SNPs and Targeted Next Generation Sequencing to Detect *RH* Variation in Asian Americans

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Faculty Disclosure

(In compliance with ACCME policy, AABB requires the following disclosures to the session audience)

- None

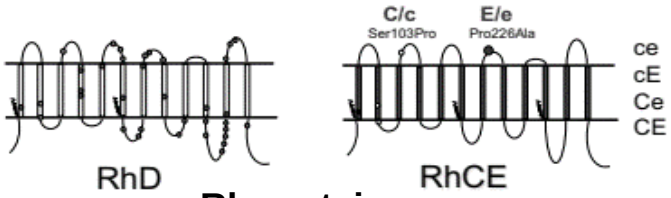
Objectives

- Distinguish between SNP assays and DNA sequencing
- Understand the genetic basis of C antigen
- Recognized causes of apparently D-negative phenotypes in individuals of Asian descent

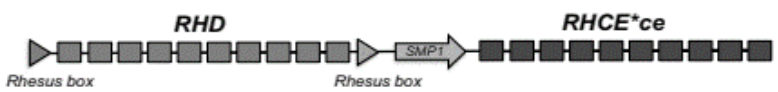
RH genetic variation and significance

Rh is encoded by 2 genes: *RHD* and *RHCE*

- 55 Rh antigens[1]
- >280 *RH* alleles sorted into 3 functional groups:
  - Partial: a protein that expresses some but not all epitopes
  - Weak: low expression of a protein that has all epitopes
  - Null: no epitopes or protein expressed



Rh proteins



***RH* genes**

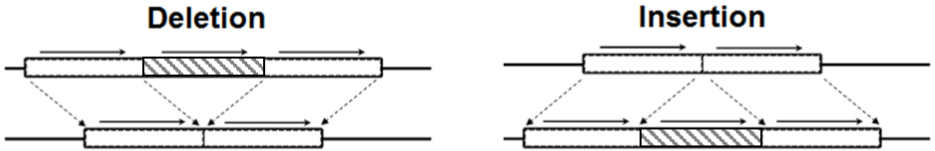
[1]ISBT 004 RHCE alleles v4.0_20180208 ; new antigens continue to be discovered

Different types of *RH* genetic variation

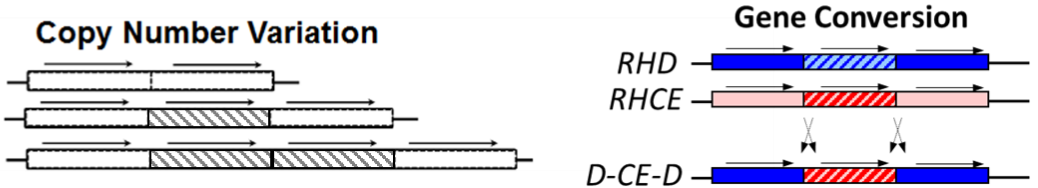
Single Nucleotide Variants (SNVs)

```
Reference  ATCCTTGGATGTTCATCAGTT
SNV        ATCCTTGGAAGTTCATCAGTT
Deletion   ATCCTTGGA___TCATCAGTT
Insertion  ATCCTTGGAGCATGTTCATCAGTT
```

Insertions and Deletions (Indels)

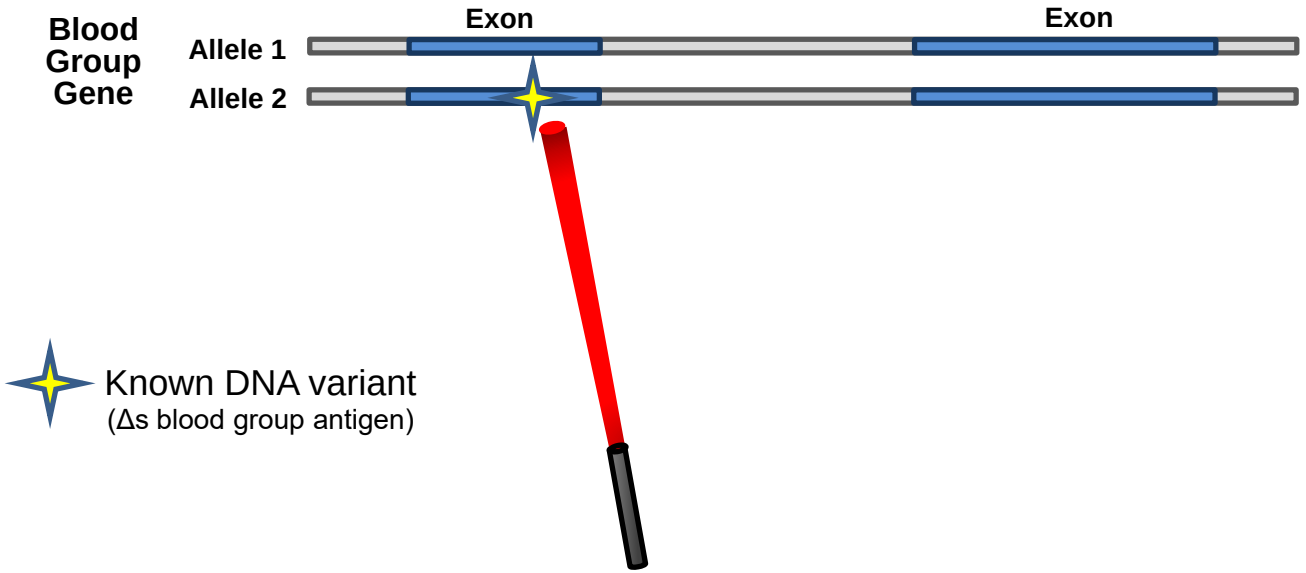


Structural Variants (SVs)

