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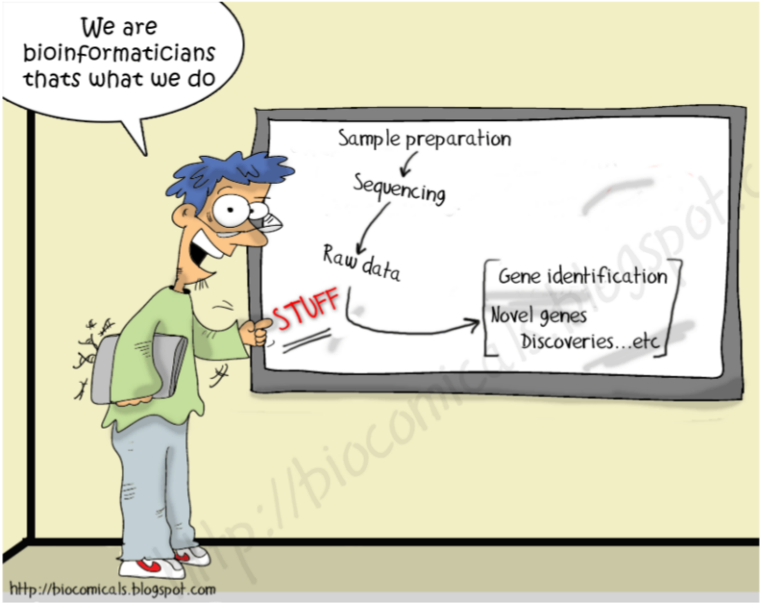
6th August 2018

Analysis of NGS data

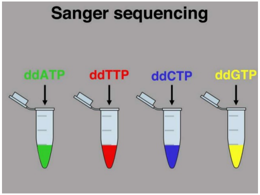
Principles of genotype and SNP calling and estimation of allele frequencies

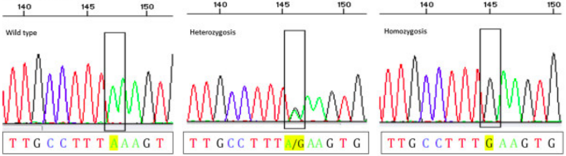
Matteo Fumagalli


We are bioinformaticians thats what we do
Sample preparation
Sequencing
Raw data
STUFF
Gene identification
Novel genes
Discoveries...etc
http://biocomicals.blogspot.com

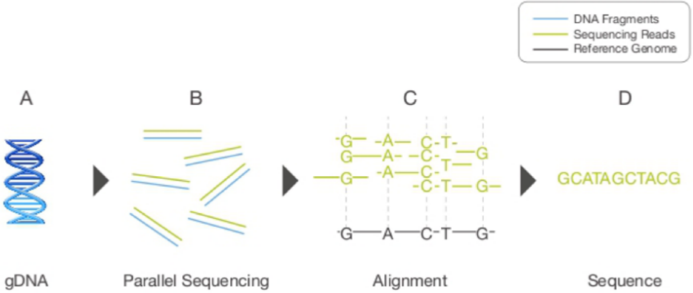
Sanger sequencing

aka first/former generation sequencing





Next Generation Sequencing



A. Extracted gDNA
B. gDNA is fragmented into a library of small segments that are each sequenced in parallel.
C. Individual sequence reads are reassembled by aligning to a reference genome
D. The whole-genome sequence is derived from the consensus of aligned reads.

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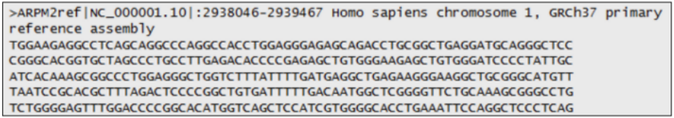
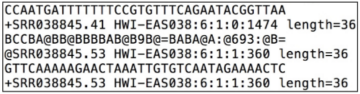
From genomes to variants

Genome (FASTA)



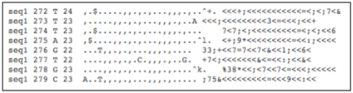
```
>ARPM2ref|NC_000001.10|:2938046-2939467 Homo sapiens chromosome 1, GRCh37 primary
reference assembly
TGGAAGAGGCCTCAGCAGGCCCAGGCCACCTGGAGGGAGAGCAGACCTGCGGCTGAGGATGCAGGGCTCC
CGGGCACGGTGCTAGCCCTGCCTTGAGACACCCCGAGAGCTGTGGGAAGAGCTGTGGGATCCCCTATTGC
ATCACAAAGCGGCCCTGGAGGGCTGGTCTTTATTTTGATGAGGCTGAGAAGGGAAGGCTGCGGGCATGTT
TAATCCGCACGCTTTAGACTCCCCGGCTGTGATTTTTGACAATGGCTCGGGGTTCTGCAAAGCGGGCCTG
TCTGGGGAGTTTGGACCCCGGCACATGGTCAGCTCCATCGTGGGGCACCTGAAATTCCAGGCTCCCTCAG
```

