Future perspectives

on genetic diagnostics

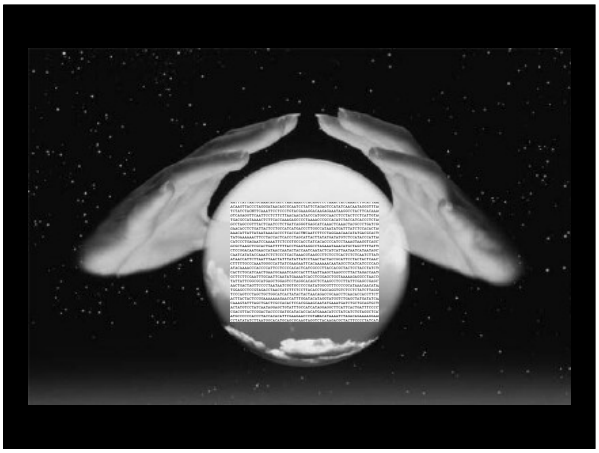
Johan den Dunnen

Human & Clinical Genetics

(Leiden University Medical Center)

CMSB

- **Genetic Disease**
  - ***neuromuscular disorders***
  - ***http:// www.DMD.nl***
  - ***diagnosis***
  - ***treatment / therapy***
- **Genome Technology**
  - ***try and apply***
  - ***facilitate***
  - ***Leiden Genome Technology Center***
  - ***http:// www.LGTC.nl /***

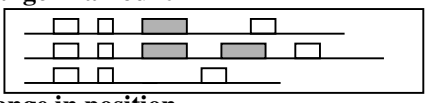


Possible variants

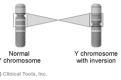
- **change in sequence**

```
ACATCAGGAGAAGATGTTC GAGACTTTGCCA
ACATCAGGAGAAGATGTTT GAGACTTTGCCA
ACATCAGGAGAAGATGTT  GAGACTTTGCCA
ACATCAGGAGAAGATGTTCCGAGACTTTGCCA
```

- **change in amount**



- **change in position**



Summary

- **change in sequence**
  - ***sequence***
- **change in amount (CNV)**
  - ***sequence & count***
    - ***paired-end > breakpoints***
- **change in position**
  - ***sequence***
    - ***paired-end > breakpoint***

Non-Invasive Prenatal diagnosis

sequence ccff DNA/RNA

paired-end > size

LGTC technologies

- **DNA**
  genomics
- **RNA**
  transcriptomics
- **protein**
  proteomics
- **metabolite**
  metabolomics

BioInformatics

LGTC equipment

sequencing
ABI3730, ABI3730xl
pyrosequence
Solexa

PCR
TaqMan 7900HT
LightCycler480
Fluidigm
…

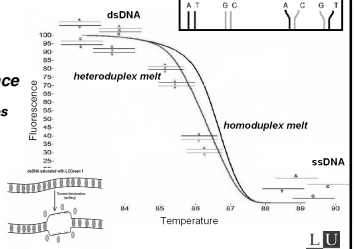
array technology
Affymetrix (incl. 4-colour)
Illumina (incl. BeadXpress)
Agilent scanner
hybstations
arrayer (OmniGrid)
FlexArrayer

other
LightScanner (Idaho)
BioAnalyzer
NanoDrop
Caliper LC-90
robotics (Tecan, Caliper)
GeneTAC G3

Melting Curve Analysis

(MCA)

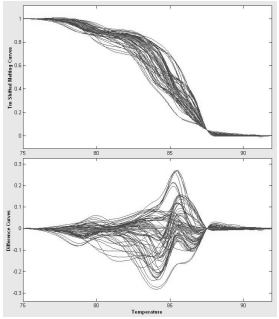
- **principle**
  sequence difference > altered melting
  mismatch in heteroduplex reduces Tm
- **detection**
  DNA + intercalating fluorescent dye
  dsDNA
  measure fluorescence while raising T
  denaturation gives decreasing fluorescence
- **closed-tube assay**



MCA applications

- **variant detection**
  BRCA1/2, DMD, …
- **SNP typing**
  melt probes
- **gel electrophoresis**
- **allelic imbalances**
  CNV confirmation
- **methylation**
- **clone identification**

