size selection.
- Sequencing either strand of the restriction fragments allows for the use of strand bias as a quality filtering criteria.

Disadvantages

- 36-bp tags could be too short to be non-ambiguously mapped in highly duplicated genomes.

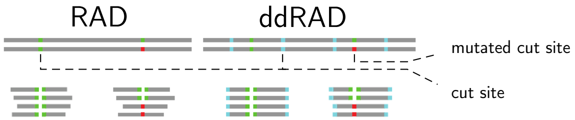
PCR duplicates and null alleles



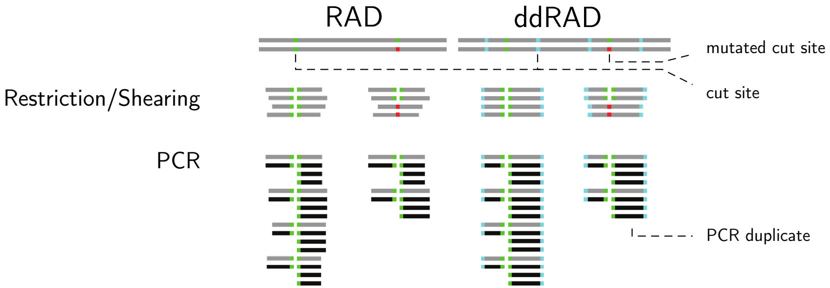
Schweyen et al., 2014

PCR duplicates and null alleles

Restriction/Shearing

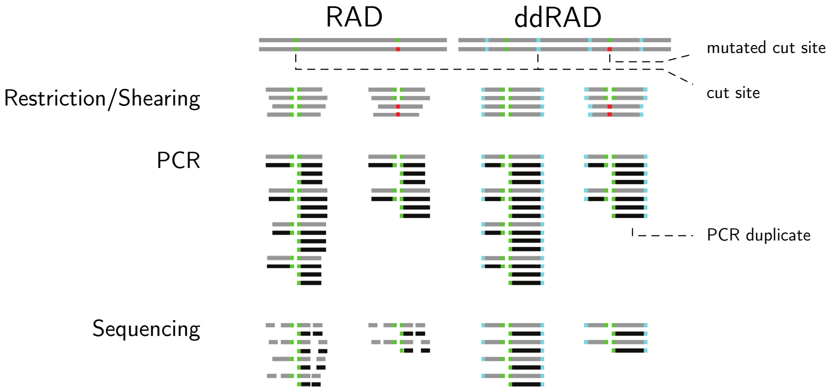


PCR duplicates and null alleles



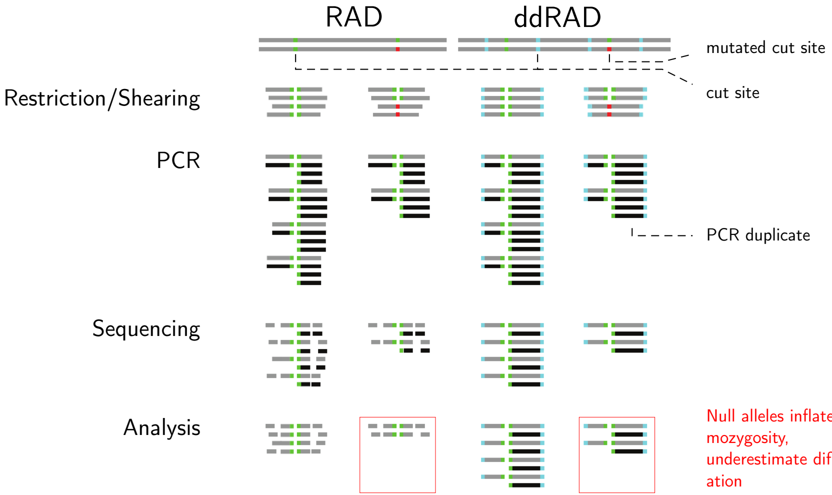
Schweyen et al., 2014

