H3ABioNet

Pan African Bioinformatics Network for H3Africa

GWAS QC -theory and steps

Introduction to Genome Wide Association Studies

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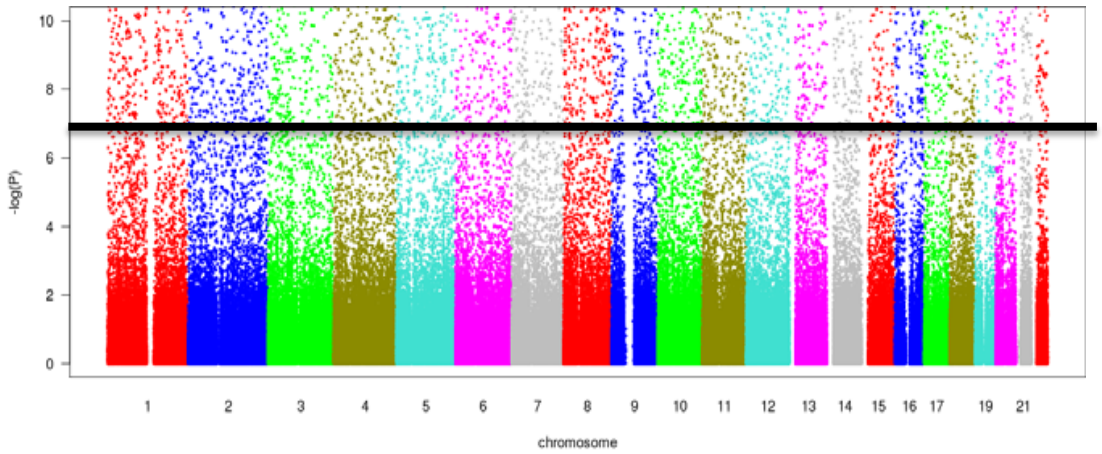
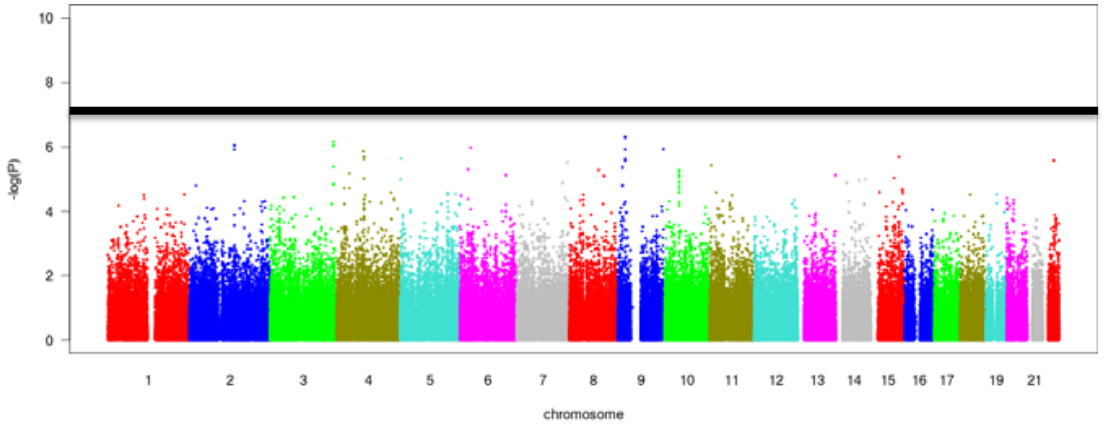
Quality control for GWAS studies

- Genotyping experiments often generate a mixed bag of results
- Errors might originate at many different steps
  - Sample selection related issues
  - Sample handling related issues
  - Genotyping chip related issues
  - Batch effect related issues
- Steps
  - QC by SNP
  - QC by sample

German MI family study Affymetrix 500K Array Set
SNPs on chips: 493,840



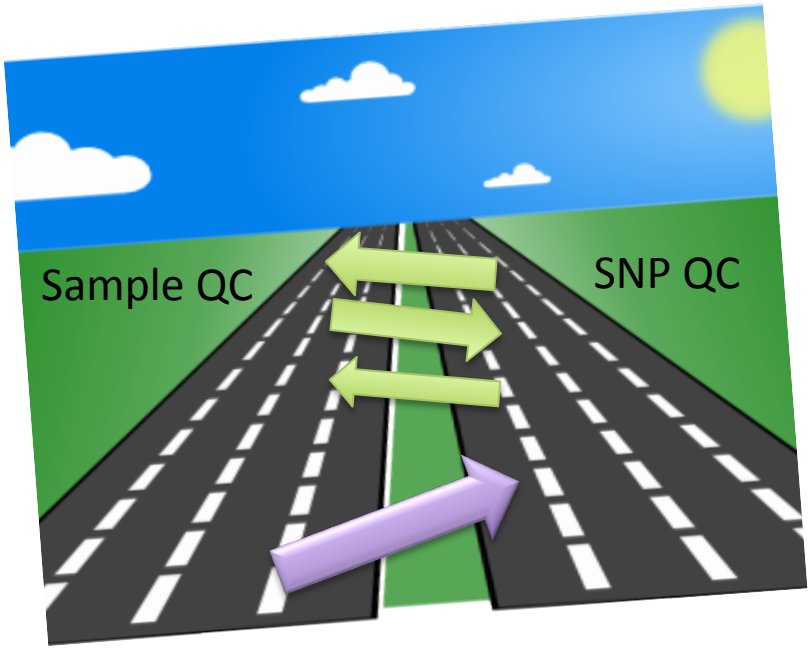
SNPs passing QC: 270,701



Samani et al. 2007 N Engl J Med 357:443-53

Roadmap

- Discordant sex information
- High Missingness
- Excess or deficiency of heterozygosity
- Duplicate or related
- Divergent ancestry



- Low minor allele frequency
- Missingness
- Differential missingness
- Hardy-Weinberg outliers


nature protocols

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NATURE PROTOCOLS | PROTOCOL

Data quality control in genetic case-control association studies

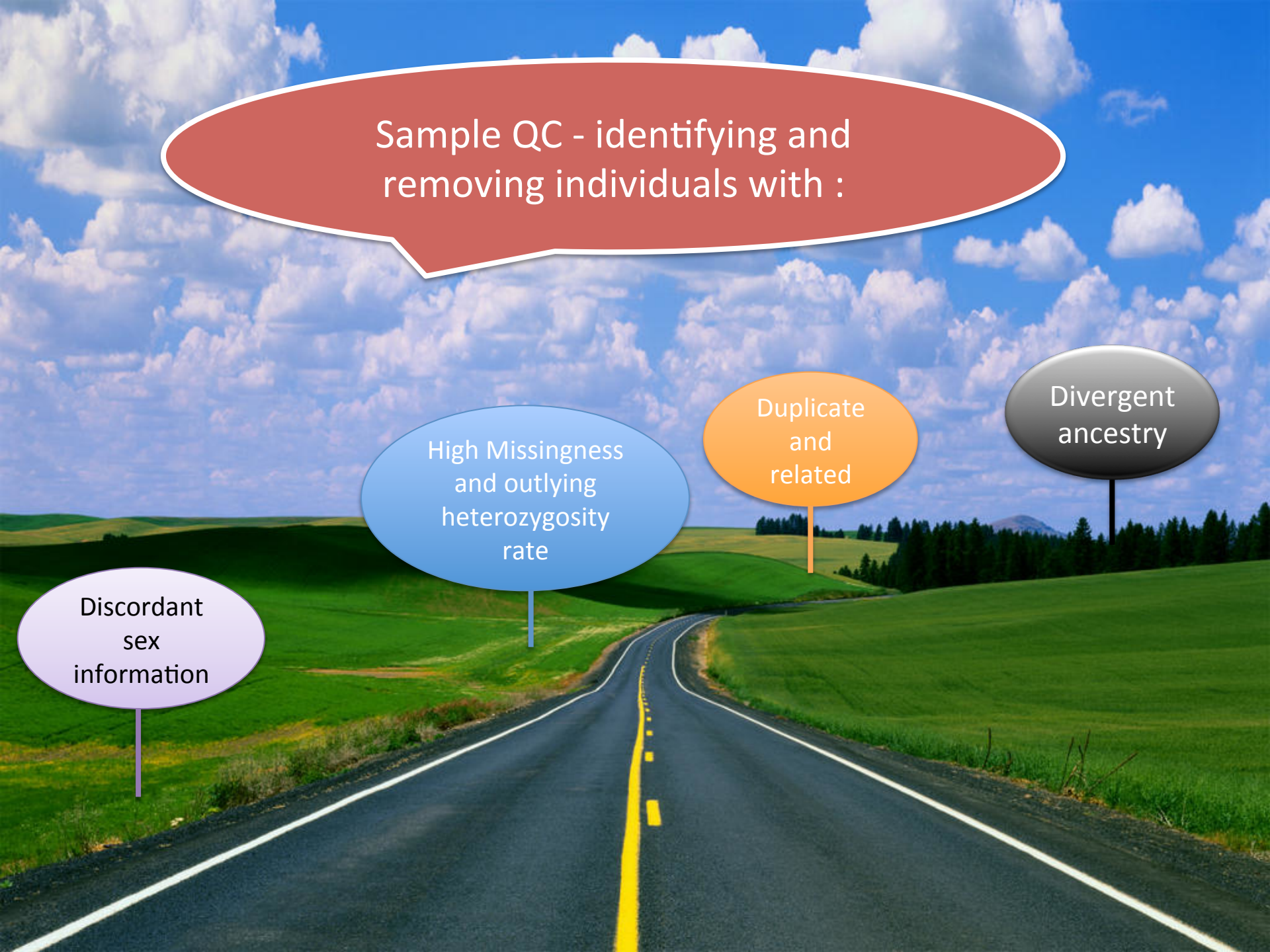
Carl A Anderson, Fredrik H Pettersson, Geraldine M Clarke, Lon R Cardon, Andrew P Morris & Krina T Zondervan

Affiliations | Contributions | Corresponding authors

Nature Protocols **5**, 1564–1573 (2010) | doi:10.1038/nprot.2010.116
Published online 26 August 2010