Clone selection



Phage-display

96 clones selected

MCA > determine complexity

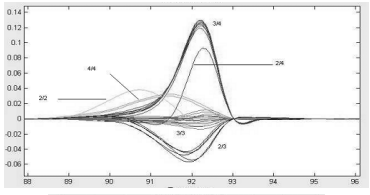
- first conclusion
- select for sequencing

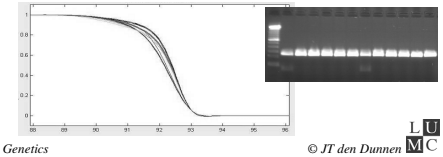
more informative then gel electrophoresis

Variant detection

ApoE
E2 / E3 / E4

dye added post-PCR





CNV & hrMCA

- **SNP-based**
  - ***required***
    - ***homozygous sample AA & BB***
    - ***test sample A?*** ***(AA, A0, AAA)***
- **assay**
  - ***amplify***
    - ***AA, BB, test sample***
    - ***1:1 mix AA / BB***
    - ***1:2 mix AA / BB***
    - ***1:1 mix BB / test (A?)***
    - ***other mixes (1:3, 3:1, etc.)***
- **result**
  - ***test = AA / BB mix > AA / BB***
  - ***test ≠ AA / BB mix > A0 / BB or AAA / BB***

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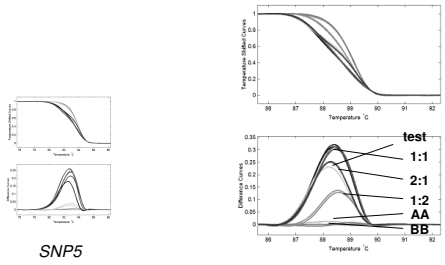
Somatic mosaicism

(high-resolution Melting Curve Analysis)



SNP5

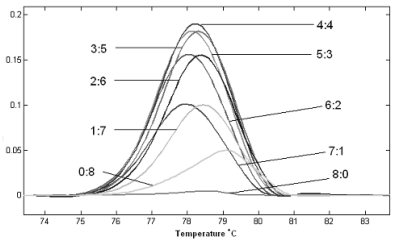
deletion confirmed

SNP4

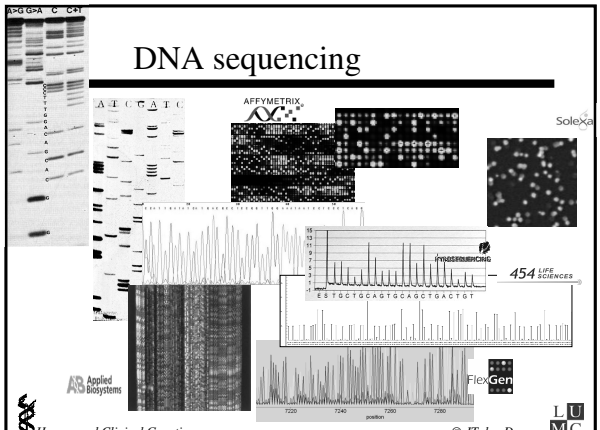
©Margot Kalf

hrMCA octaploid

(large shift variant)



DNA sequencing



LGTC / Solexa

- **LGTC characteristics**
  - ***not a large sequencing center***
  - ***no BioInformatics support***
    - ***result on CD (hard disk)***
  - ***academic customers***
    - ***hesitating new users***
  - ***many different applications***
  - ***all formats***
    - ***do it yourself / outsourcing***
    - ***collaboration ServiceXS***

Solexa 1G since Q1 2007

offer alternative technologies

Summary

- **system performs as expected**
  - ***with ups & downs***
  - ***steadily increasing performance***
    - ***0.5 MB to 2.4 Mb runs***
- **important aspects**
  - ***sample preparation !***
  - ***skilled operator***
- **data analysis**
  - ***software behind on technology***
    - ***de novo assembly***