Reads (FASTQ)



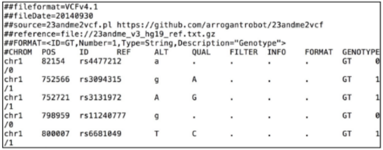
```
CCAATGATTTTTTTCCGTGTTTCAGAATACGGTTAA
+SRR038845.41 HWI-EAS038:6:1:0:1474 length=36
BCCBA@BB@BBBBAB@B9B@=BABA@A:@693:@B=
@SRR038845.53 HWI-EAS038:6:1:1:360 length=36
GTTCAAAAAGAACTAAATTGTGTCAATAGAAAACTC
+SRR038845.53 HWI-EAS038:6:1:1:360 length=36
```

Mapped Reads (mpileup, BAM)



```
seq1 272 T 24  ,.$.....,,.,.,...,,,.,..^+. <<<+;<<<<<<<<<<<=<;<;7<&
seq1 273 T 23  ,.....,,.,.,...,,,.,..A <<<;<<<<<<<<<3<=<<<;<<+
seq1 274 T 23  ,.$....,,.,.,...,,,.,...    7<7;<;<<<<<<<<<=<;<;<<6
seq1 275 A 23  ,$....,,.,.,...,,,.,...^l.  <+;9*<<<<<<<<<=<<:;<<<<
seq1 276 G 22  ...T,,.,.,...,,,.,....  33;+<<7=7<<7<&<<1;<<6<
seq1 277 T 22  ....,,.,.,.C.,,,.,..G.  +7<;<<<<<<<&<=<<:;<<&<
seq1 278 G 23  ....,,.,.,...,,,.,....^k.   %38*<<;<7<<7<=<<<;<<<<<
seq1 279 C 23  A..T,,.,.,...,,,.,..... ;75&<<<<<<<<<=<<<9<<:<<
```

Variants (VCF)



```
##fileformat=VCFv4.1
##fileDate=20140930
##source=23andme2vcf.pl https://github.com/arrogantrobot/23andme2vcf
##reference=file://23andme_v3_hg19_ref.txt.gz
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
#CHROM  POS     ID         REF     ALT     QUAL    FILTER  INFO    FORMAT  GENOTYPE
chr1    82154   rs4477212          a       .       .       .       .       GT      0
/0
chr1    752566  rs3094315          g       A       .       .       .       GT      1
/1
chr1    752721  rs3131972          A       G       .       .       .       GT      1
/1
chr1    798959  rs11240777         g       .       .       .       .       GT      0
/0
chr1    800007  rs6681049          T       C       .       .       .       GT      1
/1
```

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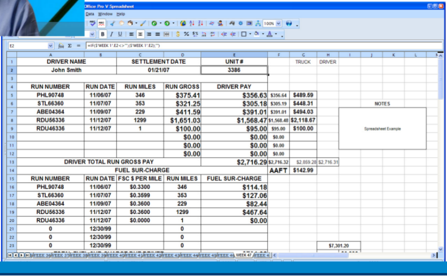
Forget about

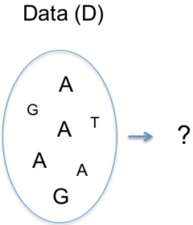


E2 =IF($'WEEK 1'.E2<>"";($'WEEK 1'.E2);"")