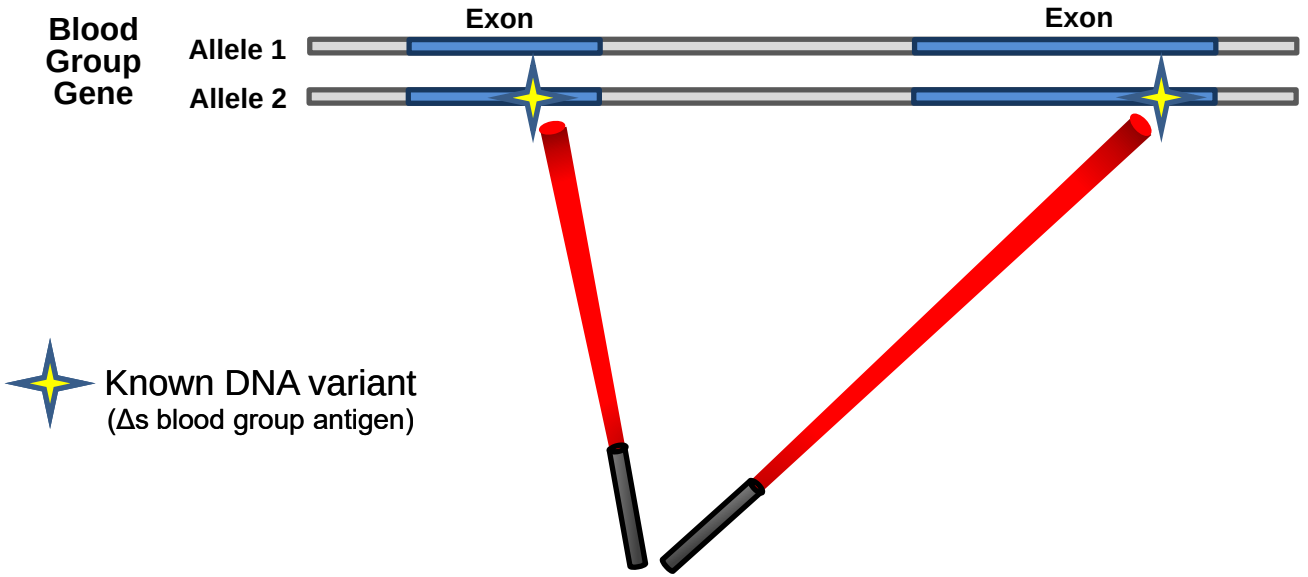


Adapted from: Johnsen et al. Blood. Nov 7;122(19):3268-75, 2013.

SNV (SNP) testing detects a known single nucleotide variant



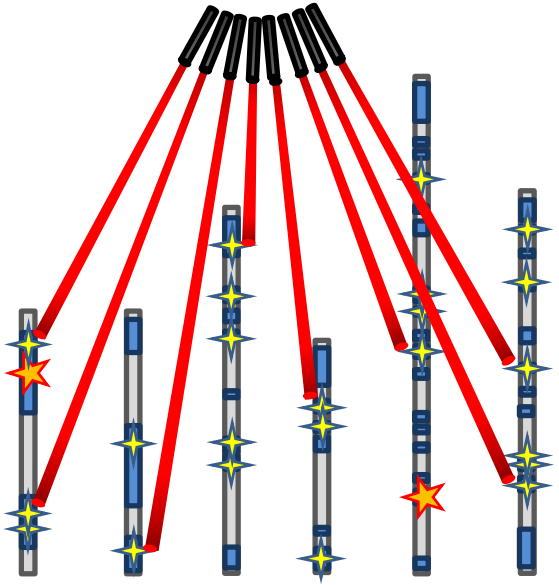
In blood group genes, more information to be gained if can detect multiple variants at once



SNP arrays can detect multiple known variants:
Capable of testing at scale

- *Cannot* directly identify any variant not in the test
  (i.e. rare, novel variants)
- Insensitive to many large variants*

*Some SNP methods can detect CNVs



Known DNA variant
(Δs blood group antigen)

Novel variant
(STOP, splice, etc.)