Yavuz Ariyurek

Sophie Greve

Leiden Genome Technology Center LGTC

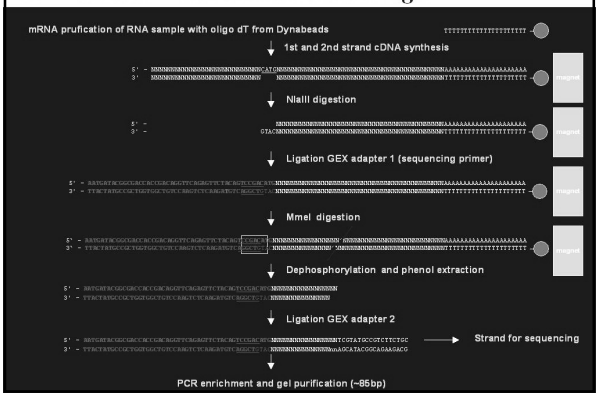
Current projects

- **replace arrray**
  - *Chromatine-IP*
  - *micro RNA profiling & discovery*
  - *gene expression profiling*
  - *SNP typing*
- **SNP-discovery**
- **genome re-sequencing**
- **disease candidate gene / gene region**
- **de novo genome sequencing**
- **….**

Expression profiling

- **model**
  - ***wt / transgenic DCLK***
    - *constitutive expression δC-doublecortin-like kinase*
    - *brain > hippocampus*
  - ***subtle behavioural abnormalities***
- **micro-array analysis**
  - ***5 platforms***
    - *> only subtle changes*
    - *> biological replicates*
- **approach**
  - ***pools (wt / transgenics)***
    - *Solexa / Illumina*
  - ***individual mice***
    - *Leiden (n=4 per group)*

Solexa – Generation SAGE tags



Results expression

	Sequence	Sample 1	Sample 2	Sum	GENE	DESCRIPTION
2	CATGATAATACATAAAAAAAA	114494	101445	215939		mitochondrial
3	CATGTATTAATAAATAGAGCA	41359	31710	73069	Tspan7	Tetraspanin 7
4	CATGTAACTTTAAGATGCTGC	28668	35622	64290	Olfm1	Olfactomedin 1
5	CATGGAATAATAAAACTATTT	30583	30787	61370	Hspa8	Heat shock protein 8
6	CATGGGCTTCGGTCTTTTTGA	22855	27311	50166	Rplp1	Ribosomal protein, large, P1
7	CATGCATCCTTGATGTTTGCC	17745	20549	38294	Ckb	Creatine kinase, brain
8	CATGTGTAAAATAACTGGATA	18165	18401	36666	2900019G14Rik	RIKEN cDNA 2900019G14 gene
9	CATGCCTTACCGCTGTAATGG	17787	17977	35764	E130013N09Rik	RIKEN cDNA E130013N09 gene
10	CATGCTGAGGAAGTGAGATAC	16401	19341	35742	Sparcl1	SPARC-like 1 (mast9, hevin)
11	CATGTAAAAAAGAAAGGATGGG	15963	17769	33732	Ndrg2	N-myc downstream regulated gene 2
12	CATGATGACAAAGAAAAAGAC	18093	15461	33554	Cplx2	Complexin 2
13	CATGTTAATAAAAGTATTCGT	16852	16598	33450	Mrfap1	Morf4 family associated protein 1
14	CATGCAAAAATAAAAGCCGCA	12881	18090	30971	Eno1	Enolase 1, alpha non-neuron
15	CATGCCTACTAACCAGAGCTG	13501	17210	30711	Aldoa	Aldolase 1, A isoform
16	CATGCAAAATACATTTATTTG	13545	16985	30530	Maged1	Melanoma antigen, family D, 1
17	CATGAGGCAGACAGTTGCTGT	14987	14380	29367	Eef1a1	Eukaryotic translation elongation factor 1 alpha 1
18	CATGTGTAGTGTAATAAAGGT	16248	13077	29325	Rps8	Ribosomal protein S8

2 samples analysed

mouse brain wt <> transgenic

~ 7.4 M tags per sample

range 36 K (0.5%) - 1

Result expression

Solexa

	A	B	C	D	E	F
1	Tag sequence	Gene-ID	sample A	sample B	Ratio	P value
2	AATTTCTTCCTCTTCCT	Mup1	2165	38	57.59	0
3	TTAATATTTAATTAGAG	CD771464	1071	7	154.62	0
4	ACAGTGGGGATTTTTTT	Ptges3	887	2783	0.32	0
5	AGAATAATATATGAATT		849	383	2.24	0.04
6	TAAATGTTGCTAGCCTC		690	2	348.64	0
7	TCCAACTCCTTTCTTTA	Mrps21	609	1458	0.42	0
8	GGGAGCGAAAAGGTTAA	Id2	380	2	192	0
9	AGTGTGACGTGACCGGG	Mapk8ip3	323	1	326.39	0
10	AAGACTCAGGACTCATC	Alb1	315	1	318.31	0
11	ATGATAATGGACTGAGC					
12	ATTTGTACTCATACCAG					
13	AGGAAAGCCTCTCAAGT					
14	TCCAACCCAAACTAATA					
15	GAAATTCTCCATTGATT					
16	CCTGATGCTACAGAAAA					
17	AAAGGATGTTAATTACA					
18	AAATTCTTCCTCTTCCT					