	A	B	C	D	E	F	G	H	I	J	K
1	DRIVER NAME		SETTLEMENT DATE		UNIT #		TRUCK	DRIVER			
2	John Smith		01/21/07		3386						
3											
4	RUN NUMBER	RUN DATE	RUN MILES	RUN GROSS	DRIVER PAY						
5	PHL90748	11/06/07	346	$375.41	$356.63	$356.64	$489.59				
6	STL66360	11/07/07	353	$321.25	$305.18	$305.19	$448.31			NOTES	
7	ABE04364	11/09/07	229	$411.59	$391.01	$391.01	$494.03				
8	RDU56336	11/12/07	1299	$1,651.03	$1,568.47	$1,568.48	$2,118.67				
9	RDU46336	11/12/07	1	$100.00	$95.00	$95.00	$100.00			Spreadsheet Example	
10				$0.00	$0.00	$0.00					
11				$0.00	$0.00	$0.00					
12				$0.00	$0.00	$0.00					
13	DRIVER TOTAL RUN GROSS PAY				$2,716.29	$2,716.32	$2,859.28	$2,716.31			
14	FUEL SUR-CHARGE					AAFT	$142.99				
15	RUN NUMBER	RUN DATE	FSC $ PER MILE	RUN MILES	FUEL SUR-CHARGE						
16	PHL90748	11/06/07	$0.3300	346	$114.18						
17	STL66360	11/07/07	$0.3599	353	$127.06						
18	ABE04364	11/09/07	$0.3600	229	$82.44						
19	RDU56336	11/12/07	$0.3600	1299	$467.64						
20	RDU46336	11/12/07	$0.0000	1	$0.00						
21	0	12/30/99		0							
22	0	12/30/99		0							
23	0	12/30/99		0				$7,301.20			

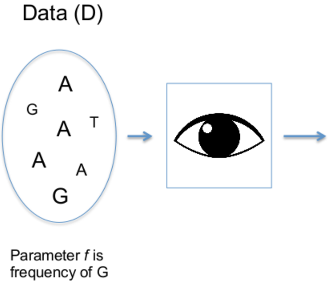
WEEK 36 WEEK 37 WEEK 38 WEEK 39 WEEK 40 WEEK 41 WEEK 42 WEEK 43 WEEK 44 WEEK 45 WEEK 46 WEEK 47 WEEK 4

Statistical inference



Parameter *f* is
frequency of G

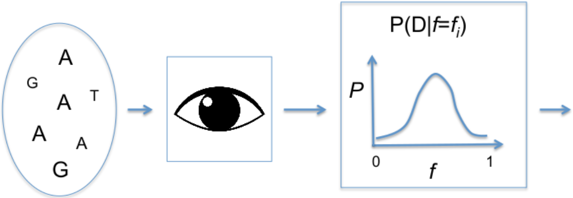
Statistical inference



Statistical inference

Data (D)

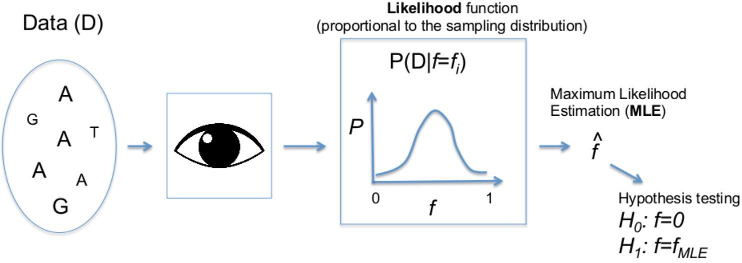
Likelihood function
(proportional to the sampling distribution)



$P(D|f{=}f_i)$

Parameter *f* is
frequency of G

Statistical inference



Data (D)

Likelihood function
(proportional to the sampling distribution)

$P(D|f=f_i)$

Maximum Likelihood
Estimation (**MLE**)

$\hat{f}$

Hypothesis testing

H_0: $f=0$

H_1: $f=f_{MLE}$

Likelihood approach:

- All the information on the parameter is in the likelihood function (we use all the data!).
- More data leads to less bias and less variance.
- Suitable for hypothesis testing.

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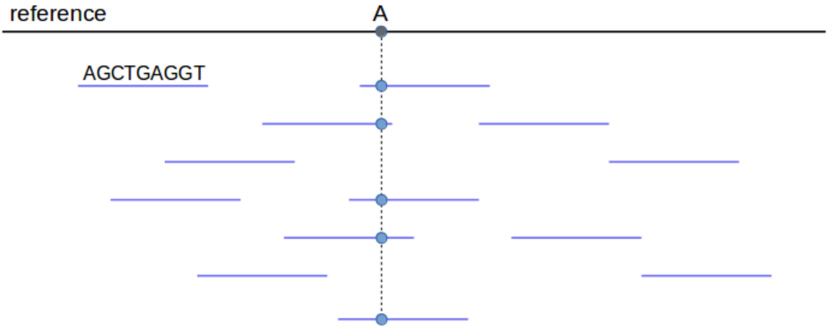
The data



● is a **nucleotide**/base/allele with a certain **quality** score

Genotype likelihoods

Likelihood

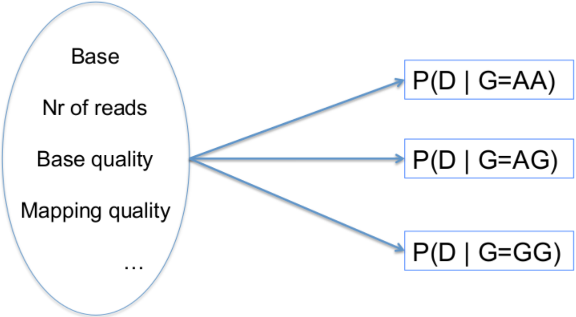
$P(D|G = \{A_1, A_2, ..., A_n\})$
with
$A_i \in \{A, C, G, T\}$ and n being the ploidy

How many genotypes likelihoods do we need to calculate for each each individual at each site?