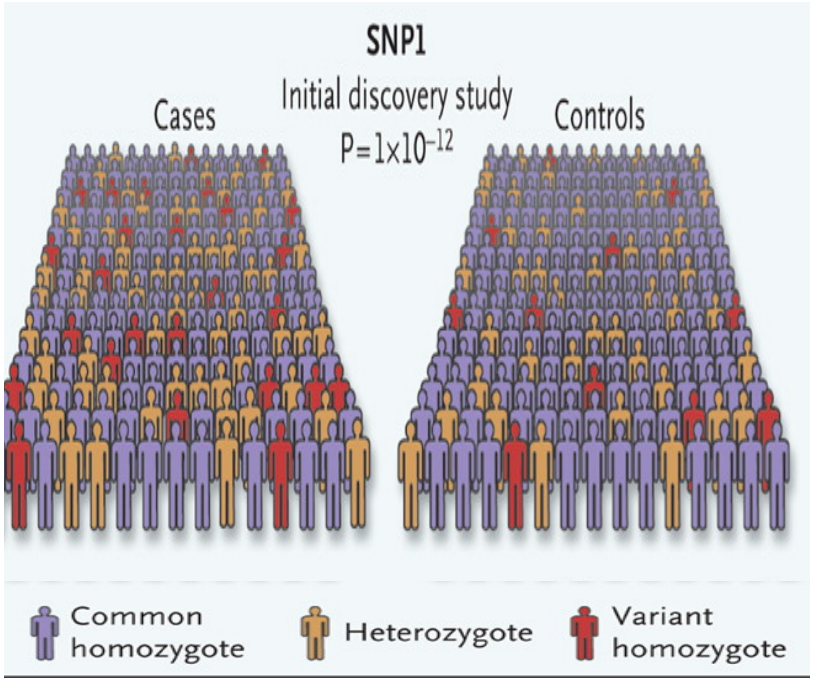
Software

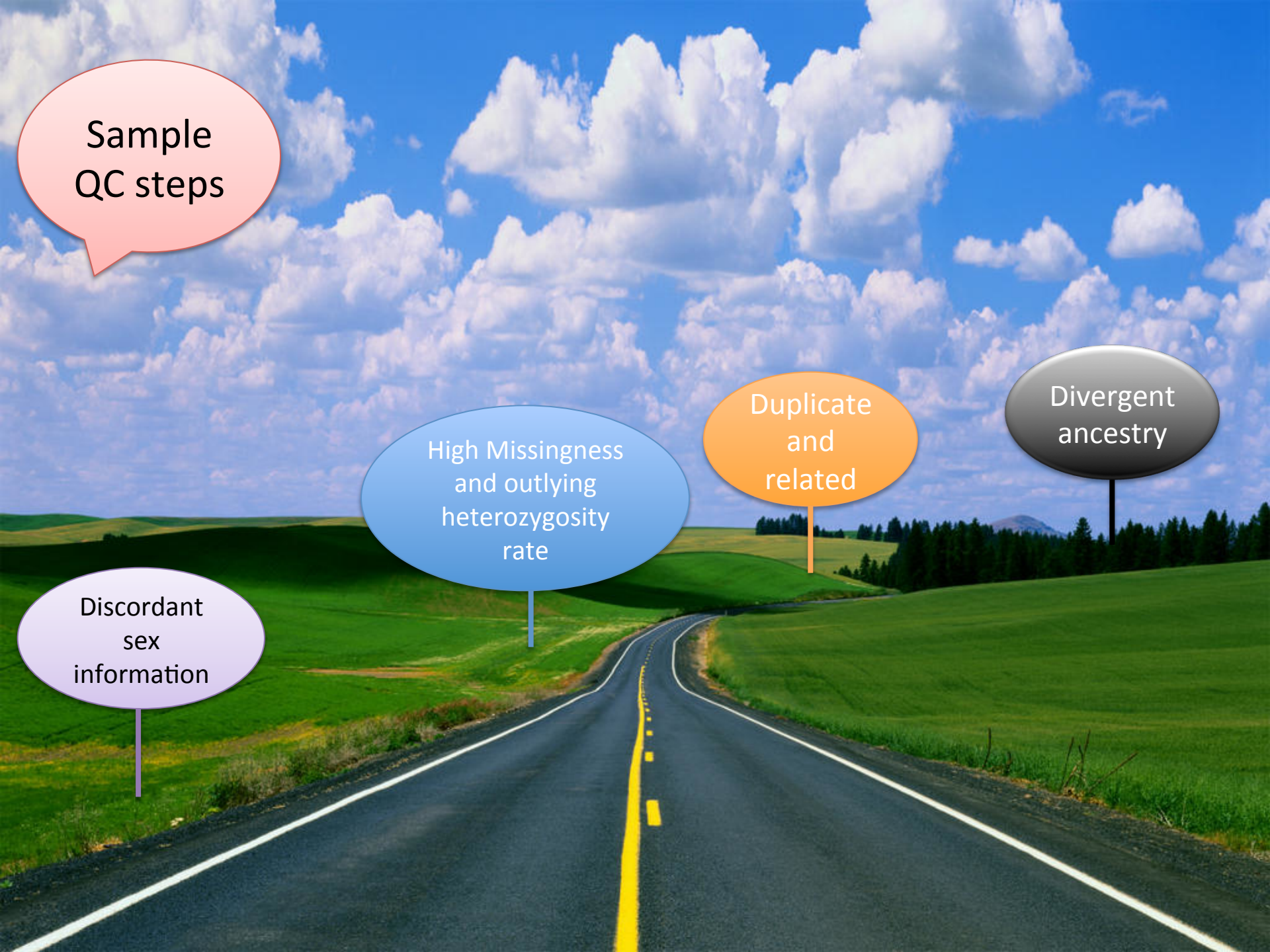
- **Programs required for QC**
  - PLINK (Purcell, 2007)
  - Scripts for processing results files
  - R (Statistical Software) for plotting results
- **Programs for population structure analysis**
  - SmartPCA, PLINK
  - Admixture


Sample QC - identifying and removing individuals with :
Discordant sex information
High Missingness and outlying heterozygosity rate
Duplicate and related
Divergent ancestry

Why quality control individuals?

- Sample handling related issues:
  - Poor DNA quality/concentration
  - Contamination
  - Error in labeling/plating
- Sample selection related issues:
  - Cryptic relatedness
  - Population structure
- Measures to remove individuals not genotyped properly




Sample QC steps
Discordant sex information
High Missingness and outlying heterozygosity rate
Duplicate and related
Divergent ancestry

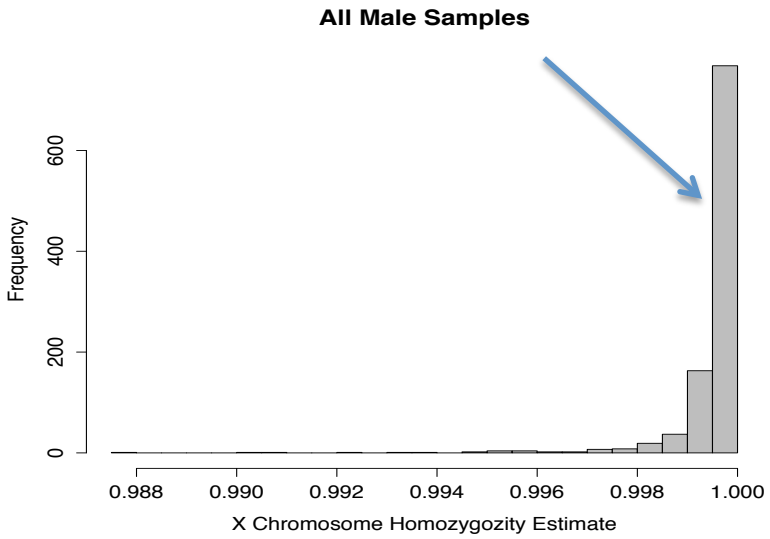
Using genotypic data to estimate sample sex

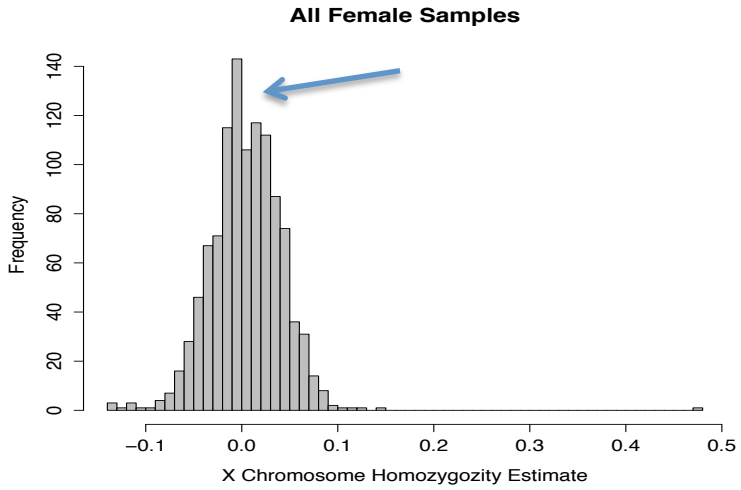
- Males have a single X chromosome and therefore can be estimated to be homozygous for all the X chromosome SNPs (other than those in the pseudo autosomal region(PAR)).
- Therefore, X chromosome homozygosity estimate for males(XHE) is 1

- Plink assigns sex based on XHE estimate (F or inbreeding coefficient) :
- Male (1) : XHE >0.80
- Female (2) : XHE <0.20
- No sex (0) : 0.20 <XHE <0.80

- Comparisons of predicted and observed sex can be used to identify miscoded sex or **sample mix-ups**, etc.

- Samples with discordant sex information are removed





Identify individuals with discordant sex information

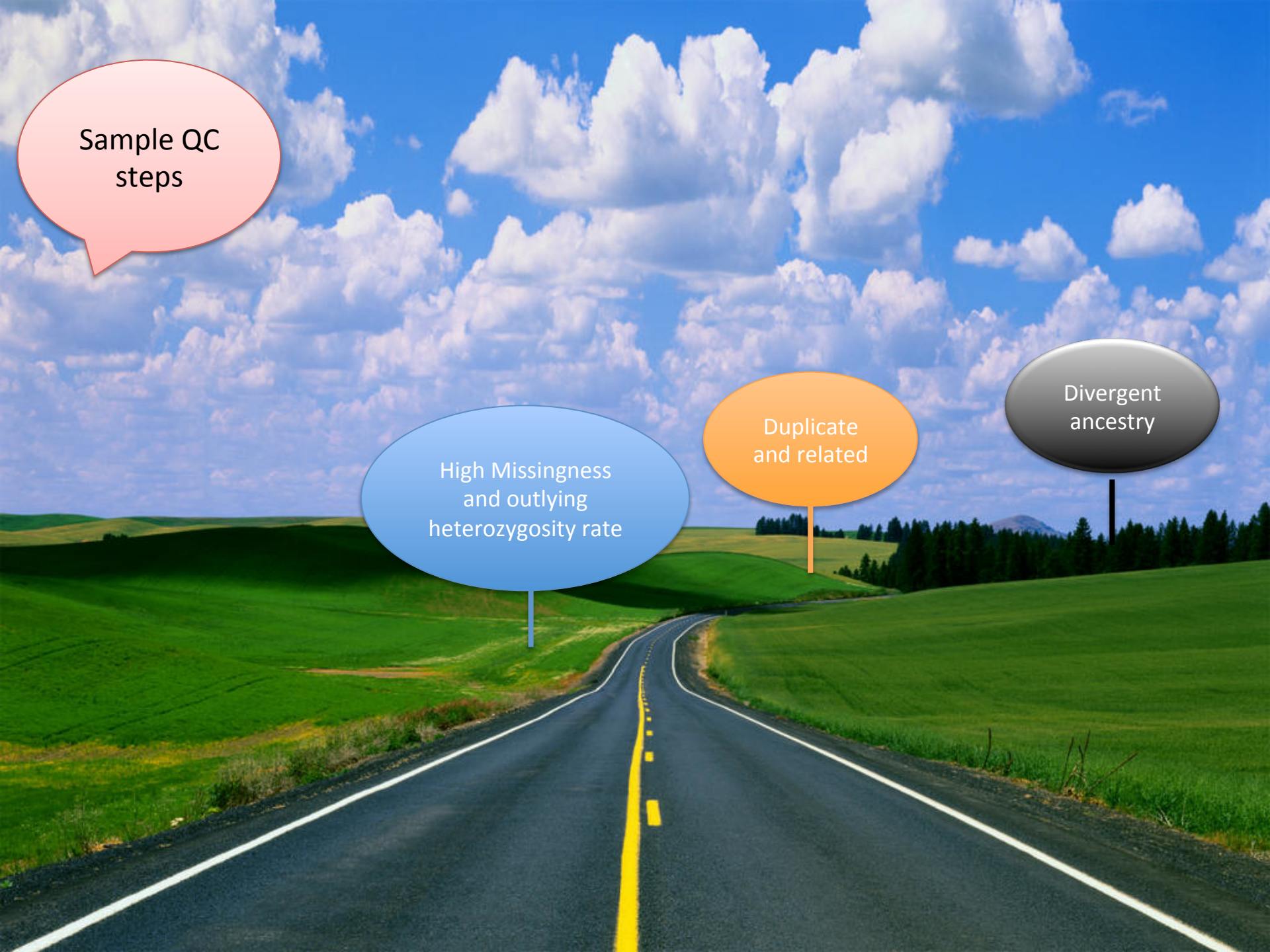
```
plink --bfile example --check-sex --out sexstat --noweb
```

Creates a file named sexstat.sexcheck

FID	IID	PEDSEX	SNPSEX	STATUS	F
P554	P554	2	2	OK	-0.02654
P555	P555	1	0	PROBLEM	0.5685
P557	P557	2	2	OK	0.1264
P558	P558	2	2	OK	-0.0007684

Select individuals with Status="PROBLEM" in the file sexstat.sexcheck
Try to identify the problem. If the problem cannot be resolved write the IDs of the individuals with discordant sex information to a file "fail_sex_check-example.txt"

```
grep "PROBLEM" sexstat.sexcheck > fail_sex_check-example.txt
```


Sample QC steps
High Missingness and outlying heterozygosity rate
Duplicate and related
Divergent ancestry