	A	B	C	D	E	F
81	CAAAATACATTTATTTA		8	54	0.15	0
82	GGCTTCGGTCTTTATGA		7	61	0.12	0
83	TTCCAGCCTTACTATGT		7	36	0.2	0.03
84	CAAATAAGCAAGGGTGG	CJ278059	6	31	0.2	0.05
85	AAAAATTTGTTTTGCTC		5	32	0.16	0.02
86	TATAGTATGTTCGATTA		4	33	0.12	0.01
87	CAATAAACTGAAAAGAC		4	26	0.16	0.04
88	CAGTTATTAATTTCTTA		3	32	0.09	0.01
89	ACCCTCTAAAGAGCAGA		3	24	0.13	0.04
90	CATCCTTGATGTTAGCC		2	40	0.05	0
91	TCCAACTTGTAACGATA		2	20	0.1	0.05
92	CCTCCTCCAGGAAAGAG		1	22	0.05	0.01
93	TCCATCCAGGTGACTTA		1	17	0.06	0.05
94	GGATTTGGCTTGTATGA		0	29	0	0
95	CTCCGTTTTGTTCAGTA		0	23	0	0
96	CAGGCCACACAAGAGCG	CJ230439	0	14	0	0.05

> 2 fold change
(P <0.05)
131 genes

Expression profiling

- **easily interpretable results**
  - *numbers*
  - *no cross-hybridization & background*
- **differential expression**
  - *4 orders of magnitude, more genes significant*
- **additional to arrays**
  - *47% genes differential polyA addition*
  - *51% genes anti-sense transcription*
- **lower variation**
  - *fewer replicates required*
  - *inter-lab data compare much better*
- **array comparison**
  - *limited overlap*
    - *all indicate disturbed GABA-ergic signaling*
  - *MPSS higher fold changes*

Identification of SNPs In Turkey using massive parallel sequencing on the Solexa sequencing platform

Martien Groenen

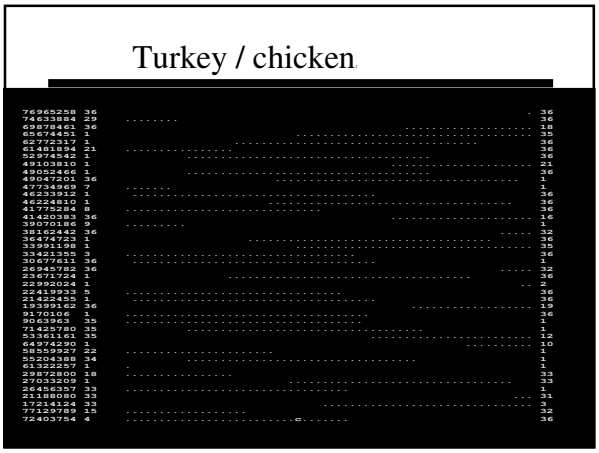
Animal Breeding & Genomics Centre

Solexa sequencing: strategy

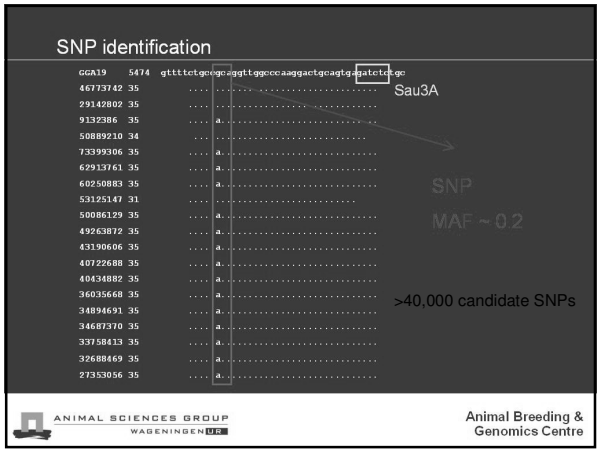
- Mix DNA from 6 individuals from 2 breeds
- DNA digested with Sau3A
- Separate on agarose gel
- Isolate 2000-4000 bp fraction
- Random shearing of fragments
- Isolate 200-250 bp fragments