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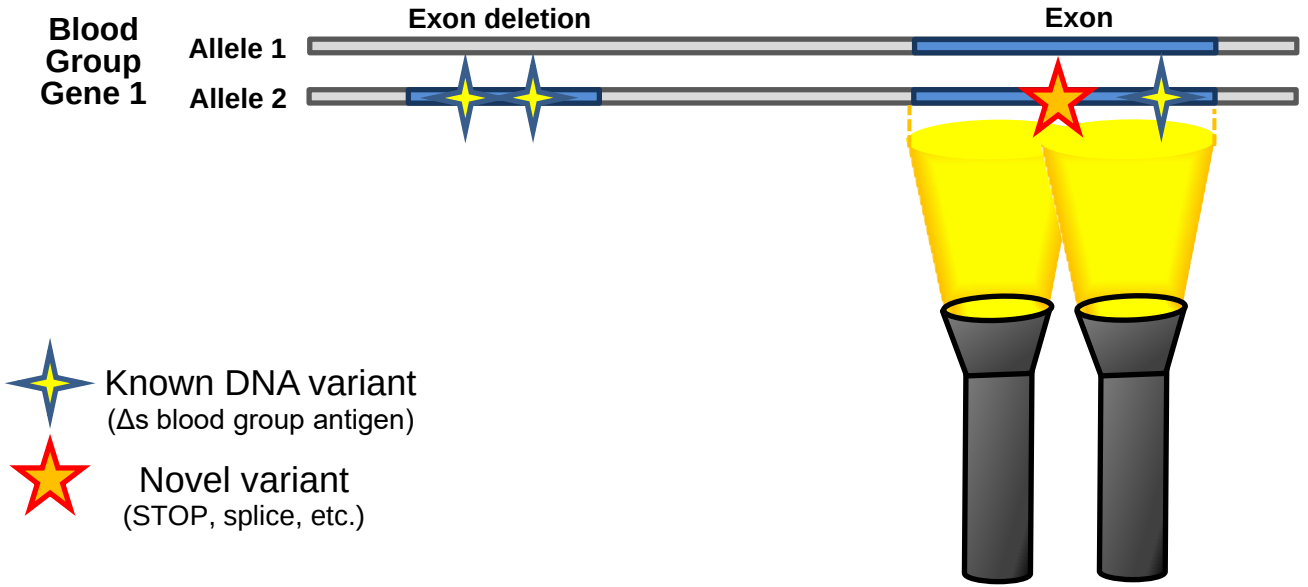
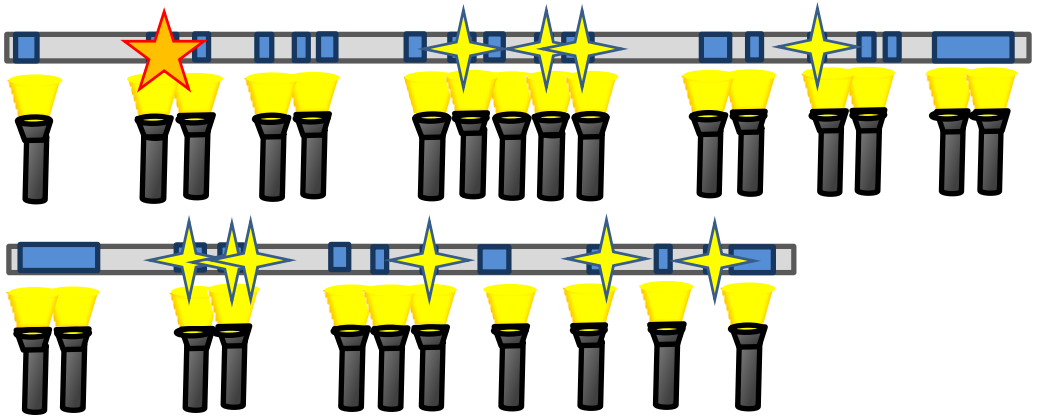
DNA sequencing can identify both known and novel variants



Targeted NGS sequencing

Examples: Exome sequencing, custom gene panels

- **Advantages**: economical, can multiplex
- **Disadvantages**: only looks at regions targeted



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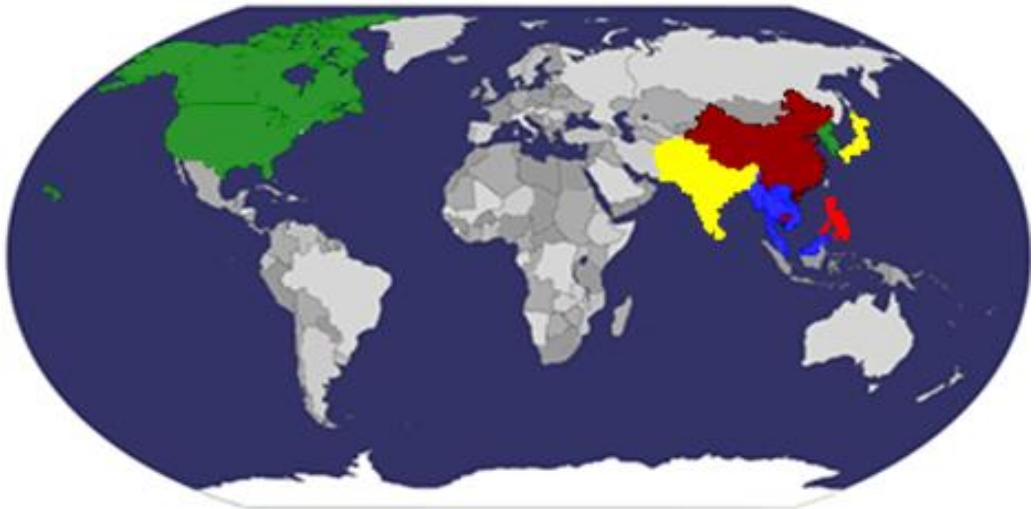
Blood group studies in Asian American donors



Self-Identified Ethnicities

Korean
Japanese
Chinese
Filipino
Southeast Asian
South Asian
Hawaiian / Pacific Islander
(Alaska Native / Aleut / Inuit)
(Native American)

[1]*Delaney M, et al. Transfusion. 2015. Oct;55(10):2369-75.*
[2]*Wheeler M., et al. Genetics in Medicine. epub 2018.*

Studies of blood group gene variation in Asian American blood donors

- SNP study[1]:
  - 9087 volunteer blood donors, clinically characterized
    - Serological typing: ABO, **D, C**, Jk^a, Jk^b, M, N
    - SNP typing (HEA Beadchip™):
      - 23 SNVs to call 32-38 blood group antigens (versions 1.1, 1.2)
      - 5 SNPs predict CcEe
- Blood group gene targeted NGS study (BloodSeq)[2]:
  - 1134 donor samples (selected from *Delaney et al.*)
  - 4 WHO RBC reference DNAs

[1]*Delaney M, et al. Transfusion. 2015. Oct;55(10):2369-75.*
[2]*Wheeler M., et al. Genetics in Medicine. epub 2018.*