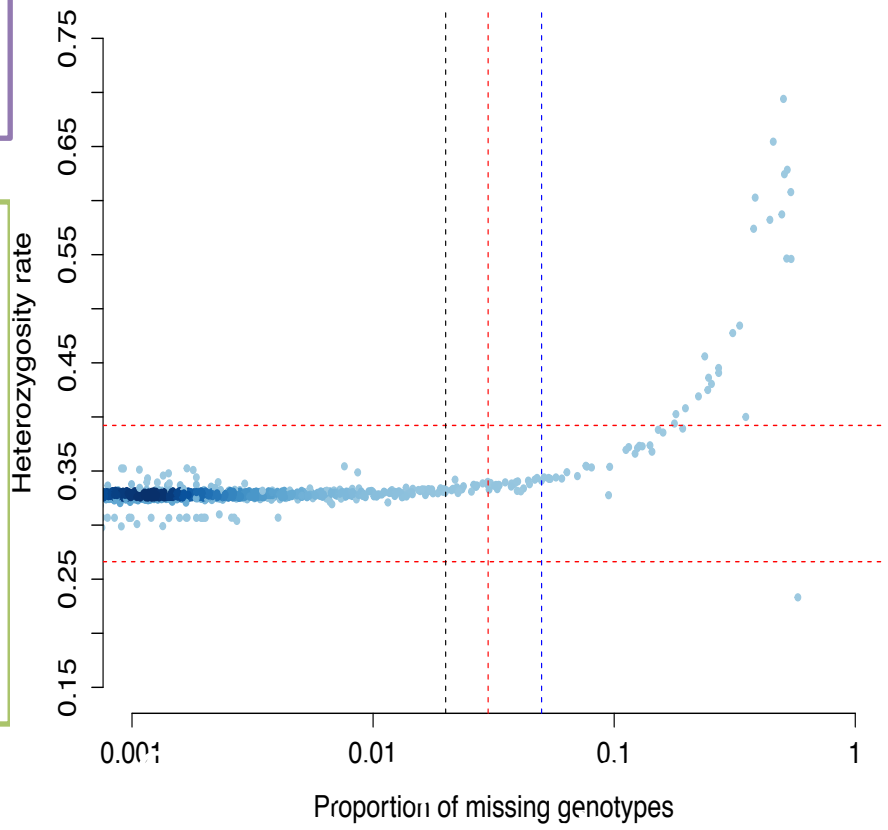
Genotyping call rate

- Per sample (individual) rate
- Number of **non-missing genotypes** divided by the total number of **genotyped** markers.
- Low genotyping call rate indicate problem with **sample DNA** like low concentration.
- Thresholds used generally vary between 3% and 7%

Heterozygosity Rate

- Per sample (individual) rate
- Number of (total non-missing genotypes(N) – homozygous(0)) genotypes divided by total non-missing genotypes(N)
- Excess heterozygosity - Possible sample **contamination**
- Less than expected heterozygosity- Possibly **inbreeding**
- Threshold for inclusion is generally Mean ± 3 std.dev. over all samples

Genotyping call rate and heterozygosity rate are generally plotted together.
Cutoffs are selected so as to identify outlier individuals based on both the statistics



Identification of individuals with elevated missing data rates

```
plink --bfile example –missing
--out example_miss
```

Missing phenotype (Y/NN)

FID	IID	MISS_PHENO	N_MISS	N_GENO	F_MISS
P554	P554	N	4096	97722	0.04191
P557	P557	N	4011	97722	0.04105
P558	P558	N	4327	97722	0.04428
P562	P562	N	4099	97722	0.04195

```
 Before frequency and genotyping pruning, there are 98604 SNPs
646 founders and 0 non-founders found
34704 heterozygous haploid genotypes; set to missing
Writing list of heterozygous haploid genotypes to [ example_miss.hh ]
3452 SNPs with no founder genotypes observed
Warning, MAF set to 0 for these SNPs (see --nonfounders)
Writing list of these SNPs to [ example_miss.nof ]
Writing individual missingness information to [ example_miss.imiss ]
Writing locus missingness information to [ example_miss.lmiss ]
```

HR	SNP	N_MISS	N_GENO	F_MISS
1	vh_1_1108138	9	646	0.01393
1	vh_1_1110294	4	646	0.006192
1	rs7515488	1	646	0.001548
1	rs6603785	9	646	0.01393

Identification of individuals with extremely high or low heterozygosity rate

```
plink --bfile example --het --out example_het
```

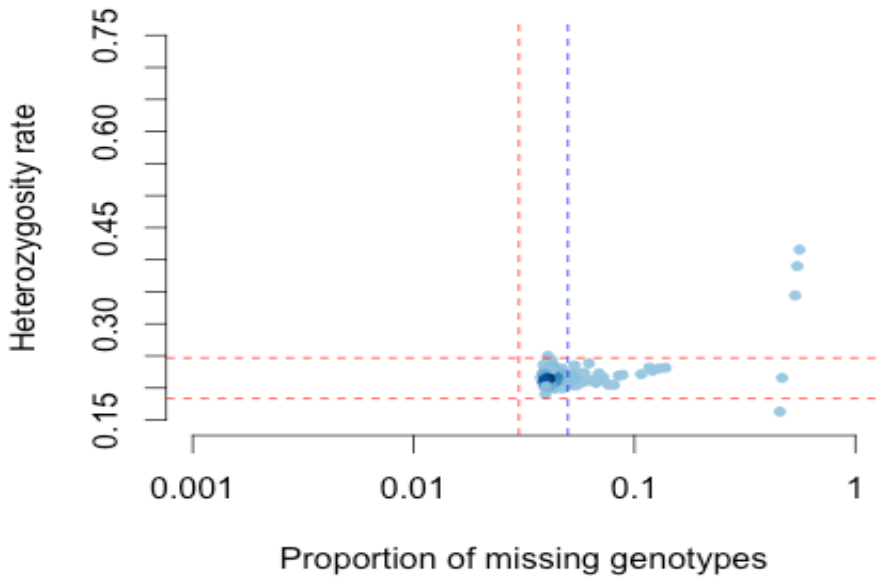
```
 Before frequency and genotyping pruning, there are 98604 SNPs
646 founders and 0 non-founders found

Detected that binary PED file is v1.00 SNP-major mode
Before frequency and genotyping pruning, there are 98604 SNPs
646 founders and 0 non-founders found
34704 heterozygous haploid genotypes; set to missing
Writing list of heterozygous haploid genotypes to [ example_het.hh ]
3452 SNPs with no founder genotypes observed
Warning, MAF set to 0 for these SNPs (see --nonfounders)
Writing individual heterozygosity information to [ example_het.het ]
```

Observed number of homozygous genotypes

Expected number of homozygous genotypes

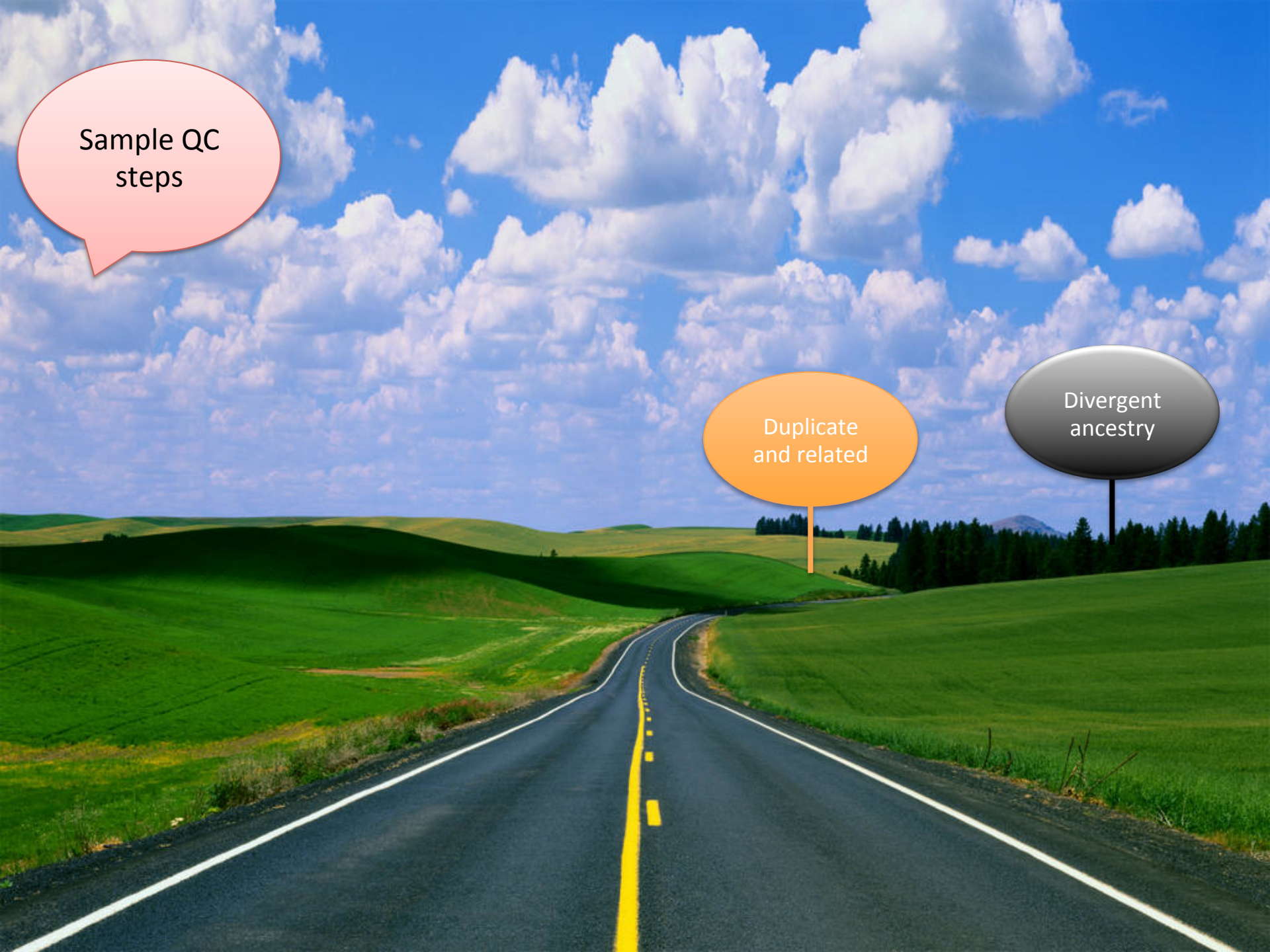
Inbreeding coefficient estimate

FID	IID	O(HOM)	E(HOM)	N(NM)	F
P554	P554	67663	6.725e+04	86305	0.02173
P557	P557	66873	6.731e+04	86388	-0.02301
P558	P558	67155	6.707e+04	86091	0.004538
P562	P562	68367	6.724e+04	86306	0.05891



Based on the plot we need to decide reasonable thresholds at which to exclude individuals based on elevated missing or extreme heterozygosity.

We decided to exclude all individuals with a genotype failure rate ≥ 0.06 and/or heterozygosity rate ± 3 standard deviations from the mean

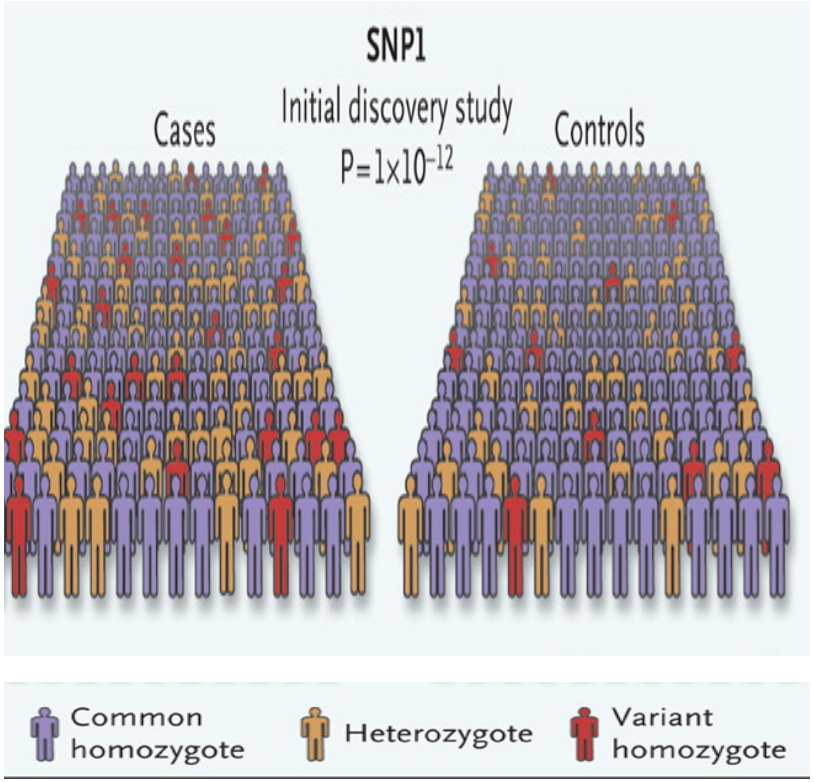

Sample QC steps
Duplicate and related
Divergent ancestry

Identify related and duplicate individuals

- A basic assumption of standard population-based case-control association studies is that all the samples are **unrelated** (i.e. the maximum relatedness between any pair of individuals is less than a second degree relative)

- Presence of duplicate and related individuals in the dataset may **introduce bias** and cause **genotypes in families** to be over-represented.