Turkey / chicken

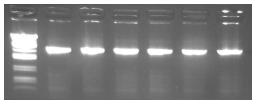
Turkey / chicken



SNP identification



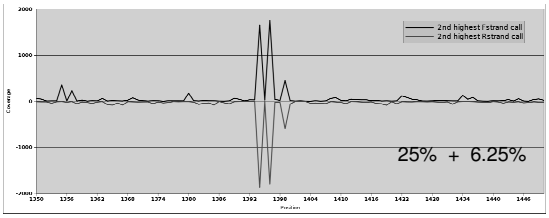
Deep sequencing



- **viral pool**
  identify low-% variants
- **sensitivity test**
  3.1 Kb fragment, plasmid cloned PCR product
  - ***high-fidelity polymerase***
  - ***gel-purified***
  - ***fragmented***
- **spike known variants**
  25% (2), 6.25%, 1.56% (2), 0.42%, 0.10% (2)

Deep sequencing

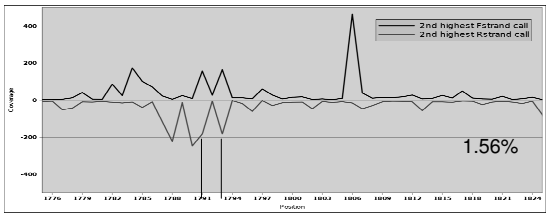
(low quality sequence run)



true variant: F + R
1394A>C + 1396A>C
1399C>T

Deep sequencing

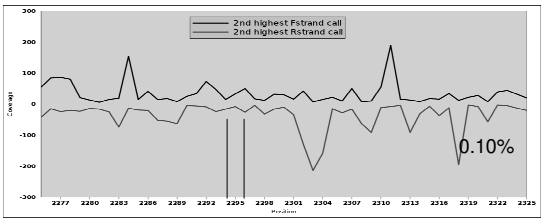
(low quality sequence run)



true variant: F + R
1791A>G + 1793C>T

Deep sequencing

(low quality sequence run)



true variant: F + R
2294C>T + 2296T>C

Targeted sequencing

(complexity reduction)

- **chromosome sorting**
- **gel separation**
  Pulsed-Field Gel-electrophoresis
- **megabase regions**
  cover by long-range PCR
  1 Mb > 100 x 10Kb fragments
  multiplex PCR
- **smaller regions**
  pool samples
  add sequence tag
- **array hyb-selection**

compare controls <> cases

Candidate gene

- **compare controls / cases**
- **long-range PCR** (8.5 Kb)

pool 176 DNA samples, then lrPCR
no reproducible result
very sensitive to DNA quality

pool 176 lrPCR products
PCR, hrMCA check (yield, purity)
reproducible result
SNP freq. 1-2%
all variants detected (1/176) + more…
error rate 0.3 - 0.5% ***(= 1/176 chromosomes)***
redundancy > useful

pool tagged (lr)PCR products
…under investigation

Deep sequencing

- **pooling is possible**
  DNA quality !
- **detection limit ~0.5 % variant**
  - mix 50-100 patients
  - mix mixes of 50-100 barcoded patients
  number of reads determines limit
  combine ~500 patients per run

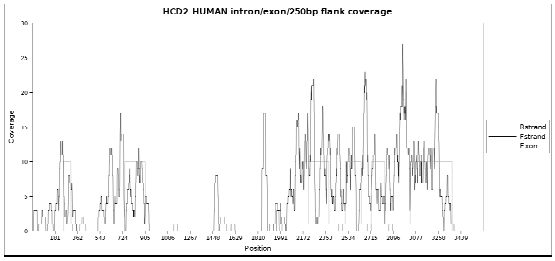
Hyb-selection

- **NimbleGen array**
  5 custom design arrays
  minimal order
  2,100,000 probes
  60-80 nt
  116 candidate genes (MR)
  exons + 100 nt intron, tiling
- **hybselection**
  Solexa sample prep
  up to seq.linkers added
  hybridize 60h
  wash, collect DNA
  qPCR check
  non-selected: 5 PCRs
  selected: 5 PCRs