Genotype likelihoods

Chrom1 272 A 24 AAAAAGGAGAGGTAAG <<<+;<<<<<<<<<<<<<=<;<;7<&

Base quality in Phred scale



Base

Nr of reads

Base quality

Mapping quality

…

P(D | G=AA)

P(D | G=AG)

P(D | G=GG)

Calculating genotype likelihoods

Likelihood function

$$P(D|G=\{A_1,A_2,...,A_N\})=\prod_{i=1}^{R}\sum_{j=1}^{N}\frac{L_{A_j,i}}{N}$$

- $L_{A_j,i}=P(D|A_G=A_j)$
- $A_i\in\{A,C,G,T\}$
- R is the depth (nr. of reads)
- N is the ploidy (nr. of chromosomes)

Example:
AAAG, all with quality score equal to 20 (in phred score)
$P(D|G=AC)=$?

Calculating genotype likelihoods

Likelihood function

$$P(D|G = \{A_1, A_2, ..., A_N\}) = \prod_{i=1}^{R} \sum_{j=1}^{N} \frac{L_{A_j,i}}{N}$$

A
A
A
G
& Q=20

$$P(D|G = \{A, C\}) = ...$$

Calculating genotype likelihoods

Likelihood function

$$P(D|G=\{A_1,A_2,...,A_N\})=\prod_{i=1}^{R}\sum_{j=1}^{N}\frac{L_{A_j,i}}{N}$$

A
A
A
G
& Q=20
$N=2; i=1; A_1=A; A_2=C$

$$P(D|G=\{A,C\})=(\frac{L_{A,1}}{2}+\frac{L_{C,1}}{2})\times ...$$

What are $L_{A,1}$ and $L_{C,1}$?

Calculating genotype likelihoods

Likelihood function

$$P(D|G=\{A_1,A_2,...,A_N\})=\prod_{i=1}^{R}\sum_{j=1}^{N}\frac{L_{A_j,i}}{N}$$

AAAG & Q=20

$$L_{C,1}=\frac{\epsilon}{3}$$

$$L_{A,1}=1-\epsilon$$

$$P(D|G=\{A,C\})=(\frac{1-\epsilon}{2}+\frac{\epsilon}{6})\times ...$$

Calculating genotype likelihoods

Likelihood function

$$P(D|G=\{A_1,A_2,...,A_N\})=\prod_{i=1}^{R}\sum_{j=1}^{N}\frac{L_{A_j,i}}{N}$$

AAAG & Q=20

$$L_{C,1}=\frac{\epsilon}{3}$$

$$L_{A,1}=1-\epsilon$$

$$P(D|G=\{A,C\})=(\frac{1-\epsilon}{2}+\frac{\epsilon}{6})^3\times\frac{\epsilon}{3}$$

What is ϵ?

Calculating genotype likelihoods

Genotype	Likelihood (log10)
AA	-2.49
AC	**-3.38**
AG	-1.22
AT	-3.38
CC	-9.91
CG	-7.74
CT	-9.91
GG	-7.44
GT	-7.74
TT	-9.91

A
A
A
G
$\epsilon = 0.01$

Genotype calling

Genotype	Likelihood (log10)
AA	-2.49
AC	-3.38
AG	-1.22
AT	-3.38
CC	-9.91
CG	-7.74
CT	-9.91
GG	-7.44
GT	-7.74
TT	-9.91

AAAG & $\epsilon = 0.01$

What is the genotype here?

Genotype calling

Genotype	Likelihood (log10)
AA	-2.49
AC	-3.38
AG	**-1.22**
AT	-3.38
CC	-9.91
CG	-7.74
CT	-9.91
GG	-7.44
GT	-7.74
TT	-9.91

AAAG & $\epsilon = 0.01$
What is the genotype?
AG.

Maximum Likelihood

The simplest genotype caller: choose the genotype with the highest likelihood.