- To identify duplicate and related individuals, a metric (identity by state, IBS) is calculated for each pair of individuals based on the average proportion of alleles shared in common at genotyped SNPs (excluding the sex chromosomes)



- The IBS method works best when only **independent SNPs** are included in the analysis.
- Independent SNP set for IBS calculation is generally prepared by removing regions of extended LD and pruning the remaining regions so that no pair of SNPs within a given window (say, 50kb) is correlated .
- Following the calculation of IBS between all pairs of individuals, duplicates are denoted as those with an IBS of 1.
- Related individuals will share more alleles IBS than expected by chance, with the degree of additional sharing proportional to the degree of relatedness.

Identity by State (IBS) = 1

How many alleles are in common?

Identity by State (IBS) = 0.5

IBS =(IBS2 + 0.5 × IBS1)/(*N* SNP pairs)

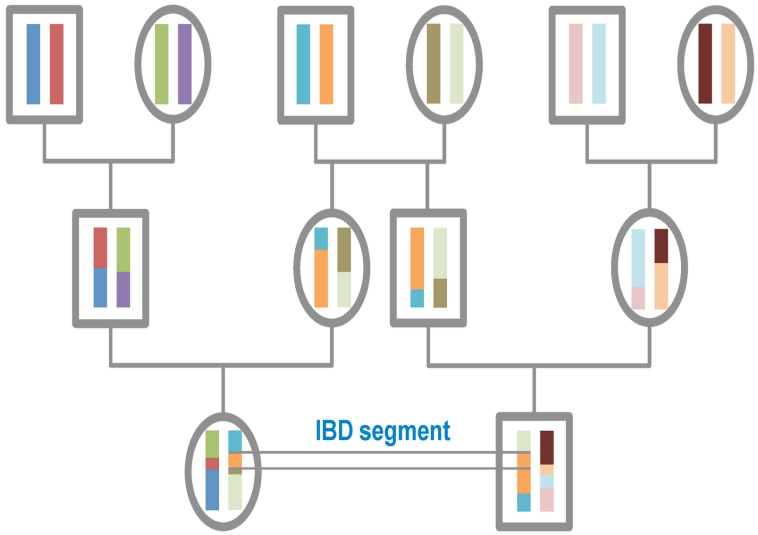
IBS2 = number of loci in which the two individuals have two alleles in common
IBS1= number of loci in which the two individuals have one allele in common,
N SNP pairs =number of common, nonmissing SNPs.

- The degree of **recent shared ancestry for a pair of individuals** (identity by descent, IBD) can be estimated using genome-wide IBS data using Plink. (IBD shown as pi_hat in plink)

- The expectation is that :
  - IBD = 1 for duplicates or monozygotic twins
  - IBD = 0.5 for first-degree relatives,
  - IBD = 0.25 for second-degree relatives
  - IBD = 0.125 for third-degree relatives

- Genotyping error, LD and population structure cause variation around these theoretical values and it is typical to remove one individual from each pair with an IBD > 0.1875 (halfway between the expected IBD for third- and second-degree relatives).

- For same reasons an IBD > 0.98 identifies duplicates.



ac

Identification of duplicated or related individuals

identify all pairs of individuals with an IBD > 0.185.
looks at the individual call rates stored in example_miss.imiss and output the ids of the individual with the lowest call-rate to 'fail_IBD_example.txt' for subsequent removal

As this step is highly computationally intensive it is a good option to remove regions of high LD (pre-calculated and stored in the file, high-LD-regions.txt) before the IBS run

creates the file example.prune.in, containing the list of SNPs to be kept in the analysis.

```
PREPROCESSING

plink --bfile example --exclude high-LD-regions.txt --range --
indep-pairwise 50 5 0.2 --out example

CALCULATING IBD
plink –bfile example --extract example.prune.in --genome --out
example

FILTERING RELATED INDIVIDUALS
perl run-IBD-QC.pl example.genome
```

creates a file example.genome containing pairwise IBS for all pairs of individuals

Population structure

- Population substructure or stratification occurs when samples have different genetic ancestries
- Can lead to spurious associations due to differences in ancestry rather than true associations
- Imperative to check for population structure within samples
- Can control for structure if identified, in downstream analysis

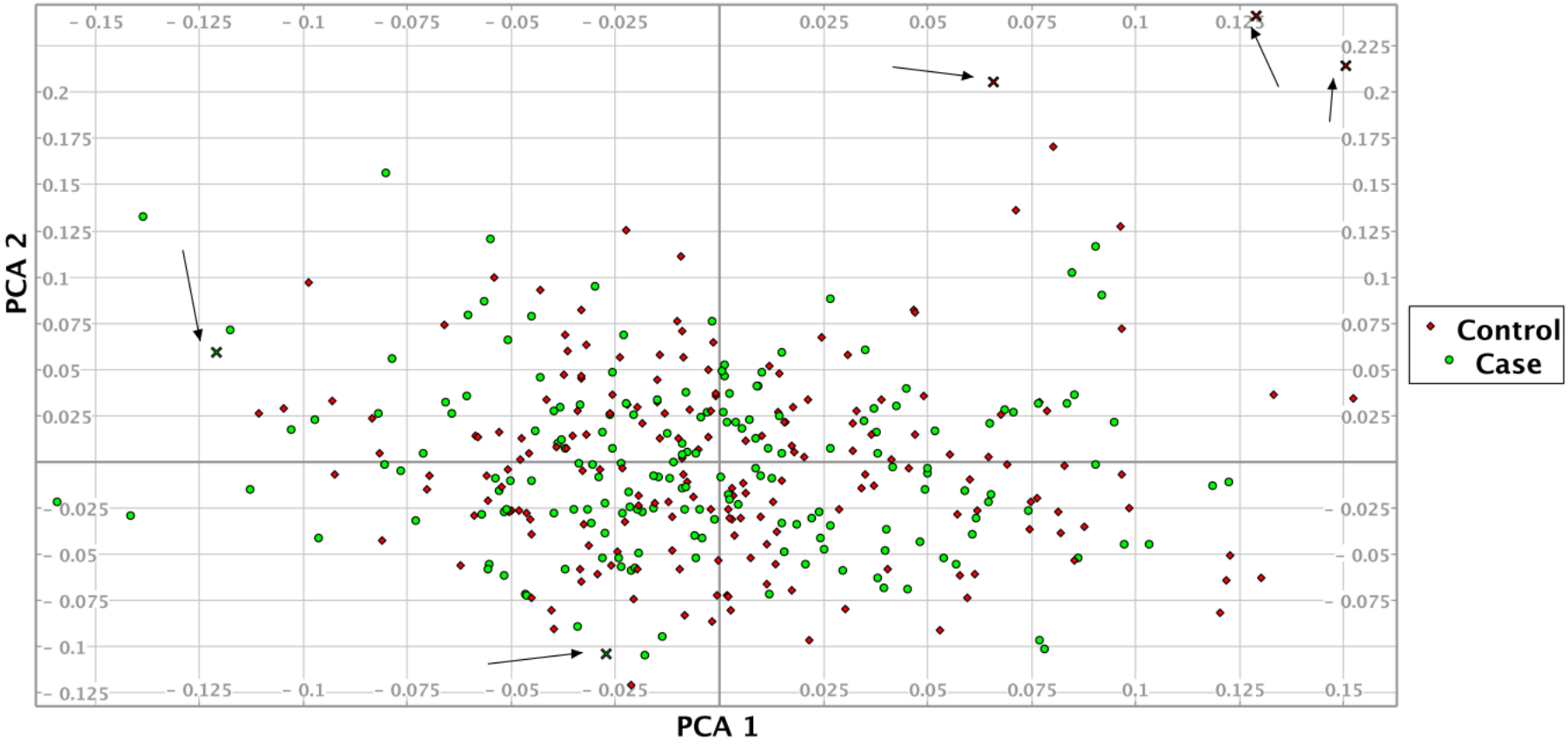
Approaches to identify population structure

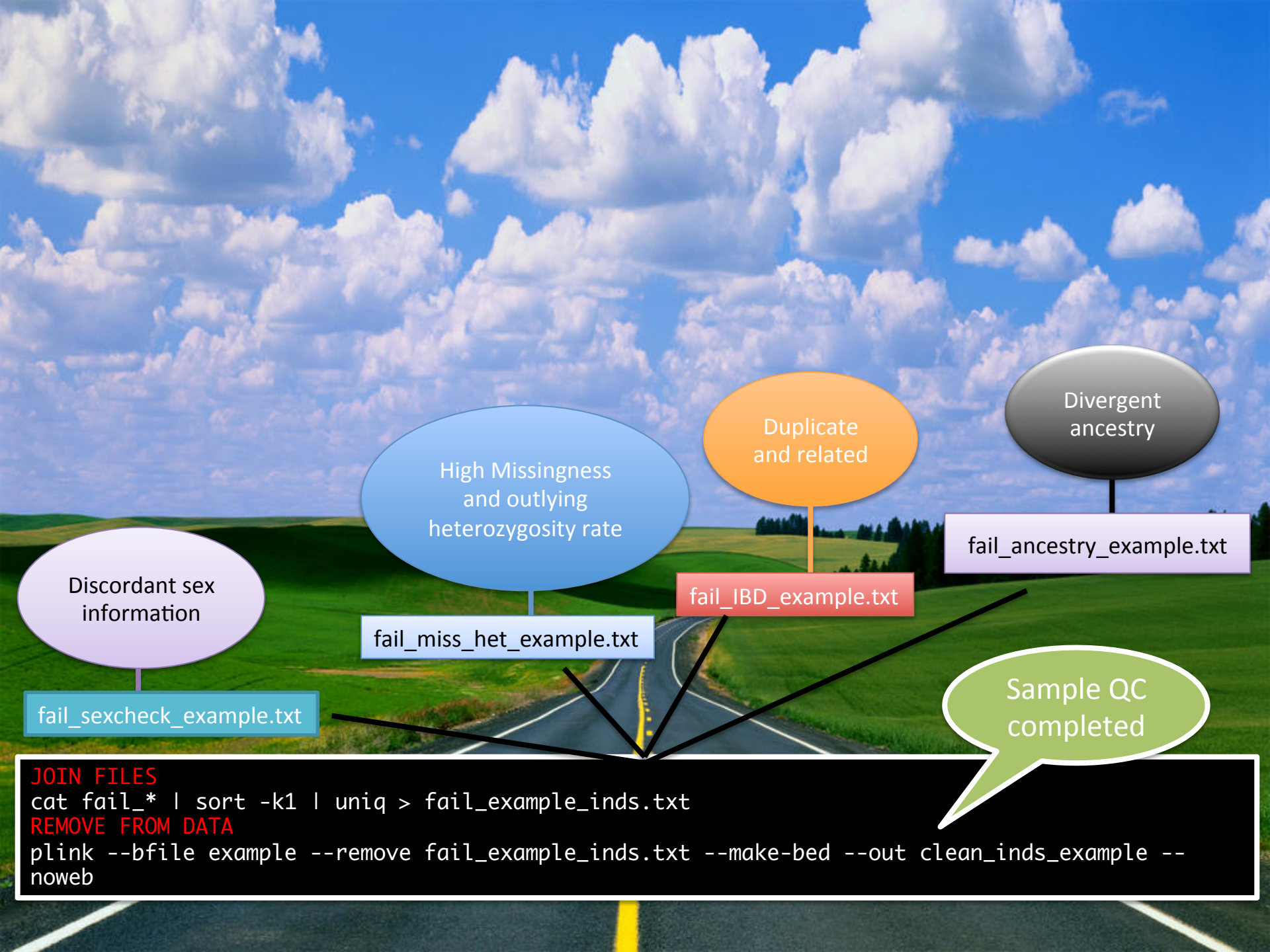
- Methods to measure the ancestry of each sample in the data
- Structure based approach:
  - Admixture, CLUMPP
- Principle component based approach
  - SmartPCA, SNPRelat, PLINK
- Comparison of cases and controls in sample
- Can also compare with other known populations