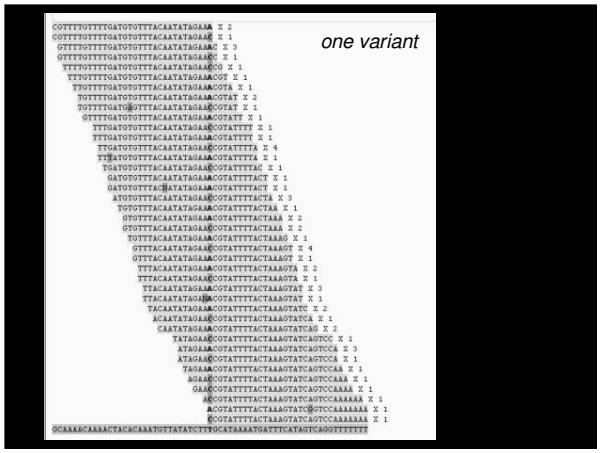
©Yavuz Ariyurek
Rowida Al-Moman

Hyb-selection

selection on NimbleGen array

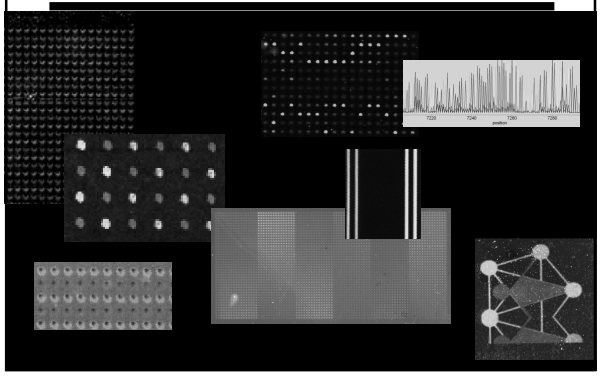


9% sequence from selected region



FlexArrayer

FlexGen arrays



Genome re-sequencing

- **bacteria**
  - ***disease-related strains***
    - *large collections*
    - *drug resistance, virulence, infectivity, …*
  - ***diagnostic typing***
  - ***genes involved***
- **viruses**
- **moulds**
- **…** *(human patients)*

Future ahead

***Soon* we will be able to sequence a complete human genome**

A human genome

(www.LUMC.nl)

- **by academic hospital**
  - *not a large genome center,*
  - *nor a company (sequence technology)*
- **Marjolein Kriek**

PhD, clinical geneticist (i.t.)

first from LUMC,
Leiden,
Nederland,
Europe
female

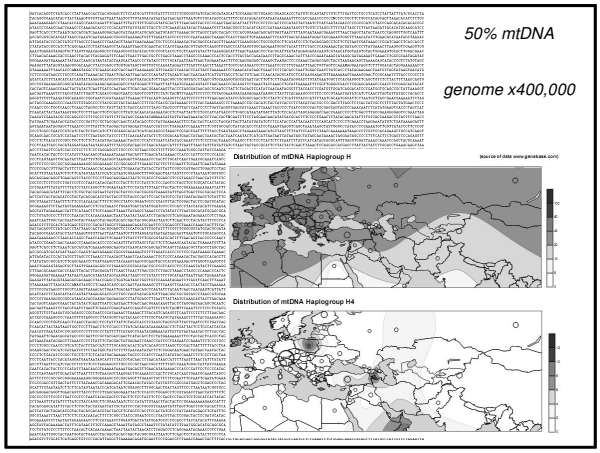
A human genome

- **why ?**
  - *show it is possible*
    - *technical, computational, analytical*
  - *to learn*
    - *technology, data floods, analysis*
  - *attractive project to tackle*
- **results**
  - *technically - no problem*
  - *computationally - at our limits*
  - *analytically - not possible*
    - *as expected*
- **>> to be applied in patients**
  - *resolve cause genetic disease*

Analyse a genome

draw DNA-based conclusions

1. a female > no Y-chromosome sequences



Analyse a genome

- **red hair…**

suggested by all

literature
MC1R gene
no LSDB

many variants
%-change red hair

Analyse a genome

- **blue eyes…**

suggested by all

literature
blue iris colour
HERC2, OCA2
no LSDBs

many variants
%-chance iris colour
analyse SNP profile


The American Journal of Human Genetics 82, 411–423, February 2008 411

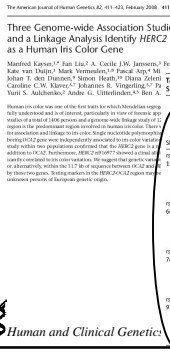
ARTICLE

Three Genome-wide Association Studies and a Linkage Analysis Identify *HERC2* as a Human Iris Color Gene