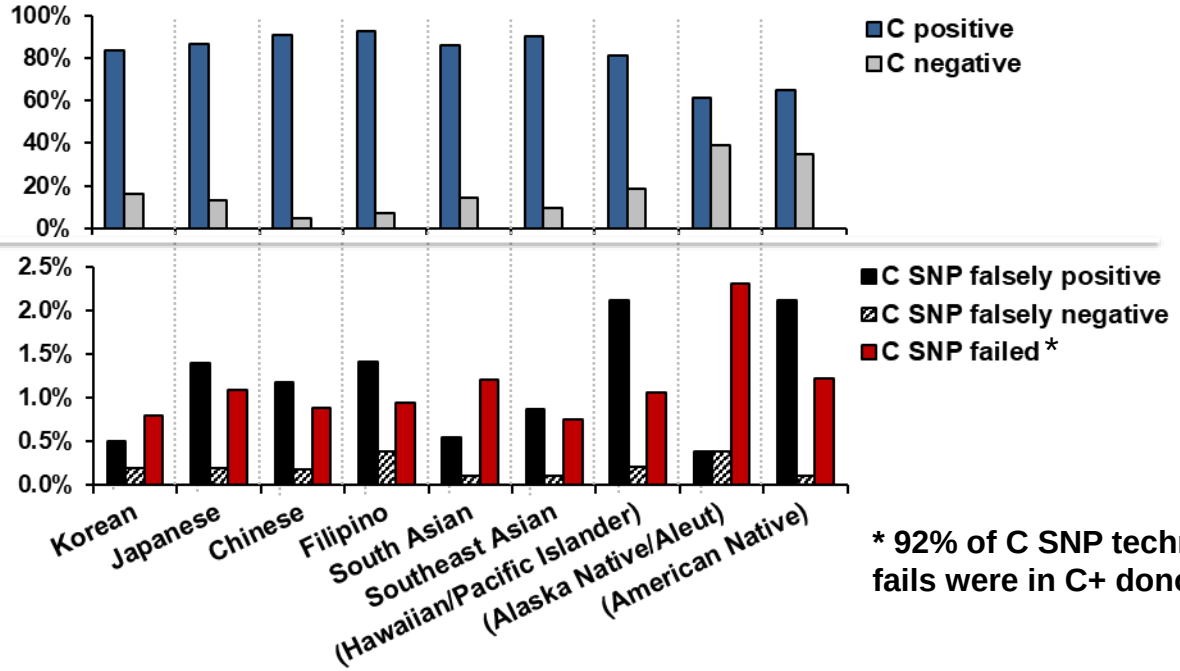
SNP array uses a common *RHCE* genotyping approach

Gene	SNP	Antigen	ISBT Phenotype	ISBT Genotype
RHCE	307 C>T	c/C	RH4 or RH2	RHCE*4 or RHCE*2
	Intron 2 109 bp insertion	e/E	RH5 or RH3	RHCE*5 or RHCE*3
	676 G>C	Vs	RH20	RHCE*01.20.01, RHCE01.20.02, RHCE*01.20.04, RHCE*01.20.05
	733 C>G, 1006 G>T	V	RH10	

Study results: C serology and C SNP

Expression of C antigen (serology)

C SNPs failed to predict C expression



*** 92% of C SNP technical fails were in C+ donors**

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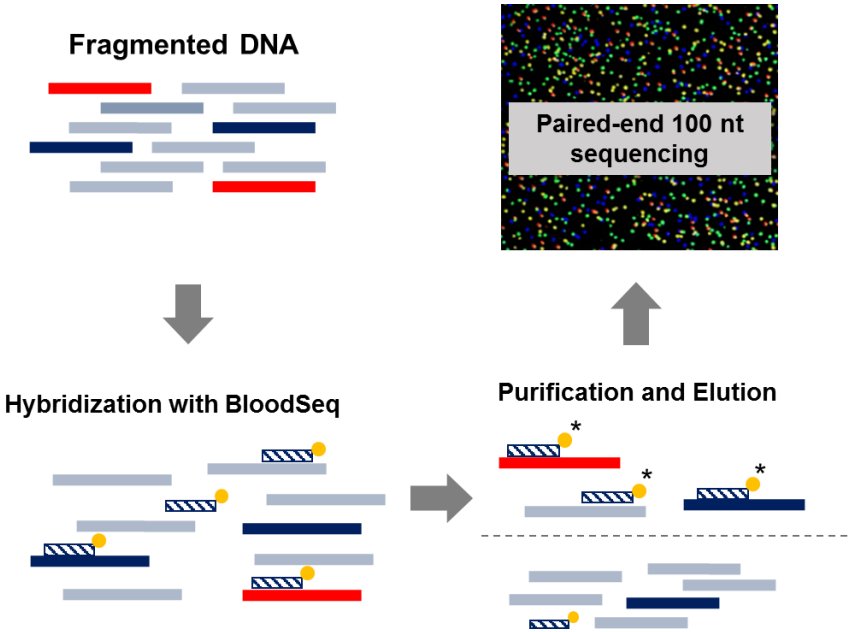
Adapted from Delaney M, et al. Transfusion. 2015. Oct;55(10):2369-75.

BloodSeq: Blood group gene targeted NGS panel

- **Custom capture panel**:
  - 41 blood group genes
  - 1473kb total target size
  - 269kb targeted *RH* region
- **Sequencing**:
  - Paired-end 100 nt
  - High coverage: ~153X
- **SNV calling**:
  - GATK HaploTypeCaller