Major and minor alleles

Likelihood function

$$\log P(D|G = A) = \sum_{i=1}^{R} \log L_{A_j,i}$$

AAAG & $\epsilon = 0.01$

Allele	Likelihood
A	**-2.49**
C	-3.38
G	**-1.22**
T	-3.38

We can reduce the genotype space to 3 entries (from 10).

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Genotype calling

AAAG & $\epsilon = 0.01$ & A,G alleles

Genotype	Likelihood
AA	-5.73
AG	-2.80
GG	-17.12

Examples varying qualities and reads... open Julia script.

Genotype likelihood ratio

$$\log_{10} \frac{L_{G(1)}}{L_{G(2)}} > t$$

i.e. $t = 1$ meaning that the most likely genotype is 10 times more likely than the second most likely one

Pros and cons?

- Yes:
- No:

Genotype likelihood ratio

$$\log_{10} \frac{L_{G(1)}}{L_{G(2)}} > t$$

i.e. $t = 1$ meaning that the most likely genotype is 10 times more likely than the second most likely one

Pros and cons?

- Yes: genotype are called with higher **confidence**
- No: more **missing** data

Practical: genotype likelihoods and (basic) genotype calling
`https://github.com/mfumagalli/Copenhagen`

Statistical thinking

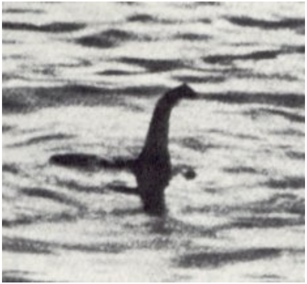

Figure 1: Nessie, the Loch Ness Monster. True or fake?

Statistical thinking

- $D = \{0, 1\}$, whether I tell you I saw Nessie or not.
- $N = \{0, 1\}$, whether Nessie exists or not.

Questions

- What are $p(D = 1|N = 1)$ and $p(D = 1|N = 0)$?
- What is a Maximum Likelihood Estimate of N?

Statistical thinking

Our inference on N, our parameter, is driven solely by our observations, given by our likelihood function.

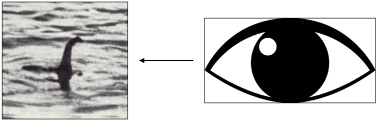

Figure 2: The eye: a "likelihood" organ.

Statistical thinking

In real life we take many decisions based not only on what we observe but also on some believes of ours.

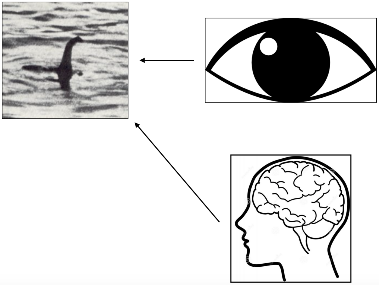

Figure 3: The brain: a ”non-likelihood” organ.

Bayesian thinking

- with "eyes only" our intuition is that $p(N|D) \approx p(D|N)$
- with "the brain" our intuition is that $p(N|D) \approx p(D|N)p(N)$

Our "belief" expresses the probability $p(N)$ **unconditional** of the data.

Question

How can we define $p(N)$?

Bayesian thinking

The ”belief” function $p(N)$ is called **prior probability** and the joint product of the likelihood $p(D|N)$ and the prior is proportional to the **posterior probability** $p(N|D)$.

The use of posterior probabilities for inferences is called Bayesian statistics.

Statistical inference

If D is the data and θ is your unknown parameter, then

- the frequentist conditions on parameters and integrates over the data, $p(D|\theta)$,
- the Bayesian conditions on the data and integrates over the parameters, $p(\theta|D)$.

Statistical inference

Bayesian *vs.* Likelihoodist

- we derive "proper" probability distributions of our parameters rather than deriving a point estimate;
- a probability is assigned to a hypothesis rather than a hypothesis is tested;
- we can "accept" the null hypothesis rather than "fail to reject" it;
- parsimony imposed in model choice rather than correcting for multiple tests.