Manfred Kayser,[1,*] Fan Liu,[2] A. Cecile J.W. Janssens,[3] Fernando Rivadeneira,[4,5] Oscar Lao,[1] Kate van Duijn,[1] Mark Vermeulen,[1,6] Pascal Arp,[4] Mila M. Jhamai,[4] Wilfred F.J. van IJcken,[6] Johan T. den Dunnen,[6] Simon Heath,[10] Diana Zelenika,[10] Dominiek D.G. Despriet,[5,7] Caroline C.W. Klaver,[5,7] Johannes R. Vingerling,[5,7] Paulus T.V.M. de Jong,[5,11,12] Albert Hofman,[5] Yurii S. Aulchenko,[2] Andre G. Uitterlinden,[4,5] Ben A. Oostra,[2] and Cornelia M. van Duijn[2]

Human iris color was one of the first traits for which Mendelian segregation was established. To date, the genetics of iris color is still not fully understood and is of interest, particularly in view of forensic applications. In three independent genome-wide association (GWA) studies of a total of 1406 persons and a genome-wide linkage study of 1292 relatives, all from the Netherlands, we found that the 15q13.1 region is the predominant region involved in human iris color. There were no other regions showing consistent genome-wide evidence for association and linkage to iris color. Single nucleotide polymorphisms (SNPs) in the *HERC2* gene and, to a lesser extent, in the neighboring *OCA2* gene were independently associated to iris color variation. *OCA2* has been implicated in iris color previously. A replication study within two populations confirmed that the *HERC2* gene is a new and significant determinant of human iris color variation, in addition to *OCA2*. Furthermore, *HERC2* rs916977 showed a clinal allele distribution across 23 European populations, which was significantly correlated to iris color variation. We suggest that genetic variants regulating expression of the *OCA2* gene exist in the *HERC2* gene or, alternatively, within the 11.7 kb of sequence between *OCA2* and *HERC2*, and that most iris color variation in Europeans is explained by those two genes. Testing markers in the *HERC2-OCA2* region may be useful in forensic applications to predict eye color phenotypes of unknown persons of European genetic origin.

Analyse a genome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ARTICLE

Three Genome-wide Association Studies and a Linkage Analysis Identify *HERC2* as a Human Iris Color Gene

Manfred Kayser,[1,*] Fan Liu,[2] A. Cecile J.W. Janssens,[3] Fernando Rivadeneira,[4,5] Oscar Lao,[1] Kate van Duijn,[1] Mark Vermeulen,[1,6] Pascal Arp,[4] Mila M. Jhamai,[4] Wilfred F.J. van IJcken,[6] Johan T. den Dunnen,[6] Simon Heath,[10] Diana Zelenika,[10] Dominiek D.G. Despriet,[5,7] Caroline C.W. Klaver,[5,7] Johannes R. Vingerling,[5,7] Paulus T.V.M. de Jong,[5,11,12] Albert Hofman,[5] Yurii S. Aulchenko,[2] Andre G. Uitterlinden,[4,5] Ben A. Oostra,[2] and Cornelia M. van Duijn[2]