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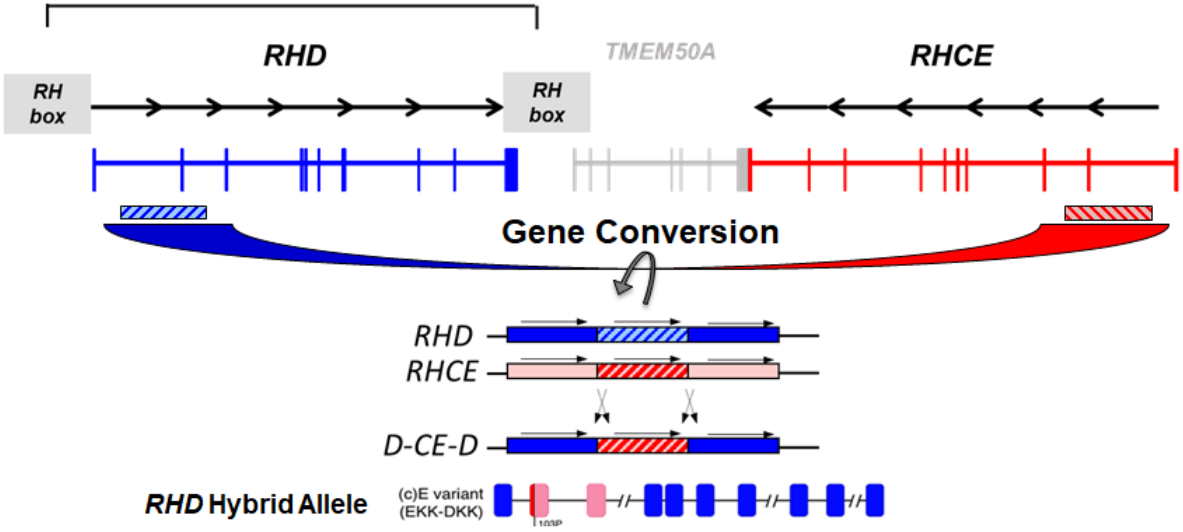
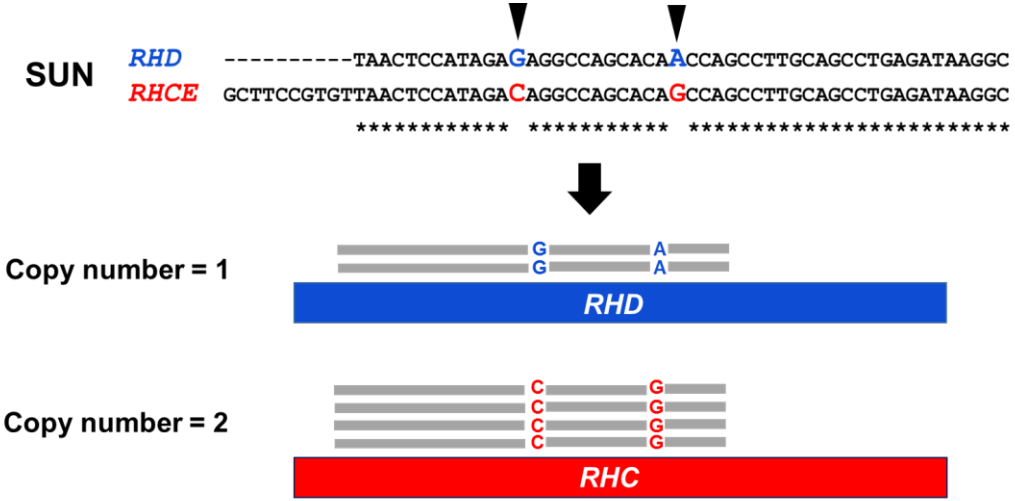
Wheeler M., et al. Genetics in Medicine. epub 2018.

RH structural variants (SVs) :
Confounds standard NGS analytical methods

- *RHD* and *RHCE* genes are 97% identical
  - Conversion events occur between the genes (hybrid alleles)



Custom analysis for *RH* SV (hybrid alleles):
Singly unique nucleotides (SUNs)



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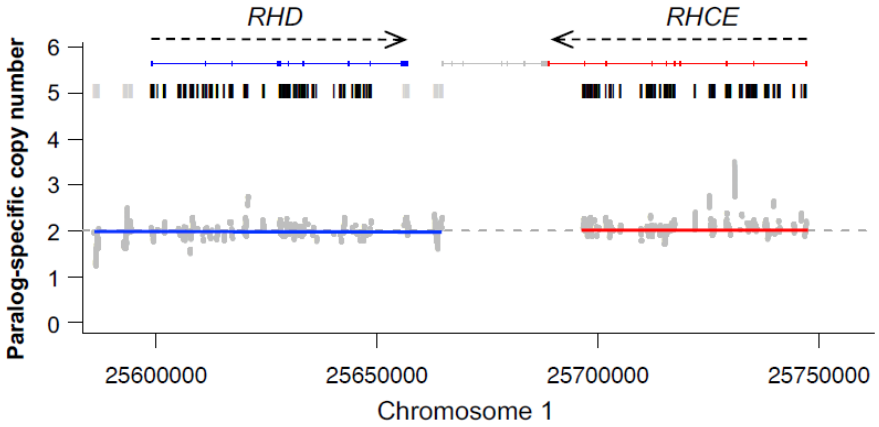
Wheeler M., et al. Genetics in Medicine. epub 2018.