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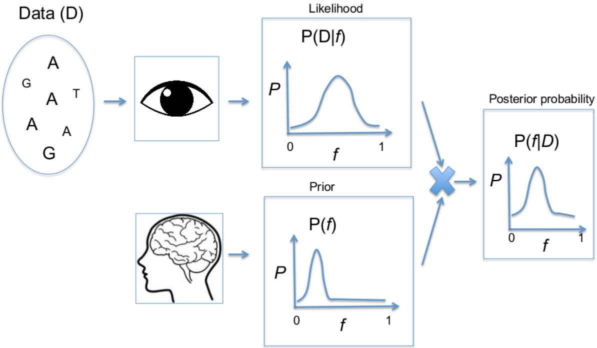
Bayesian inference



Bayesian concepts

Bayes' Theorem

$$p(\vec{\theta}|\vec{y}) = \frac{f(\vec{y}|\vec{\theta})\pi(\vec{\theta})}{m(\vec{y})} = \frac{f(\vec{y}|\vec{\theta})\pi(\vec{\theta})}{\int f(\vec{y}|\vec{\theta})\pi(\vec{\theta})d\vec{\theta}} \quad (1)$$

- $\vec{\theta}$ is not a fixed parameter but a random quantity with prior distribution $\pi(\vec{\theta})$
- $p(\vec{\theta}|\vec{y})$ is the posterior probability distribution of $\vec{\theta}$
- $\int p(\vec{\theta}|\vec{y})d\vec{\theta} = 1$

Genotype posterior probability

A

A

A

G

$\epsilon = 0.01$

A,G alleles

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73		
AG	-2.80		
GG	-17.12		

Genotype posterior probability

AAAG & $\epsilon = 0.01$ & A,G alleles

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73	1/3	0.05
AG	-2.80	1/3	0.95
GG	-17.12	1/3	0

Only call genotypes if the largest probability is above a certain threshold (e.g. 0.95).

Genotype posterior probability

AAAG & $\epsilon = 0.01$ & A,G alleles & **A is the reference allele**
$P(AA) > P(AG) > P(GG)$

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73	0.80	0.22
AG	-2.80	0.15	0.78
GG	-17.12	0.05	0

The reference allele is just one of the possible alleles, often chosen arbitrarily: why give it so much weight?

Genotype posterior probability

AAAG & $\epsilon = 0.01$ & A,G alleles & $f(A) = 0.7$ from a reference panel
$P(AA) = ?$; $P(AG) = ?$; $P(GG) = ?$

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73		
AG	-2.80		
GG	-17.12		

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Genotype posterior probability

AAAG & $\epsilon = 0.01$ & A,G alleles & $f(A) = 0.7$ from a reference panel

$P(AA) = ?$; $P(AG) = ?$; $P(GG) = ?$

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73	0.49	0.06
AG	-2.80	0.42	0.94
GG	-17.12	0.09	0

If the assumption of HWE can be reasonably met.

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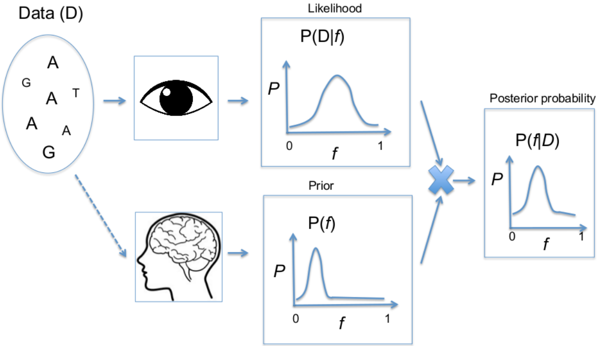
Empirical Bayesian inference



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Genotype posterior probability

AAAG & $\epsilon = 0.01$ & A,G alleles & $f(A) = 0.6$ from the data itself

$P(AA) = ?$; $P(AG) = ?$; $P(GG) = ?$

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73	0.49	0.04
AG	-2.80	0.42	0.96
GG	-17.12	0.09	0

- if the assumption of HWE can be reasonably met
- if you have enough samples to have a robust estimate of the allele frequencies

Practical: genotype calling

`https://github.com/mfumagalli/Copenhagen`

Genotype posterior probability

AAAG & $\epsilon = 0.01$ & A,G alleles & $f(A) = 0.6$ from the data itself

Genotype	Likelihood (log)	Prior	Posterior
AA	-5.73	0.49	0.04
AG	-2.80	0.42	0.96
GG	-17.12	0.09	0

- if the assumption of HWE can be reasonably met
- if you have enough samples to have a robust estimate of the allele frequencies

How can we estimate allele frequencies?

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Estimating allele frequencies

Assuming 2 alleles (A,G) with true allele frequency of 0.50

Sample	True genotype	Reads allele A	Read allele G
1	AA	7	0
2	AA	25	1
3	AG	5	3
4	AG	4	4
5	GG	0	2
6	GG	0	4

What is the simplest estimator of allele frequencies?