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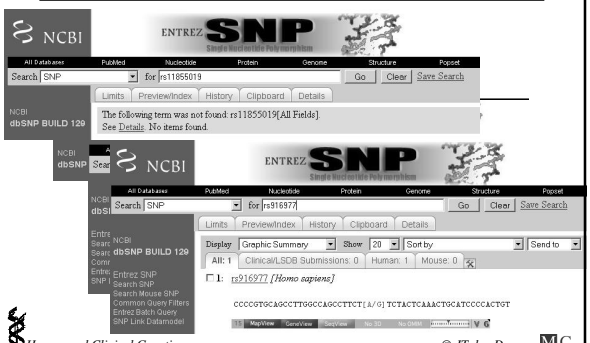
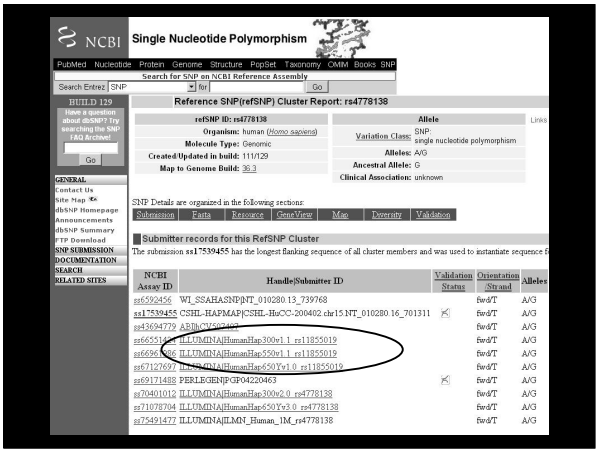
Table 3. Confirmation of Iris Color Association of Four SNPs from the *OCA2-HERC2* Region in the Total ERF and Rotterdam Studies, Stage 2, and HapMap Genotype Frequencies for the SNPs Studied

SNP	Geno type	ERF Study (n = 2217) n (%)	Blue	Intermediate	Brown	p Value	Rotterdam Study (n = 6054) n (%)	Blue	Intermediate	Brown	p Value	HapMap Europeans n	Freq. (%)	East Asians n	Freq. (%)	Africans n	Freq. (%)
rs 11855019	TT	1440 (66.7)	88.4	72.1	40.0	4.4×10^{-186}	4580 (77.3)	88.1	69.4	47.9	5.4×10^{-215}	49	81.7	5	5.5	3	5.0
	TC	655 (30.3)	11.1	27.0	53.1		1250 (21.1)	11.5	29.0	46.6		11	18.3	32	35.6	26	43.3
	CC	66 (3.0)	0.5	0.9	6.9		97 (1.6)	0.4	1.6	5.5		0	0	53	58.9	31	51.7
rs 6497268[a,b]	GG	1441 (66.7)	86.8	68.1	44.2	5.0×10^{-75}	4233 (72.1)	85.7	57.5	38.0	2.8×10^{-167}	45	75.0	2	2.2	8	13.3
	GT	652 (30.2)	12.6	29.7	49.5		1486 (25.3)	13.6	39.5	54.0		15	25.0	24	26.7	30	50.0
	TT	67 (3.1)	0.6	2.2	6.3		151 (2.6)	0.7	3.0	8.0		0	0	64	71.1	22	36.7
rs 7495174[a]	TT	1749 (81.4)	98.4	82.8	62.3	4.3×10^{-79}	5273 (88.4)	97.4	82.2	64.3	1.4×10^{-219}	54	90.0	7	7.8	43	71.7
	TC	382 (17.8)	1.5	17.2	35.7		659 (11.0)	2.6	17.5	33.4		6	10.0	38	42.2	16	26.7
	CC	17 (0.8)	0.1	0	2.0		34 (0.6)	0	0.3	2.3		0	0	45	50.0	1	1.6
rs 916977[a]	CC	1543 (70.9)	94.4	71.9	44.8	1.9×10^{-103}	4572 (77.4)	94.2	58.6	35.2	$<1.0 \times 10^{-308}$	44	73.3	2	2.2	0	0
	CT	573 (26.3)	5.4	27.7	48.4		1229 (20.8)	5.6	39.8	58.1		16	26.7	28	31.1	6	10.0
	TT	60 (2.8)	0.2	0.4	6.8		105 (1.8)	0.2	1.6	6.7		0	0	60	66.7	54	90.0

Analyse a genome





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A human genome

…what do all these variants mean ??

- **few tools available**
- **data scattered over the web**
  - *databases, incl. LSDBs*
  - *many formats*
  - *little phenotype information*
- **most data in drawers**
- **…tools are there**
  - *gene variant databases (LSDBs)*
- **change in attitude required**

Future ahead

***Soon* we will be able to sequence a complete human genome,**

but if we can not make sense out of the variants detected, as to whether they are "pathogenic or not", this information is useless and misinterpretation….

Gene variant databases

Submit <u>all</u> the changes

you have, NOW

(without errors)

LOVD Leiden Open Variation Database

GEN2PHEN

Summary

Sequencing has the future

As clinical lab,
do not buy a system yet,
use that of your colleague,
but start saving money,
in 3-5 years you need it…