PCR duplicates and null alleles



Schweyen et al., 2014

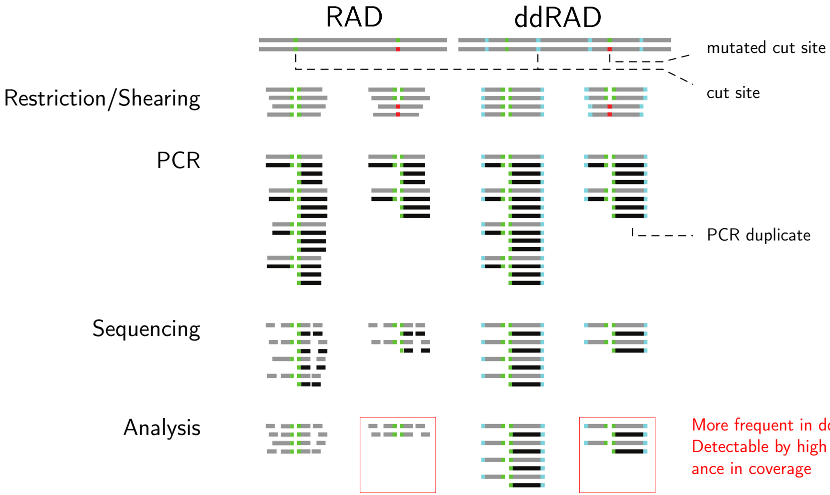
PCR duplicates and null alleles



Null alleles inflate homozygosity, underestimate differentiation

Schweyen et al., 2014

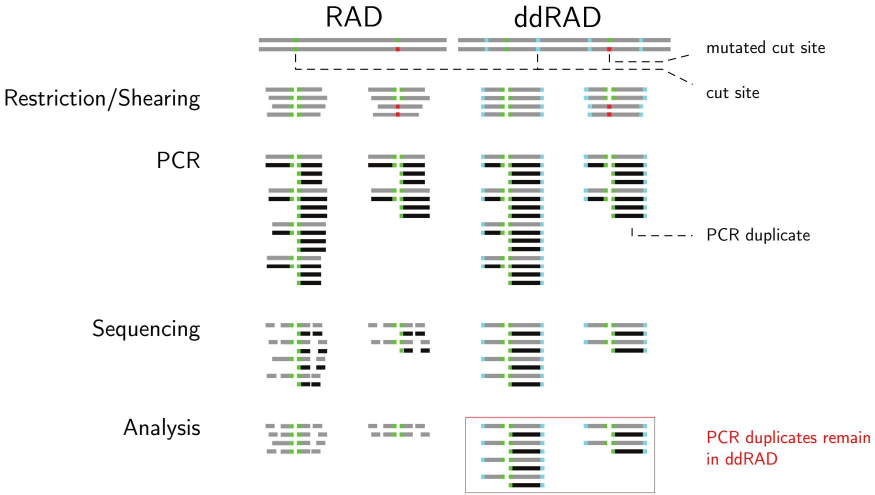
PCR duplicates and null alleles



More frequent in ddRAD
Detectable by high variance in coverage

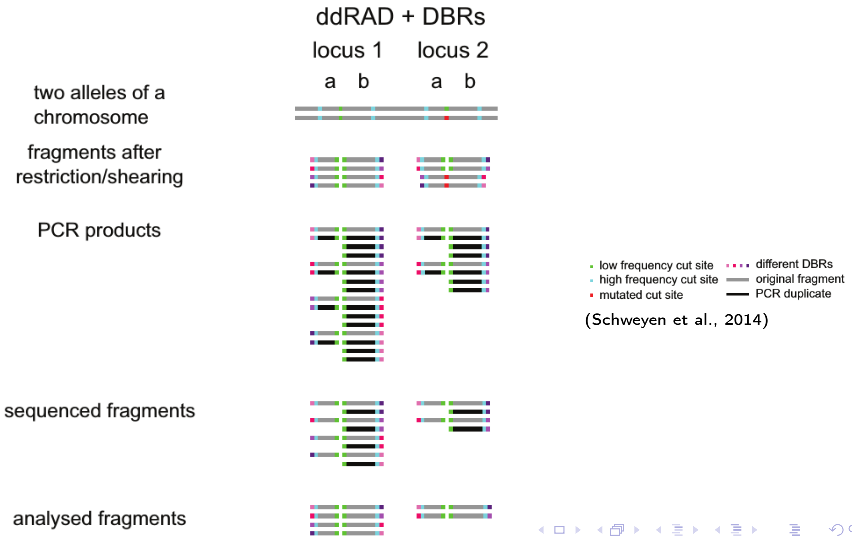
Schweyen et al., 2014

PCR duplicates and null alleles



Schweyen et al., 2014

Degenerate base regions detect PCR duplicates in ddRAD ((Schweyen et al., 2014; Tin et al., 2015))