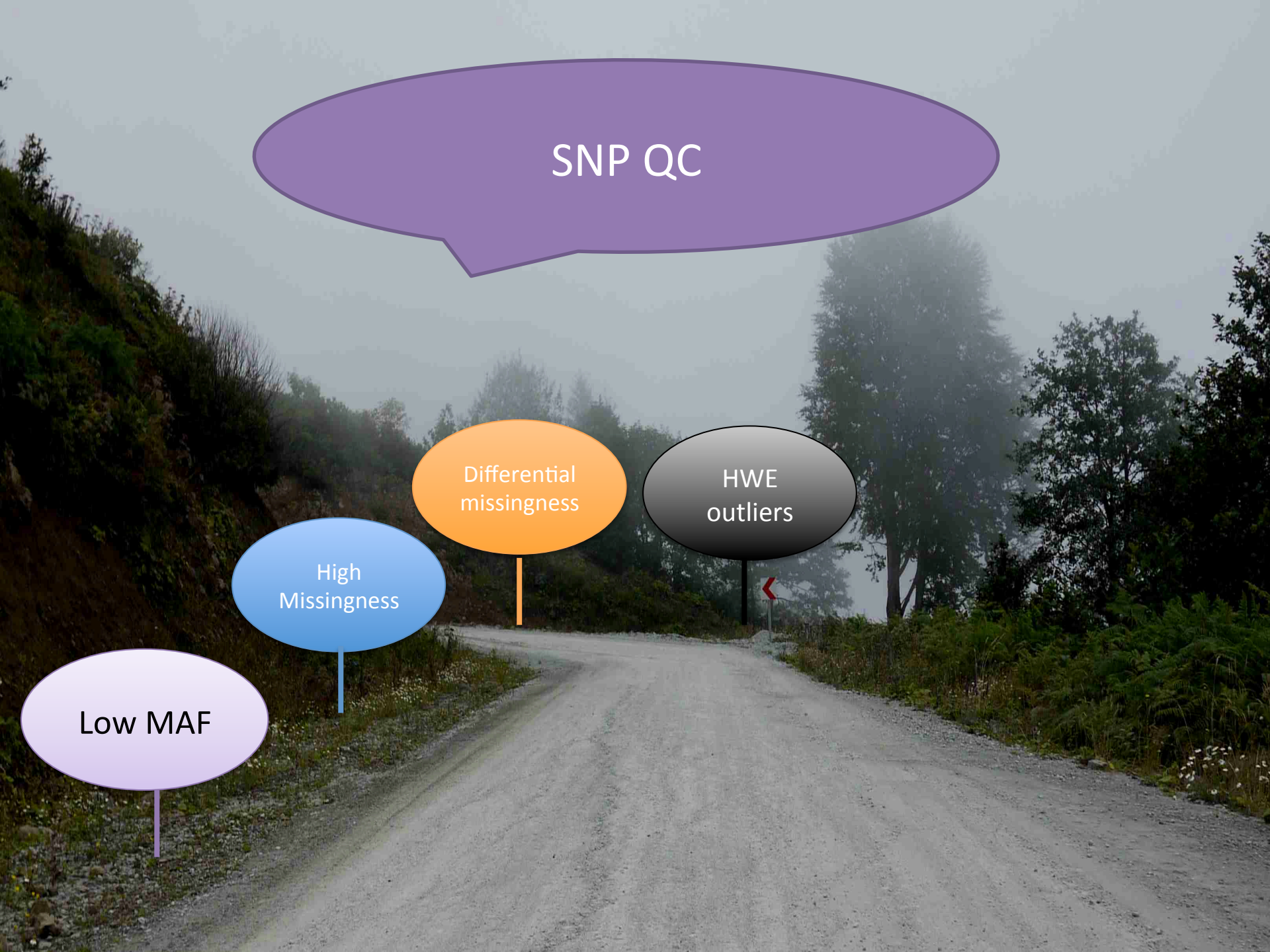
Population structure

- Outcome of approaches
  - Identify if there is population structure in the dataset – apply appropriate measures to control for this in association test/selection of association test
  - Identification of samples that are significant outliers in the dataset based on population ancestry – exclude those individuals

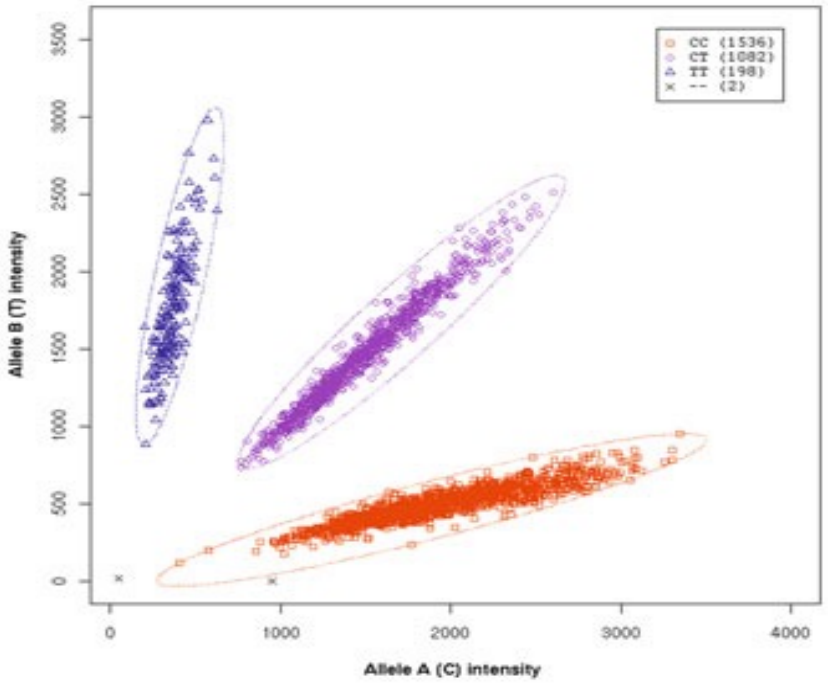
Population structure - PCA



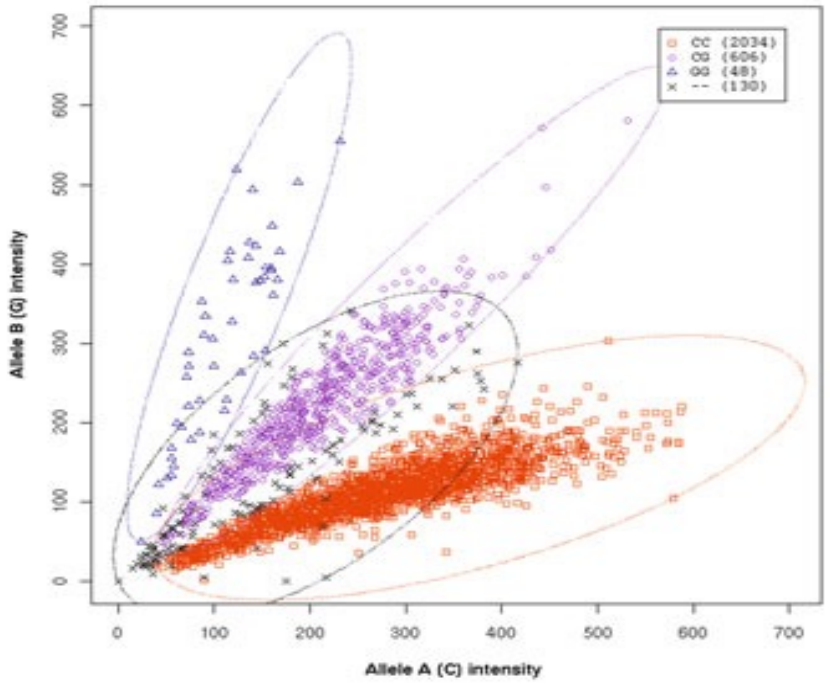

Discordant sex information
High Missingness and outlying heterozygosity rate
Duplicate and related
Divergent ancestry
fail_sexcheck_example.txt
fail_miss_het_example.txt
fail_IBD_example.txt
fail_ancestry_example.txt
Sample QC completed
JOIN FILES
cat fail_* | sort -k1 | uniq > fail_example_inds.txt
REMOVE FROM DATA
plink --bfile example --remove fail_example_inds.txt --make-bed --out clean_inds_example --noweb


SNP QC
Differential missingness
HWE outliers
High Missingness
Low MAF

Genotype resolution is often challenging



Good calls!

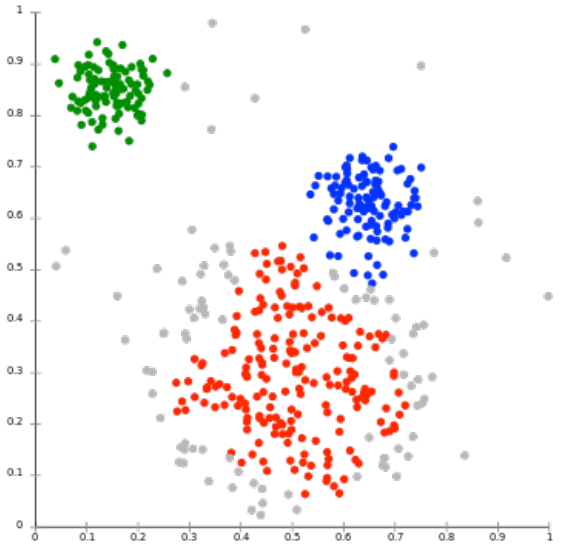


Bad calls!

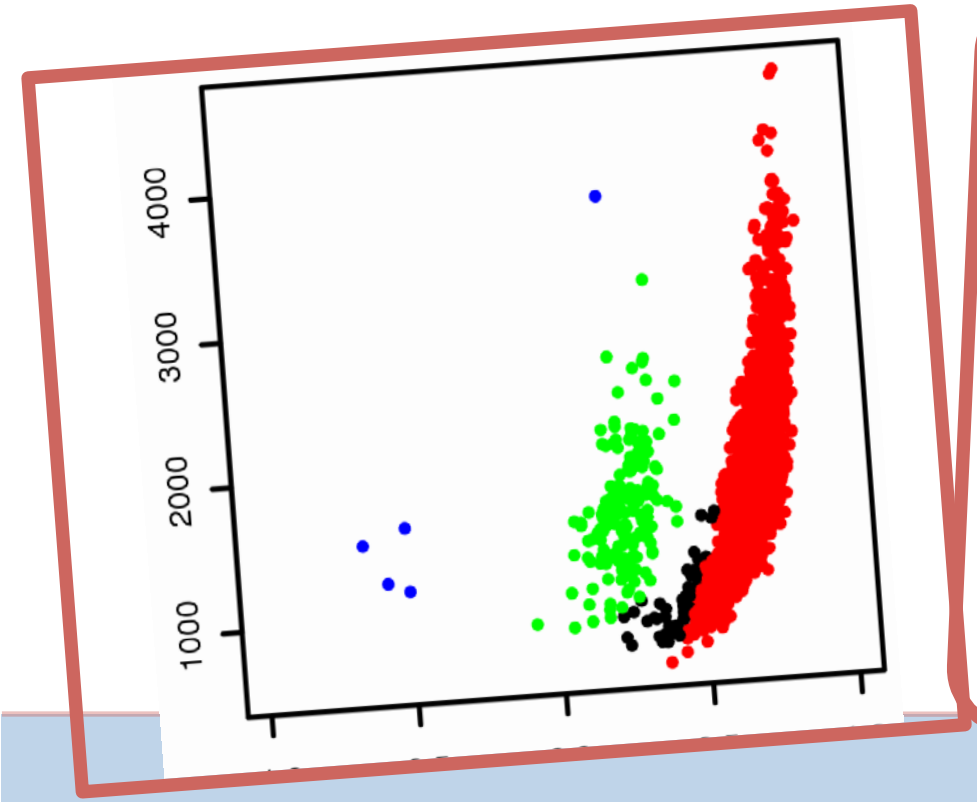
Bad calls can lead to false associations !