Estimating allele frequencies

Assuming 2 alleles (A,G) with true allele frequency of 0.50

Sample	True genotype	Reads allele A	Read allele G
1	AA	7	0
2	AA	25	1
3	AG	5	3
4	AG	4	4
5	GG	0	2
6	GG	0	4
Total		41	14

$$\hat{f} = \frac{\sum_{i=1}^{N} n_{A,i}}{\sum_{i=1}^{N}(n_{A,i} + n_{G,i})}$$

$\hat{f} = 0.75$

What is wrong with this estimator?

Estimating allele frequencies

Assuming 2 alleles (A,G) with true allele frequency of 0.50

Sample	True genotype	Reads allele A	Read allele G
1	AA	7	0
2	AA	25	1
3	AG	5	3
4	AG	4	4
5	GG	0	2
6	GG	0	4
Total		41	14

$$\hat{n_A} = \sum_{i=1}^{N} (1-\epsilon) n_{A,i} + \epsilon n_{G,i} - \epsilon n_{A,i} - (1-\epsilon) n_{G,i}$$

$\hat{f} = 0.77$

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Estimating allele frequencies

Maximum Likelihood estimator

$$P(D|f) = \prod_{i=1}^{N} \sum_{g \in \{0,1,2\}} P(D|G=g)P(G=g|f)$$

Estimating allele frequencies

Maximum Likelihood estimator

$$P(D|f) = \prod_{i=1}^{N} \sum_{g \in \{0,1,2\}} P(D|G=g)P(G=g|f)$$

$P(D|G=g)$ is the genotype likelihood and $P(G=g|f)$ is given by HWE (for instance).

In our previous example, $\hat{f} = 0.46$ which is much closer to the true value than previous estimators.

SNP calling

Challenges

- If high levels of missing data, then genotypes can be lost.
- Rare variants are hard to detect.
- Trade off between false positive and false negative rates.

How to call SNPs?

- If at least one heterozygous genotype has been called.
- If the estimated allele frequency is above a certain threshold.

SNP calling

Call a SNP if

$$\hat{f} \geq t$$

where t can be the minimum sample allele frequency detectable (e.g. $t = 1/2N$ with N diploids).

Likelihood Ratio Test

A Likelihood Ratio Test (LRT) compares the goodness of fit between the null and the alternative model:

- Null model: $f = 0$
- Alternative model: $f \neq 0$

$$T = -2 \log \frac{L(f = 0)}{L(f = \hat{f}_{MLE})}$$

where T is χ^2 distributed with 1 degree of freedom.

Practical: allele frequencies and SNP calling
`https://github.com/mfumagalli/Copenhagen`

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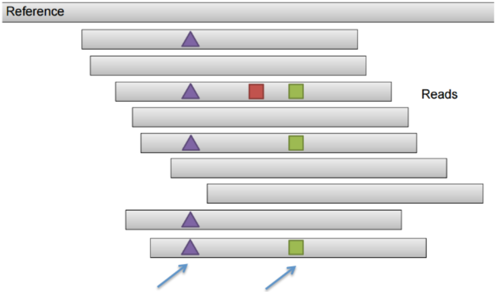
SNP calling procedures

- Alignment-based caller



We completely rely on how reads have been mapped

Figure from Erik Garrison

SNP calling procedures

- Assembly-based caller (as in GATK)

Local re-alignment around putative variants; better resolution for INDELs detection.

- Haplotype-based caller (as in freebayes)

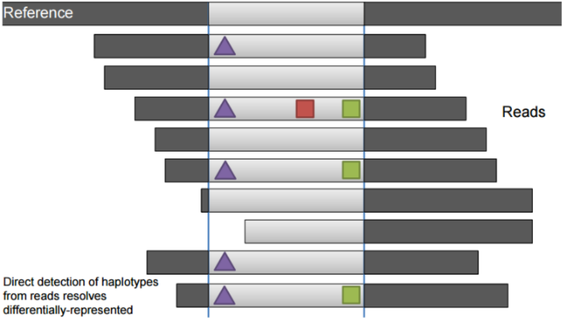
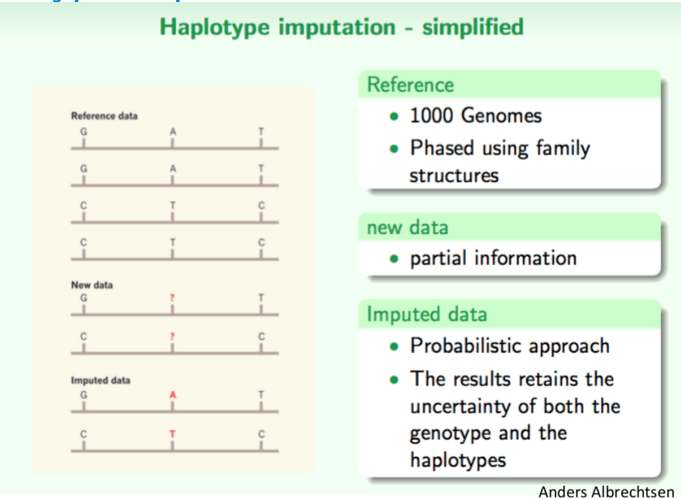