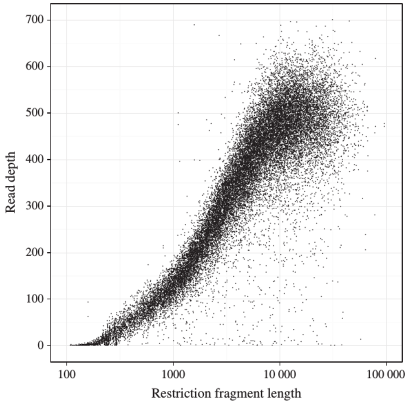
(Schweyen et al., 2014)

Avoid PCR duplicates

- Reduce occurrence by lowering PCR steps
- Avoid PCR duplicates in ezRAD with Illumina PCR-free kits

Shearing introduces bias in coverage

Bias in sequencing depth towards larger fragment sizes

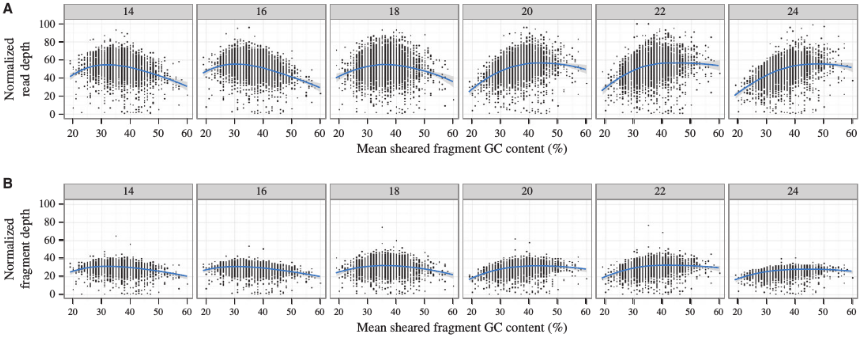


(Davey et al., 2013)

Reason: Fragments of <10 kb shear with lower efficiency

Amplification bias in favor of high GC content

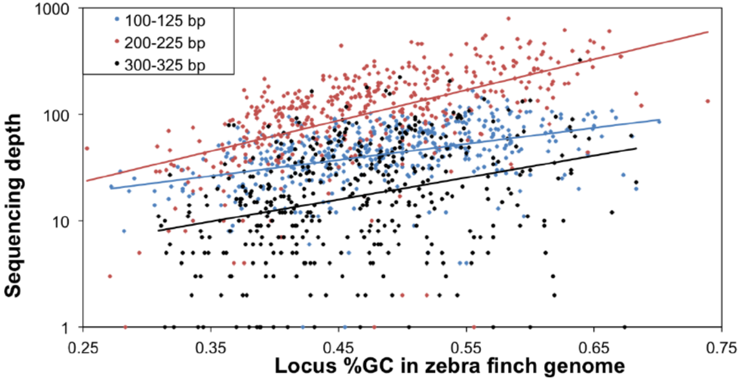
Read depths are influenced by GC content and number of PCR cycles, with (A) or without PCR duplicates (B).