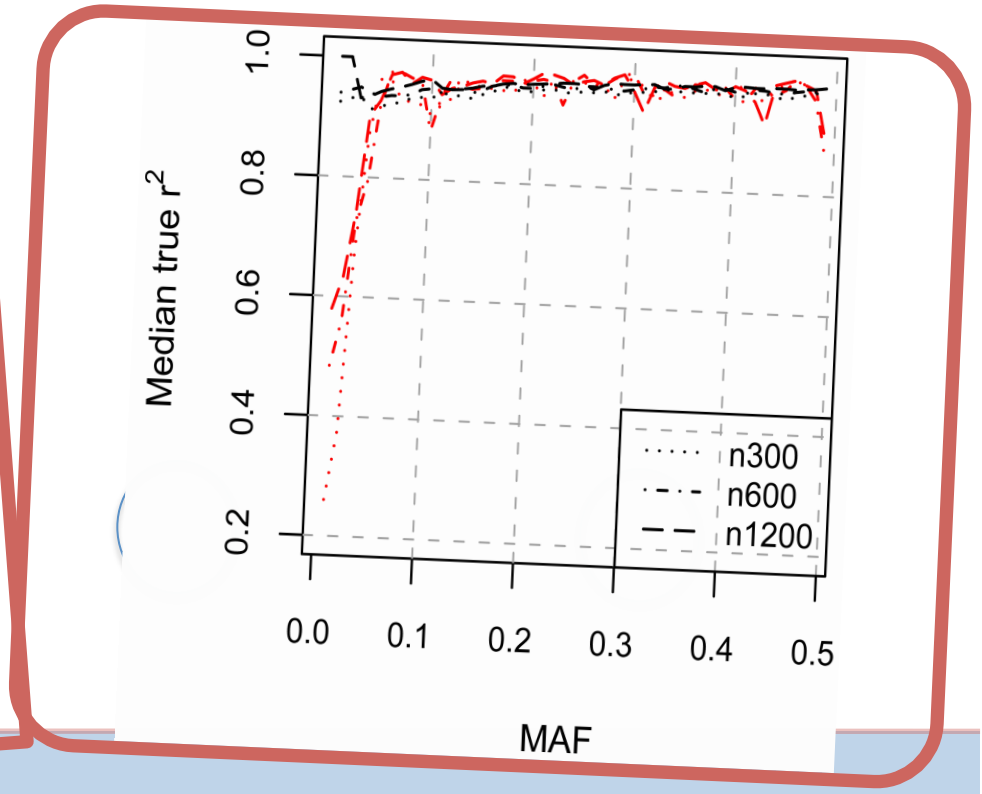
What causes low quality SNP genotyping?

- Genotype clusters of many SNPs demonstrate low quality genotyping due to :
  - Low DNA concentration
  - Poor binding and competitive binding by other sequences
  - Structural and copy number variants
- Not possible to QC each SNP manually
- Measures to remove low quality SNPs are required




SNP/Marker
QC steps
Low MAF
High
Missingness
Differential
missingness
HWE
outliers





Low minor allele frequency SNPs

- Genotype calling algorithms **perform poorly** for SNPs with low MAF
- Clustering depends largely on **sample size** for low MAF SNPs
- **Power** for detecting associations to SNPs with low MAF is low (unless the sample size is very large).
- Low MAF SNPs are therefore excluded
- An often used exclusion threshold is MAF 1% to 2%

Identify low minor allele frequency SNPs

GET ALLELE FREQUENCIES

```
plink --bfile clean-inds-example --freq --
out clean_inds_example_freq
```

Generates the file “clean_inds_example_freq.frq” containing minor allele frequency of each SNP

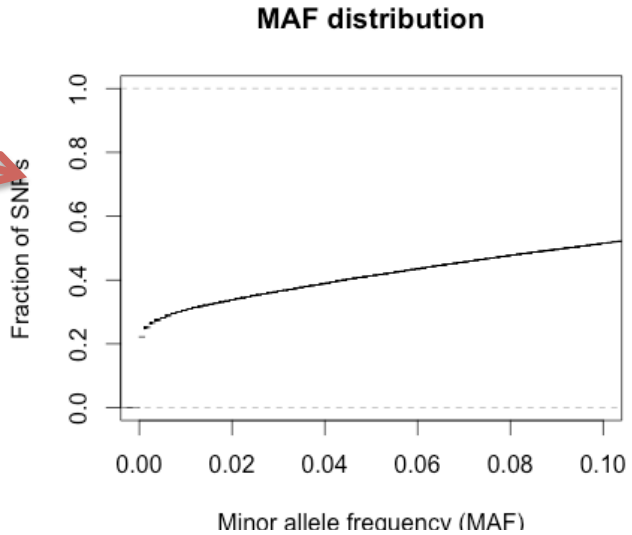
GENERATE MAF PLOT USING R SCRIPT

maf_plot.R

CHOOSE STANDARD MAF CUTOFF (MAF>0.01)

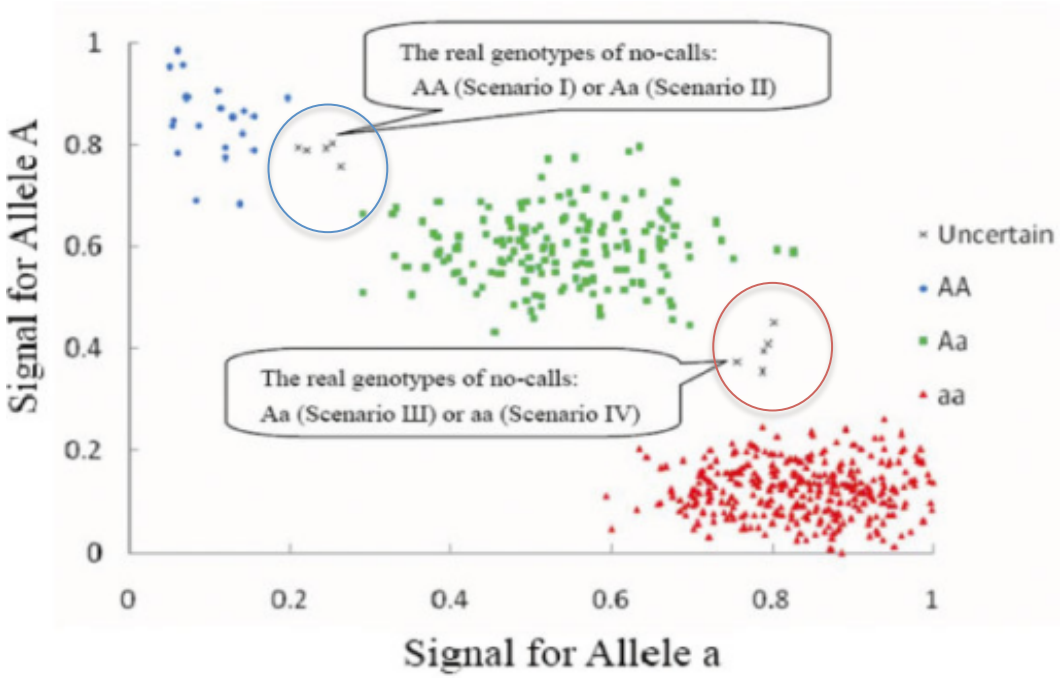
OR SELECT ONE ON THE BASIS OF THE PLOT

CHR	SNP	A1	A2	MAF	NCHROBS
1	vh_1_1108138	A	G	0.4443	1292
1	vh_1_1110294	A	G	0.362	1304
1	rs7515488	A	G	0.2893	1310
1	rs6603785	T	A	0.4985	1292
1	rs6603788	G	A	0.2221	1306
1	1_1209245	G	C	0.04609	1150
1	rs2274264	A	G	0.1336	1302
1	rs12103	G	A	0.09862	1308




SNP/Marker
QC steps
High
Missingness
Differential
missingness
HWE
outliers
Low MAF

Missingness frequency



- SNPs which cannot be assigned definitively to a cluster are assigned **"missing"** status during genotype calling.
- **Missing frequency** (also termed 1 minus SNP call rate) is the fraction of total genotype calls for a SNP which has been assigned missing status
- High missingness often implies that **the cluster separation** for a particular SNP has been **poor** and the SNP needs to be removed
- A missingness cutoff of 1%-5% is generally used.

Identify SNPS with high missingness

GET ALLELE FREQUENCIES

```
plink --bfile clean_inds_example --missing
--out clean_inds_example_missing --noweb
```

Generates the file “clean_inds_example_mising.lmiss” containing missingness value for each SNP