Custom NGS analysis detects *RH* SV

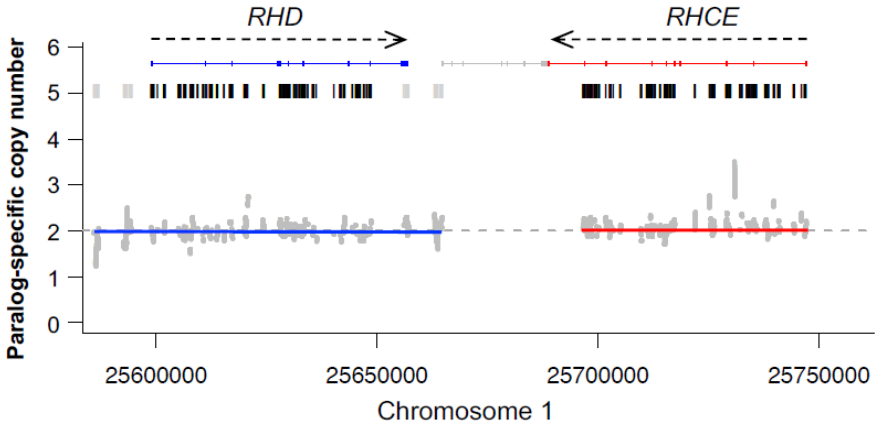
No *RH* SV



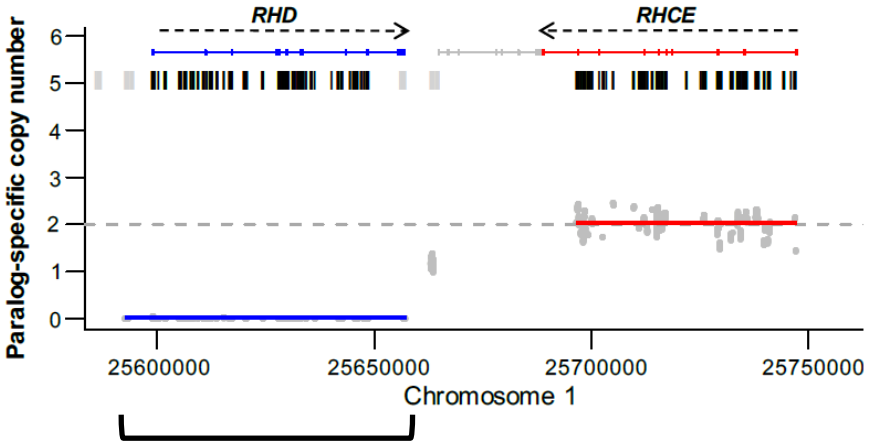
Wheeler M., et al. Genetics in Medicine. epub 2018.

Custom NGS analysis detects *RH* SV

No *RH* SV



Homozygous *RHD* gene deletion (*RHD*01N.01*)



***RHD* deletion**

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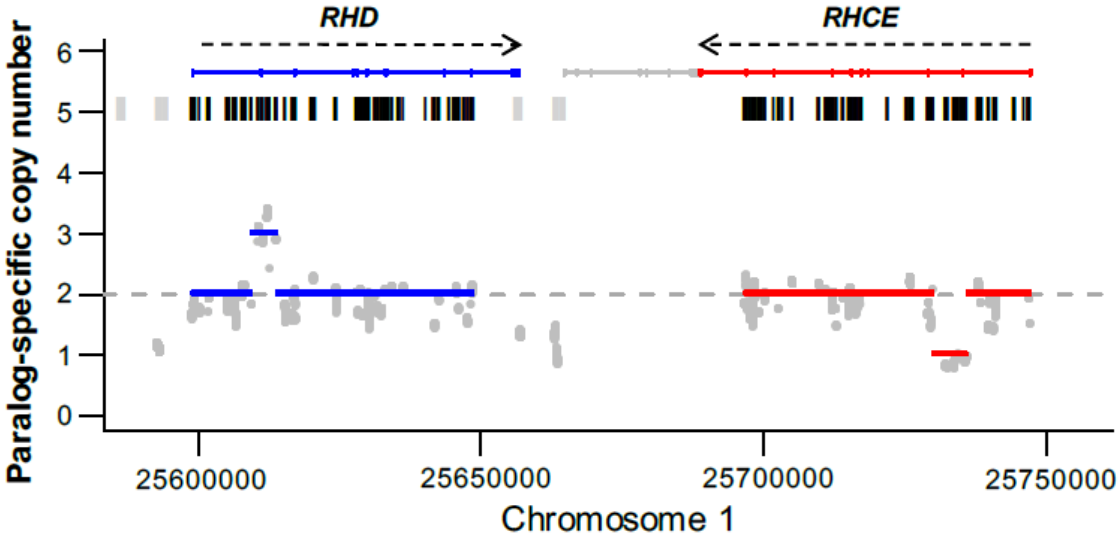
Wheeler M., et al. Genetics in Medicine. epub 2018.

*RHCE*CE-D(2)-CE* hybrid alleles

Accurately predicts C antigen expression (>99%)



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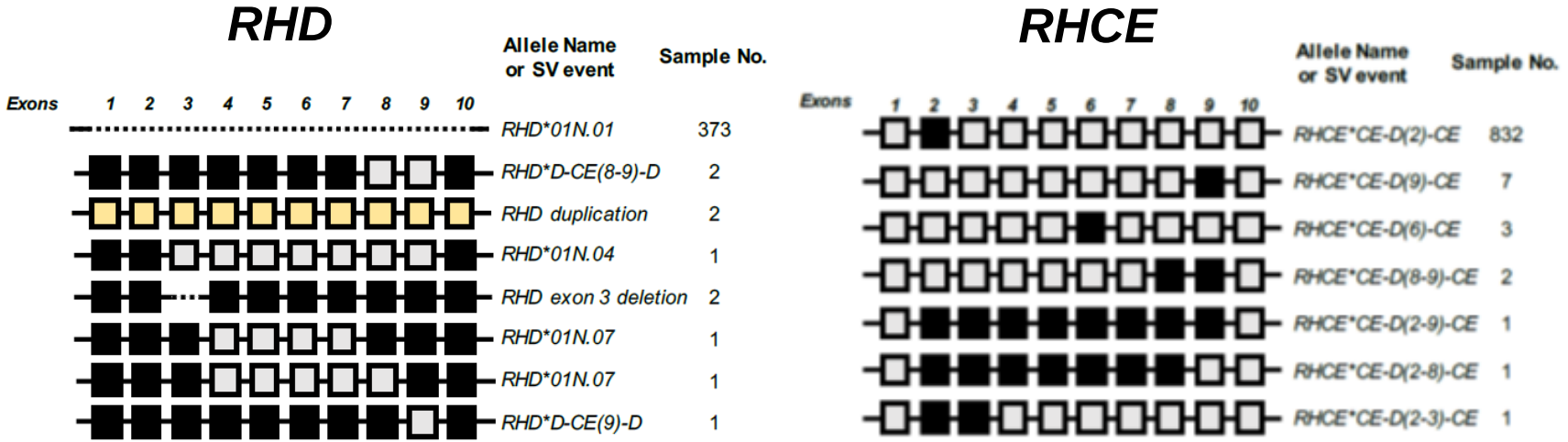
Wheeler M., et al. Genetics in Medicine. epub 2018.