(Davey et al., 2013)

Modifications of PCR enrichment can help (see (Benjamini and Speed, 2012; Puritz et al., 2014b))

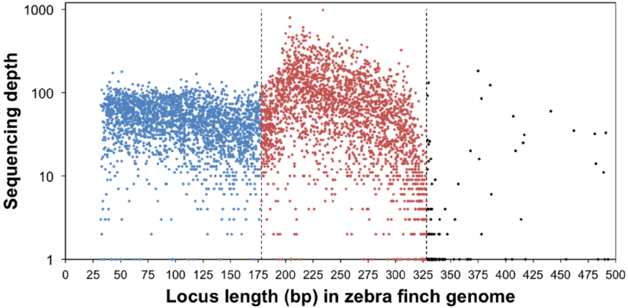
Sequencing depth bias in favor of loci with high GC content



(DaCosta and Sorenson, 2014)

- Combined with a GC-rich recognition sequence, this can result in an overrepresentation of GC-rich portions of the genome

Amplification and, thus, depth decreases with fragment length



(DaCosta and Sorenson, 2014)

- Affects ddRAD more than RAD-seq (each locus different fragment lengths) or 2bRAD (all loci same fragment length)
- Bias reduced by precise size selection (Pippin Prep instrument) (DaCosta and Sorenson, 2014).

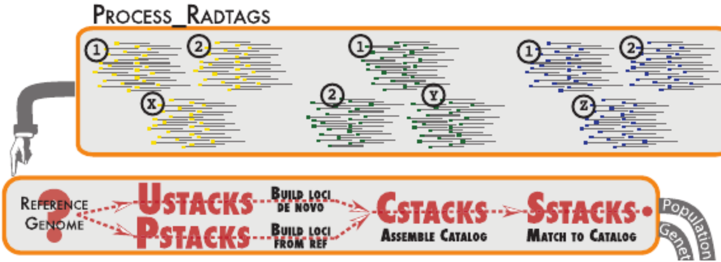
STACKS (Puritz et al., 2014a)

Stacks: Building and Genotyping Loci *De Novo* From Short-Read Sequences

Julian M. Catchen,* Angel Amores,† Paul Hohenlohe,* William Cresko,* and John H. Postlethwait†,[1]
*Center for Ecology and Evolutionary Biology and †Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403

STACKS - basic pipeline for RAD-Seq

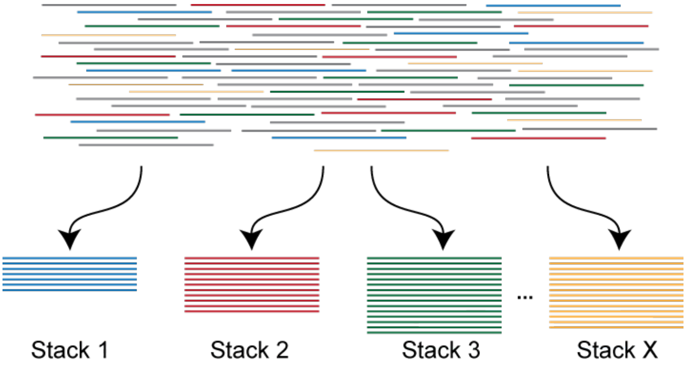
STACKS - software pipleine to build loci from RADseq reads and use them for population genomics and phylogeographic analyses.



(Catchen et al., 2013)

STACKS - Ustacks *de novo* assembly step 1

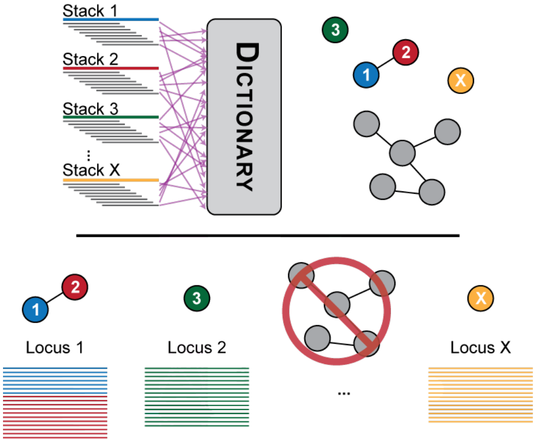
- Only exact matches are assembled
- Secondary reads are set aside
- The minimum stack depth parameter controls the number of raw reads required to form an initial stack



(Catchen et al., 2013)