GENERATE PLOT USING R SCRIPT

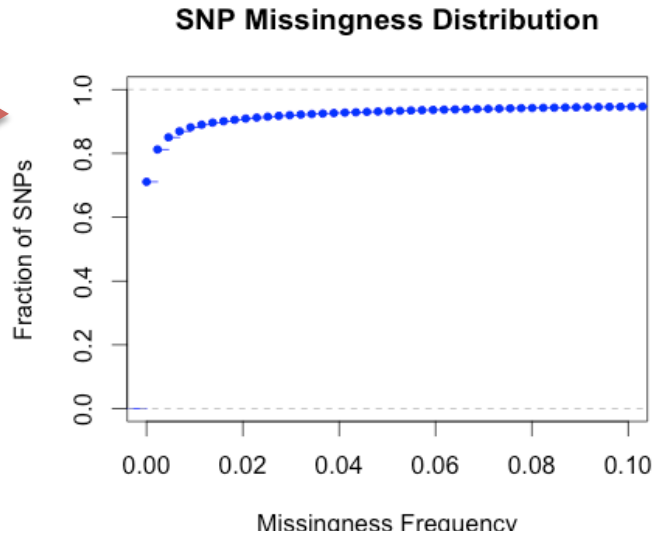
snpmiss_plot.R

CHOOSE THE STRANDARD MISSINGNESS (F_MISS) CUTOFF >0.05

OR

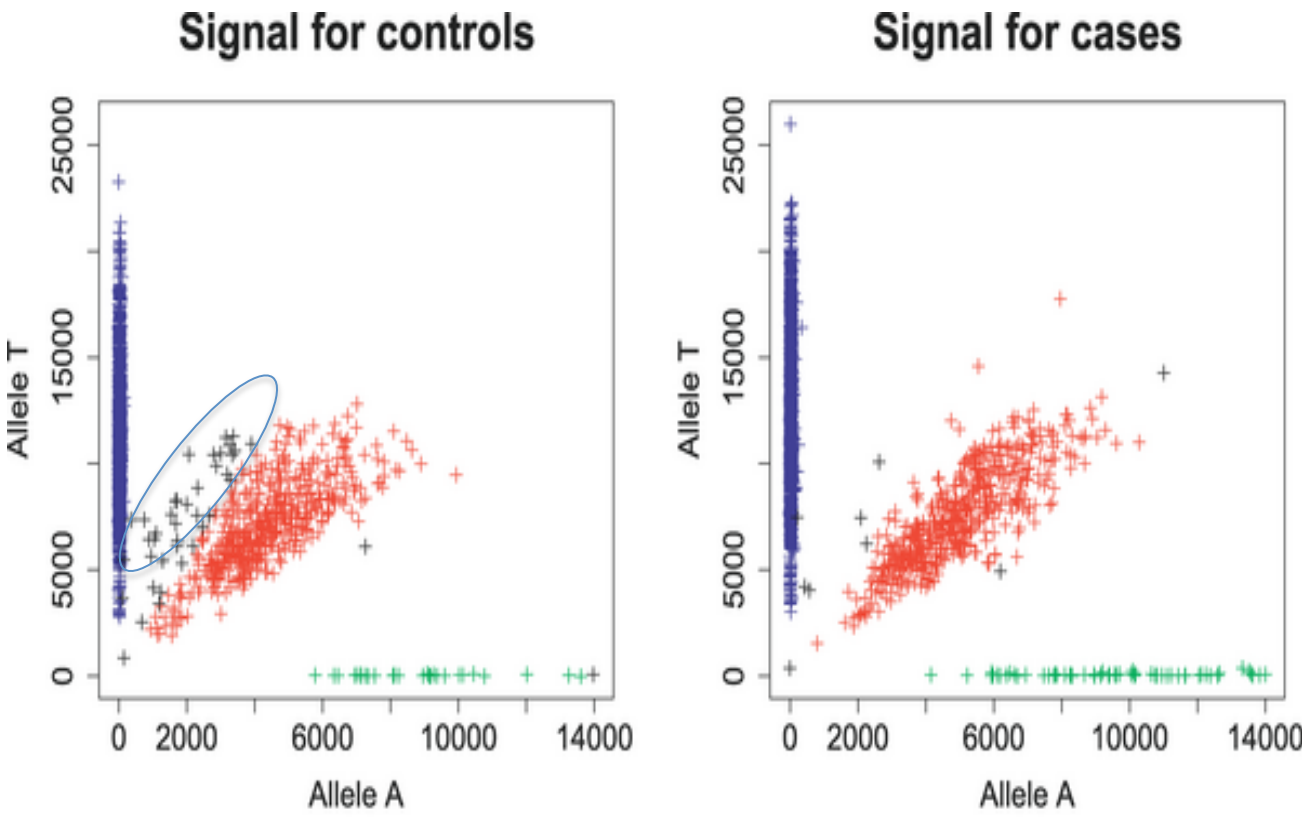
CHOOSE CUTOFF ON THE BASIS OF THE PLOT

CHR	SNP	N_MISS	N_GENO	F_MISS
1	vh_1_1108138	10	656	0.01524
1	vh_1_1110294	4	656	0.006098
1	rs7515488	1	656	0.001524
1	rs6603785	10	656	0.01524
1	rs6603788	3	656	0.004573
1	1_1209245	81	656	0.1235
1	rs2274264	5	656	0.007622
1	rs12103	2	656	0.003049
1	rs12142199	7	656	0.01067
1	rs880051	2	656	0.003049




SNP/Marker
QC steps
Differential
missingness
HWE
outliers
High
Missingness
Low MAF

Differential missingness



- Missing frequency is also assessed separately in cases and in controls because differential missingness is a common source of false positive associations.
- SNPs showing highly differential missingness (P<0.00001) are excluded

Identify SNPS with high differential missingness in case and controls

GET ALLELE FREQUENCIES

```
plink --bfile clean_inds_example --test-missing --
out clean_inds_example_test_missing --noweb
```

Generates the file “example_test_missing.missing” containing differential missingness statistics for each SNP

GENERATE PLOT USING R SCRIPT

diffmiss_plot.R

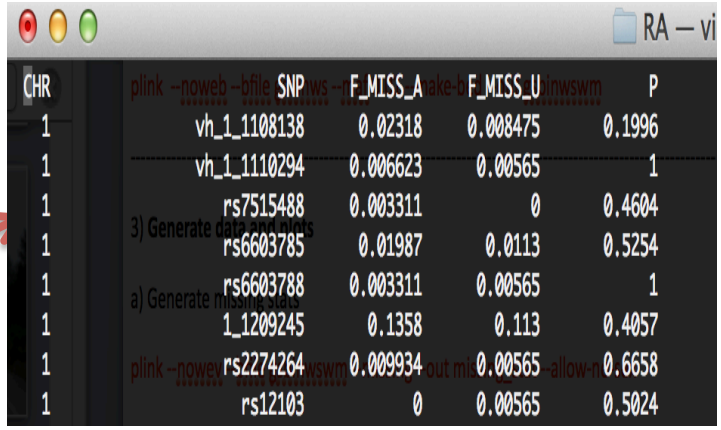
CHOOSE STANDARD DIFFERENTAIL MISSINGNESS P-VALUE CUTOFF (0.00001)

OR CHOOSE ON THE BASIS OF THE PLOT

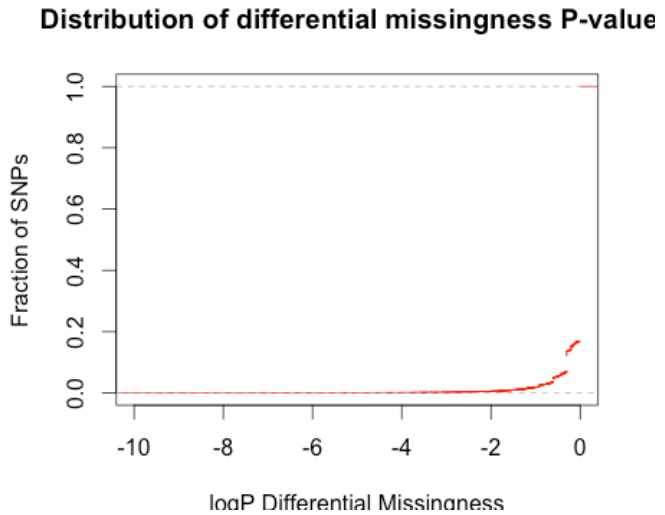
To identify SNPs showing differential missingness P-value greater than cutoff :

perl run_difmiss.pl clean-Inds_example

Creates the file “fail_diffmiss_example.txt”



CHR	SNP	F_MISS_A	F_MISS_U	P
1	vh_1_1108138	0.02318	0.008475	0.1996
1	vh_1_1110294	0.006623	0.00565	1
1	rs7515488	0.003311	0	0.4604
1	rs6603785	0.01987	0.0113	0.5254
1	rs6603788	0.003311	0.00565	1
1	1_1209245	0.1358	0.113	0.4057
1	rs2274264	0.009934	0.00565	0.6658
1	rs12103	0	0.00565	0.5024

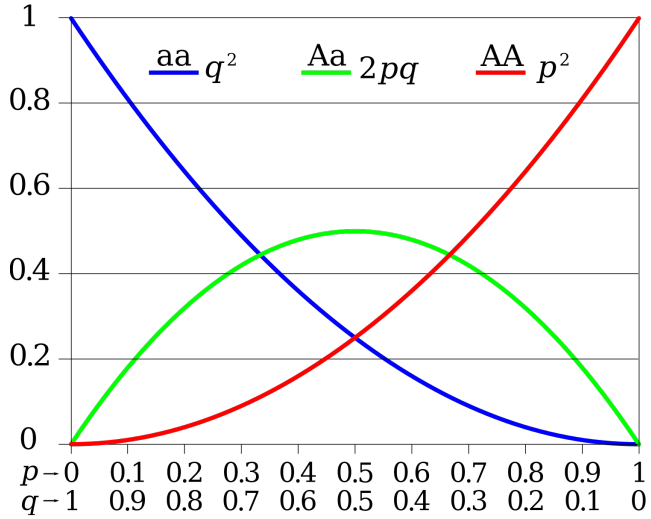



SNP/Marker
QC steps
Low MAF
High
Missingness
Differential
missingness
HWE
outliers

Hardy Weinberg Equilibrium

Assumptions

- Diploid organisms
- Infinite population size
- Non-overlapping generations
- Random mating
- No selection, mutation or migration



Testing for HWE

- Calculate the allele frequency (p)
  - Using observed genotype counts
- Calculate the expected genotype counts
  - Using the allele frequency (p)
- Compare the observed to the expected counts
  - χ^2 test

HWE Example

Step I

- Observed Genotypes

Genotype	*GG*	*Gg*	*gg*
Frequency	*12*	*2*	*8*

1. *Calculate the allele frequency (p):*

$$p = \frac{2(12)+ 2}{2(22)} = 0.59$$

Step II

Genotype	*GG*	*Gg*	*gg*
Frequency	*12*	*2*	*8*

- Observed Genotypes

2. Calculate the expected genotype counts:

$$E(GG) = np^2 = 22(0.59^2) = 7.66$$

$$E(Gg) = n2pq = 22(0.59)(1-0.59) = 10.64$$

$$E(gg) = nq^2 = 22((1-0.59)^2 = 3.68$$

Step III

Genotype	*GG*	*Gg*	*gg*
Frequency	*12*	*2*	*8*

- Observed Genotypes

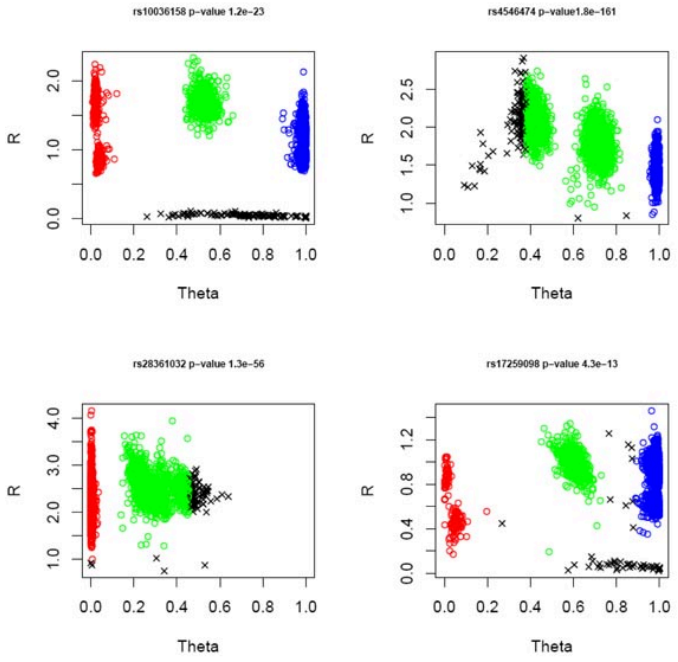
3. Compare the observed and expected counts:

$$\chi_1^2 = \frac{(12-7.66)^2}{7.66} + \frac{(2-10.64)^2}{10.64} + \frac{(8-3.69)^2}{3.69} = 14.50$$

REJECT THE NULL!

Reasons for HW Deviations

- **Genotyping Error**
- Subdivided Population
  - Excess homozygotes = "Wahlund Effect"
- Excess homozygotes= "Allele dropout in old samples"
- Any violations of the HW assumptions



GENEVA alcohol-dependence project: Quality control report

- SNPs are excluded if substantially more or fewer samples heterozygous at a SNP than expected (excess heterozygosity or heterozygote deficiency)
- Threshold for significance 10^{-3} to 10^{-6}

Identify SNPS which show extreme HWE deviations

GET ALLELE FREQUENCIES

```
plink --bfile clean_inds_example --hardy --out
clean_inds_example_hwe --noweb
```

Generates the file "clean_inds_example_hwe.hwe" containing Hardy Weinberg statistics for each SNP separately in cases, controls and all. samples

SELECT UNAFFECTED

```
head -1 clean_inds_example_hwe.hwe >
example_clean_inds_example_hweu.hwe | grep
"UNAFF" clean_inds_example_hwe.hwe >>
example_clean_inds_example_hweu.hwe
```

GENERATE PLOT USING R SCRIPT

hwe_plot.R

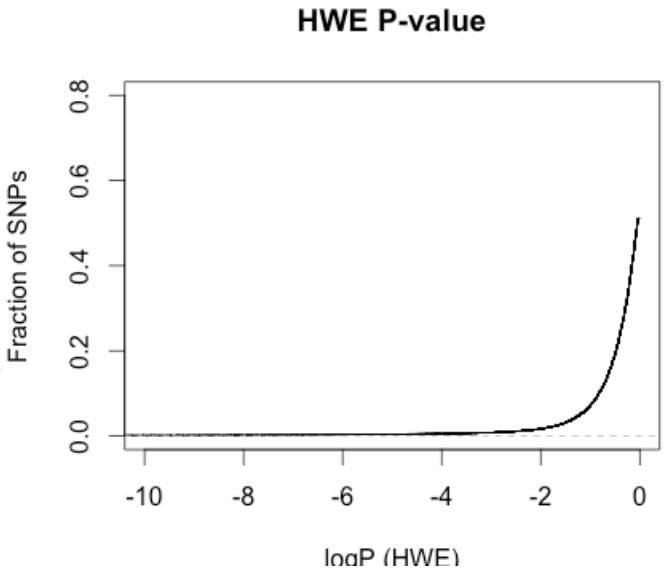
(based only on controls)