RH hybrid alleles in Asian American donors



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Wheeler M., et al. Genetics in Medicine. epub 2018.

RH SNVs detected in Asian American donors

RHD

	Allele Name in ISBT v2.0[1]	cDNA[1]	Phenotype[1]	Allele No.[2]
Null	*RHD*01N.16*	711delC	D null	2
	*RHD*01N.20*	941G>T	D null	1
Weak	*RHD*01W.1*	809T>G	Weak D Type 1	2
	*RHD*01W.2*	1154 G>C	Weak D Type 2	3
	*RHD*01W.33*	520G>A	Weak D Type 33	2
	RHD[602C>G; 667T>G][4]*	602C>G; 667T>G	Weak D Type 40, DFV	1
	*RHD*01W.45*	1195G>A	Weak D Type 45	2
	*RHD*01W.66*	916G>A	Weak D Type 66	2
DEL	*RHD*DEL1*	1227G>A	DEL	6
	*RHD*05.04*	697G>C	DV Type 4	1
	*RHD*08.01*	667T>G	DFV	6
	RHD[667T>G; 1136C>T][4]*	667T>G; 1136C>T	-	1
	*RHD*10.00[5]*	1136C>T	DAU0	140
	*RHD*16.01*	667G>T; 676G>C	DCS1	1
	*RHD*33*	1073T>C	DWI	1

RHCE

Allele Name in ISBT v2.0[1]	cDNA[1]	Phenotype[1]	Allele No.[2]
*RHCE*01.01*	48G>C	e weak	1188
RHCE[48G>C; 676G>C][4]*	48G>C; 676G>C	e weak, E	72
*RHCE*01.06*	254C>G	partial e	2
*RHCE*01.07*	48G>C; 667G>T	partial e partial c	3
*RHCE*01.20.01*	733C>G	partial e partial c	3
*RHCE*01.20.02*	48G>C; 733C>G	partial e partial c	4
*RHCE*01.20.05*	48G>C; 1006G>T	partial e	1
*RHCE*01.21*	341G>A	RH:48 (JAL+)	1
RHCE[48G>C; 122A>G][4]*	48G>C; 122A>G	e weak, partial C	9
*RHCE*03*	676G>C	E[3]	507

Common East Asian DEL allele

- **usually tests D-negative**
- **associated with a very weak D**
- **reports of DEL donors sensitizing D-negative patients**

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Wheeler M., et al. Genetics in Medicine. epub 2018.

Summary

- *RHD* and *RHCE* DNA variation is common
- SNP tests can detect known, simple DNA variation well
- NGS detects small (SNP) and large (indel, hybrid) variants at *RH*
- C is best predicted by directly detecting the *RHCE*CE-(D2)-CE* hybrid
- *RHD*01N.01* (*RHD* gene deletion) is a common cause of D-negative in admixed Asian American donors
- *RHD*01EL.01* (*DEL1*) is a common cause of D-neg in East Asia

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Genome Sciences UNIVERSITY OF WASHINGTON

The Rh Blood Group: Diversity and Molecular Techniques in a Global Context

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Disclosure

No conflict of interest in which I disclose any relevant or financial interest to topic discussed for this presentation.

Blood Groups

- Blood group system are expressed on the surface of red blood cells.
- Direct clinical impact:
  - Transfusion.
  - Transplantation.
  - Hemolytic disease of the new born medicine.
- Most are glycoproteins.
- Majority of genes that encoded all the blood group systems are very well characterized.
- High genetics complexity , varying degree of polymorphism and associated with production of a specific alloantibody.
- Population specific heterogeneity.
- Varied clinical significance of red blood cells antibodies

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Ref: Storry JR. *ISBT Sci Ser . 2016*

Blood Groups

The majority of blood group antigens are:

1. Missense mutation and consequences of single nucleotide variation (SNV).
2. Insertion/deletion and slice site variants which will have a qualitative and /or quantities impact on antigen expression.
3. RH and MNS, exhibit an additional criteria of genetics variations because system comprises homologous genes (gene crossover or gene conversion events have generated complex antigenic profiles).

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Rh Blood Group System

- The most clinically significant blood group system after ABO.
- Highly immunogenic antigens.
- Exhibiting complex structural variation (SV)
- Currently >50 reported antigens
- 5 common (major) antigens
- Polymorphic RhD (D), and Polymorphic CE (C, E, c, and e) antigens C/c and E/e are antithetical.

– Make 8 most common haplotypes:
  - DCe(R_1) Ce(r′)
  - DcE(R_2) cE(r′′)
  - Dce(R_0) ce(r)
  - DCE(R_Z) CE(rY)

The prevalence of given antigen expression is different in different population, so it depends on what population you are studying.

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Rh system (ISBT 004): antigens RH8 to RH62

ISBT # / Name	Preva-lence	ISBT # / Name	Preva-lence	ISBT # / Name	Preva-lence	ISBT # / Name	Preva-lence
RH8 / C^W	Low	RH26 / c-like	Poly	RH39 / Rh39	High	RH51 / MAR	High
RH9 / C^X	Low	RH27 / cE	Poly	RH40 / Tar	Low	RH52 / BARC	Low
RH10 / V	Low	RH28 / hr^H	Low	RH41 / Rh41	Poly	RH53 / JAHK	Low
RH11 / E^W	Low	RH29 / Rh29	High	RH42 / Rh42	Low	RH54 / DAK	Low
RH12 / G	Poly	RH30 / Go^a	Low	RH43 / Crawford	Low	RH55 / LOCR	Low
RH17 / Hr_o	High	RH31 / hr^B	High	RH44 / Nou	High	RH56 / CENR	High
RH18 / Hr	High	RH32 / Rh32	Low	RH45 / Riv	Low	RH57 / CEST	High
RH19 / hr^S	High	RH33 / Rh33	Low	RH46 / Sec	High	RH58 / CELO	High
RH20 / VS	Low	RH34 / Hr^B	High	RH47 / Dav	High	RH59 / CEAG	High
RH21 / C^G	Poly	RH35 / Rh35	Low	RH48 / JAL	Low	RH60 / PARG	Low
RH22 / CE	Low	RH36 / Be^a	Low	RH49 / STEM	Low	RH61 / CEVF	High
RH23 / D^W	Low	RH37 / Evans	Low	RH50 / FPTT	Low	RH62 / CEWA	High

Missing numbers caused by antigens becoming obsolete

Low prevalence in one population is common in another population. Some population is/are not yet very well characterized.

www.isbt.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology.