Figure from Erik Garrison

Haplotype imputation



Haplotype imputation - simplified

Reference

- 1000 Genomes
- Phased using family structures

new data

- partial information

Imputed data

- Probabilistic approach
- The results retains the uncertainty of both the genotype and the haplotypes

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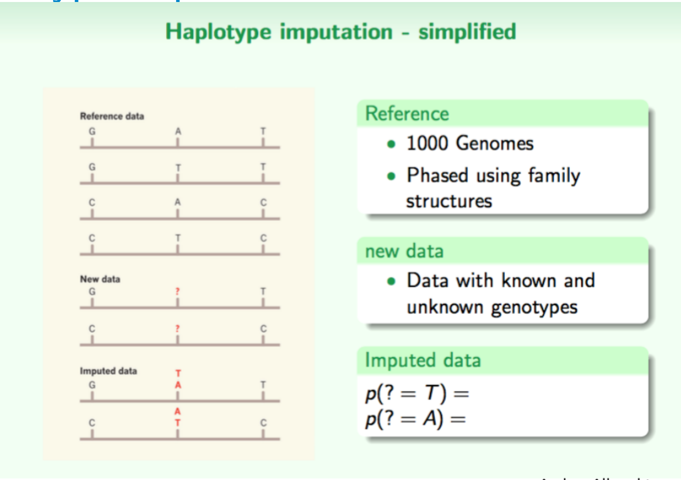
Haplotype imputation



Haplotype imputation - simplified

Reference

- 1000 Genomes
- Phased using family structures

new data

- Data with known and unknown genotypes

Imputed data

$p(? = T) =$
$p(? = A) =$

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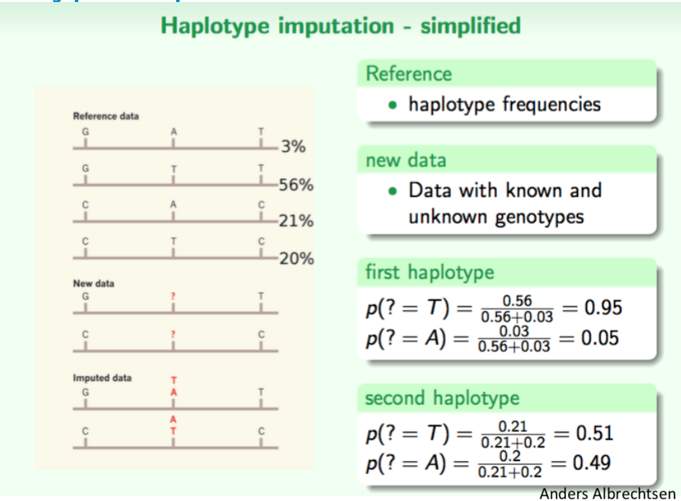
Haplotype imputation



Haplotype imputation - simplified

Reference

- haplotype frequencies

new data

- Data with known and unknown genotypes

first haplotype

$$p(? = T) = \frac{0.56}{0.56+0.03} = 0.95$$
$$p(? = A) = \frac{0.03}{0.56+0.03} = 0.05$$

second haplotype

$$p(? = T) = \frac{0.21}{0.21+0.2} = 0.51$$
$$p(? = A) = \frac{0.2}{0.21+0.2} = 0.49$$

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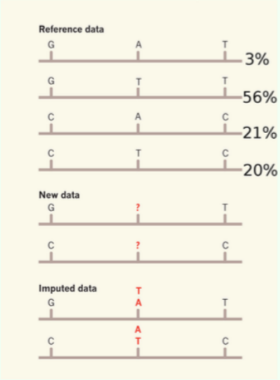
Haplotype imputation

Haplotype imputation - simplified



Bayes formula

$$p(H=h|f,G) = \frac{P(G|H=h)P(H=h|f)}{\sum_{h'} P(G|H=h')P(H=h'|f)}$$

$P(G|H=h)$

1 if consistent

0 otherwise

first haplotype

$$p(?=T) = \frac{0.56}{0.56+0.03} = 0.95$$
$$p(?=A) = \frac{0.03}{0.56+0.03} = 0.05$$

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