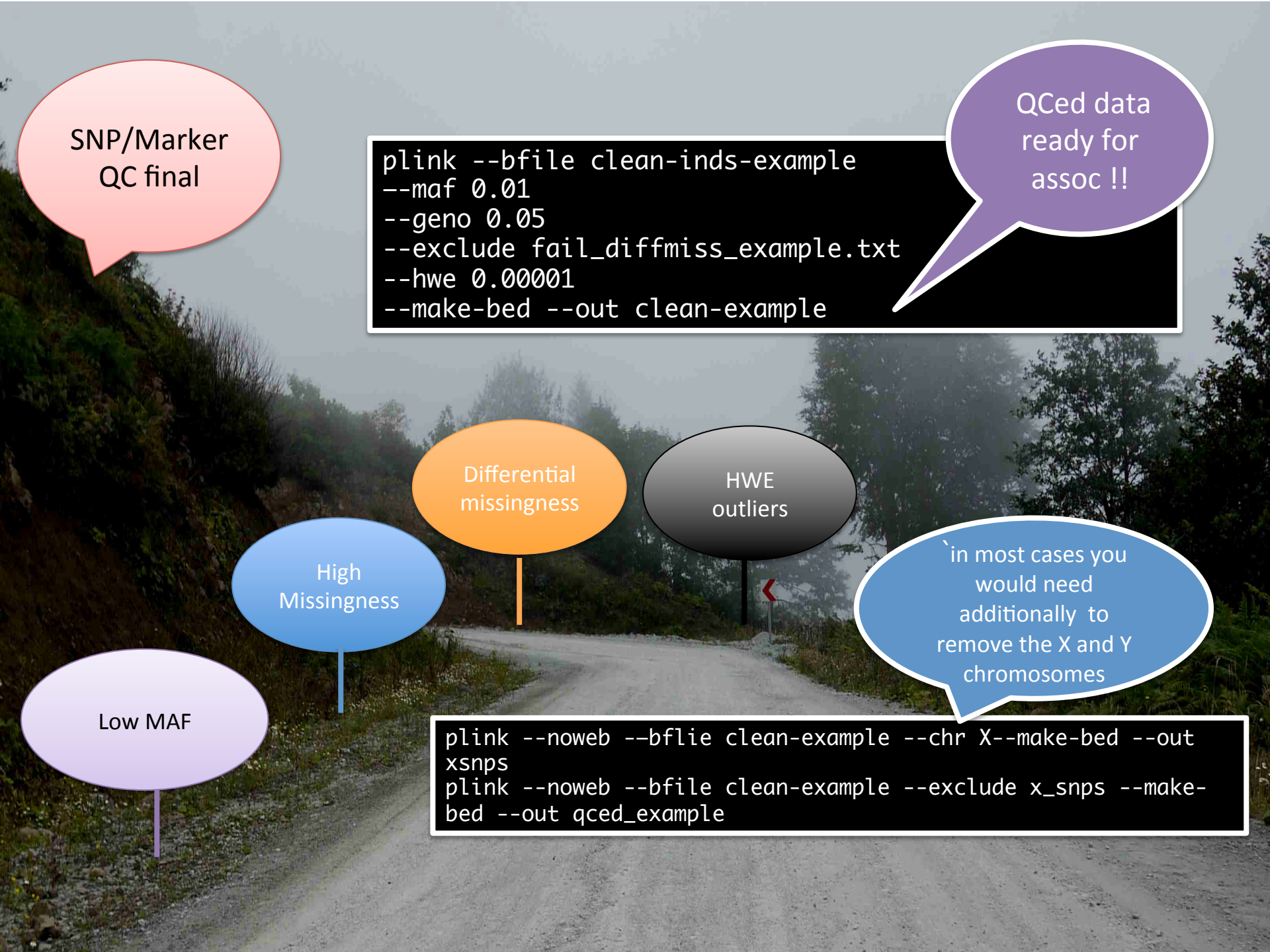
CHOOSE THE STANDARD HWE P-VALUE CUTOFF (0.00001)

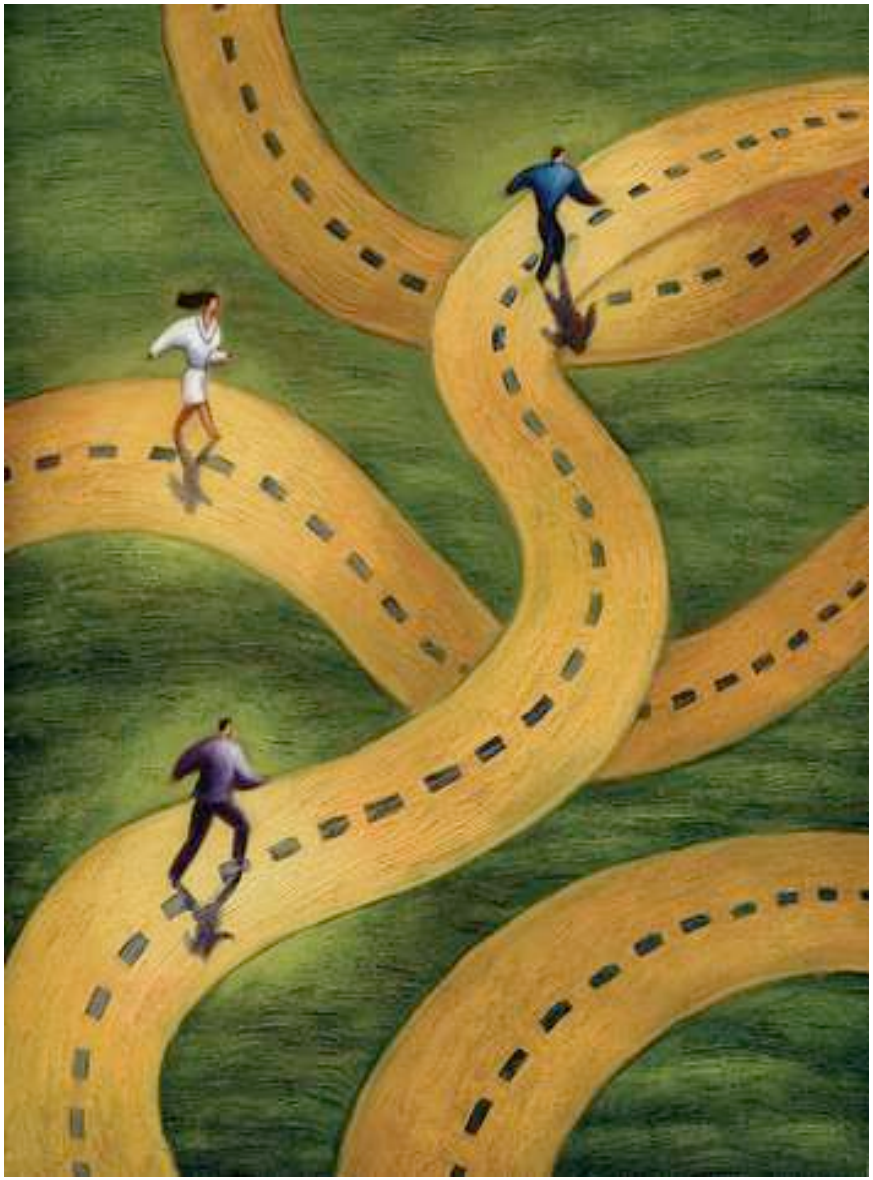
OR SELECT ONE ON THE BASIS OF THE PLOT

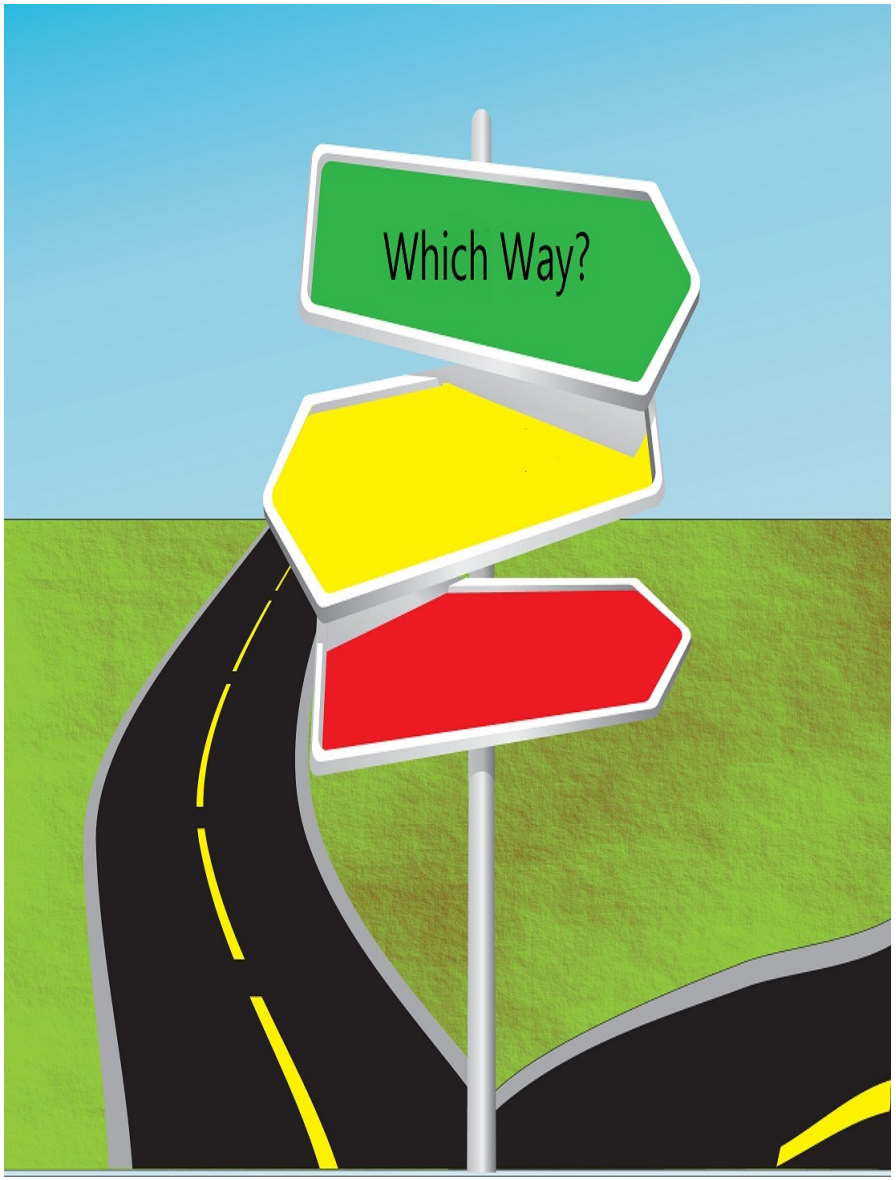
RA — vim — 158x37

CHR	SNP	TEST	A1	A2	GENO	O(HET)	E(HET)	P
1	vh_1_1108138	ALL	A	G	134/306/206	0.4737	0.4938	0.301
1	vh_1_1108138	AFF	A	G	63/135/97	0.4576	0.4934	0.2376
1	vh_1_1108138	UNAFF	A	G	71/171/109	0.4872	0.4941	0.829
1	vh_1_1110294	ALL	A	G	92/288/272	0.4417	0.4619	0.2706
1	vh_1_1110294	AFF	A	G	37/136/127	0.4533	0.455	1
1	vh_1_1110294	UNAFF	A	G	55/152/145	0.4318	0.4673	0.1707
1	rs7515488	ALL	A	G	50/279/326	0.426	0.4112	0.3931




SNP/Marker QC final
plink --bfile clean-inds-example
--maf 0.01
--geno 0.05
--exclude fail_diffmiss_example.txt
--hwe 0.00001
--make-bed --out clean-example
QCed data ready for assoc !!
Differential missingness
HWE outliers
High Missingness
Low MAF
`in most cases you would need additionally to remove the X and Y chromosomes
plink --noweb --bflie clean-example --chr X--make-bed --out xsnps
plink --noweb --bfile clean-example --exclude x_snps --make-bed --out qced_example




Which Way?


thank you!