STACKS - Ustacks *de novo* assembly step 2

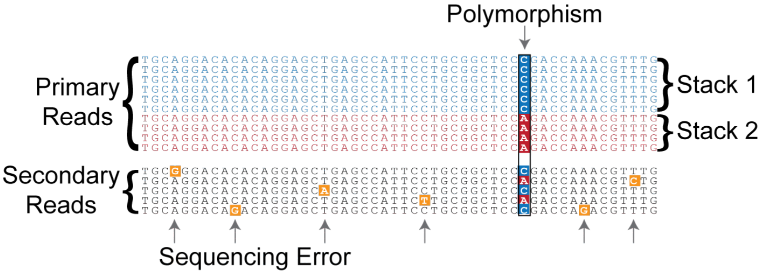
- Stacks with few nucleotide differences are merged.
- Repetitive sequences with many alleles are excluded



(Catchen et al., 2013)

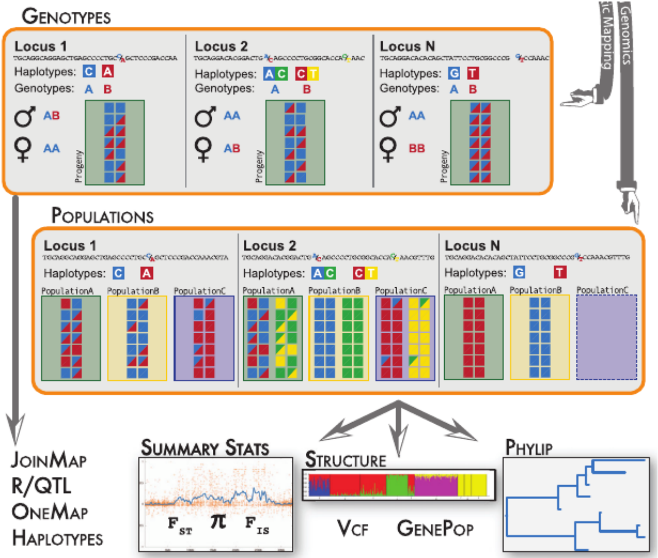
STACKS - Ustacks *de novo* assembly step 3

- Alignment of secondary reads (those not indcluded in stacks) against stacks.
- Alleles are discriminated from sequencing errors by their frequency.



(Catchen et al., 2013)

STACKS - populations or genotypes pipeline



(Catchen et al., 2013)

DDocent (Puritz et al., 2014a)

PeerJ

dDocent: a RADseq, variant-calling pipeline designed for population genomics of non-model organisms

Jonathan B. Puritz, Christopher M. Hollenbeck and John R. Gold

Marine Genomics Laboratory, Harte Research Institute, Texas A&M University-Corpus Christi, Corpus Christi, TX, USA

DDocent (Puritz et al., 2014a)

Uses stand-alone software packages to perform

- quality trimming
- adapter removal
- *de novo* assembly of RAD loci
- read mapping
- SNP and InDel calling
- data filtering.

Identifies more SNPs at a higher coverage than STACKS, due to

- simulatneous use of forward and reverse reads during alignment to reference instead of clustering
- quality trimming instead of removing entire reads

AftrRAD

MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2015) **15**, 1163–1171 doi: 10.1111/1755-0998.12378

AftrRAD: a pipeline for accurate and efficient *de novo* assembly of RADseq data

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PyRAD

Bioinformatics Advance Access published March 20, 2014

BIOINFORMATICS **ORIGINAL PAPER**

2014, pages 1–6
doi:10.1093/bioinformatics/btu121

Phylogenetics

Advance Access publication March 5, 2014

PyRAD: assembly of *de novo* RADseq loci for phylogenetic analyses

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Associate Editor: David Posada

Important considerations

- Degraded DNA interfers with cutted DNA in methods with enzyme-unspecific adaptors
- Higher amount of starting DNA can reduce number of PCR cycles and thus minimize PCR duplicates.
- RADseq libraries are low-diversity libraries as they all start with the same cutting site and can cause problems in cluster generation for Illumina sequencing.
  - Solution: Reduce cluster density and spike-in PhiX control or use dark-cycling.

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