Why the Rh is Complex?

- The terminology (Fisher Race vs. Wiener vs. Tippett).
- Several antigens, phenotypes and alleles
- RHD and RHCE genes exhibits 92% similarity between the coding regions. Homologous nucleotide sequences complicate the mapping of the sequencing reads to correct genes
- One phenotype can come from several genotypes
- Most warm autoantibodies have Rh specificities (e.g. anti-e).
- Differentiating between auto-antibodies and alloantibody when the patient is recently transfused.
- Making an alloantibody to an antigen expressed on their RBCs = partial antigens. Positive serology.
- Comprises homologous genes in which gene crossover or gene conversion events have generated complex antigen profiles. e.g. RHD*-CE-D gene variants, generating partial D and D- phenotypes.

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RH Terminology

Fisher-Race	Wiener
D	Rh_0
C	rh’
E	rh’’
c	hr’
e	hr’
3 separate genes encode D, C or c, E or e	1 gene encodes 1 agglutinogen

- Two systems of nomenclature developed prior to advances in molecular genetics.
- Based on serologic observations and inheritance theories based on family studies.
- Used interchangeably.

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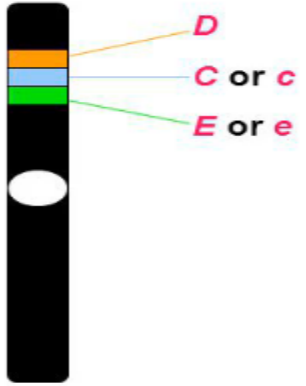
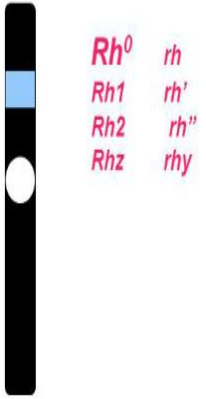
Fisher-Race: CDE Terminology



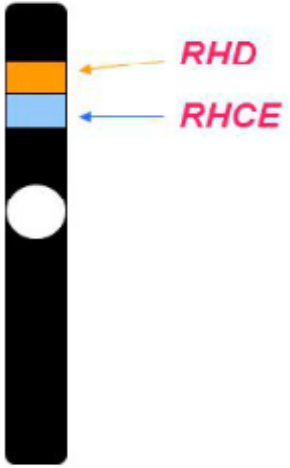
- Fisher-Race
  - 3 separate genes that encoded the antigen.
  - Each gene complex carries D or its absence (d), C or c, E or e.
  - Each gene (except d) causes production of an antigen.

Wiener Theory



- Postulated that TWO genes, one on each chromosome pair, controls the entire express of Rh system.
- Each gene produces a structure on the red cell called an agglutinogen.
- Eight (8) major alleles (agglutinogens): R_0, R_1, R_2, R_z, r, r', r" and $r_{y.}$

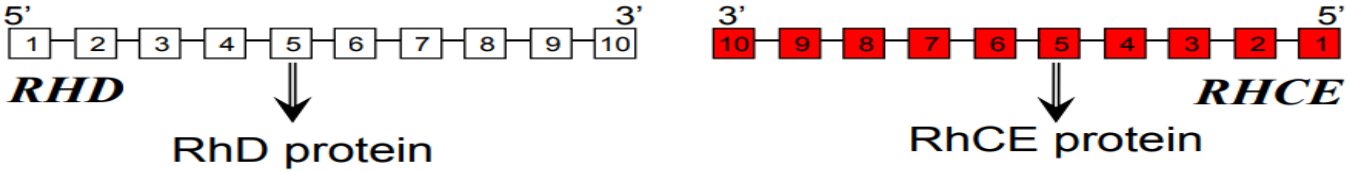
Rh Inheritance: Tippett



- Using molecular techniques (DNA) Tippett showed in 1986 that Rh inheritance comes from two genes.
  - RHD gene controls production of the D antigen.
  - RHCE gene controls production of CcEe antigen combinations.

RH Genes on Chromosome 1 at 1p36.11

- – RHD and RHCE, each consisting of 10 exons expressed on opposite orientation
  - – Gene duplication; RHCE*ce is the ancestral gene
  - – Genes are homologous (>95% identity at nucleotide and at the amino acid level, gene duplication)

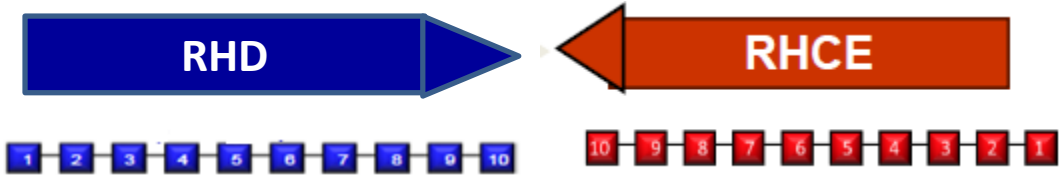


The genetics diversity originates from the genetics variation in two homologous paralogs, RHD and RHCE, lies in close proximity at the RH locus.

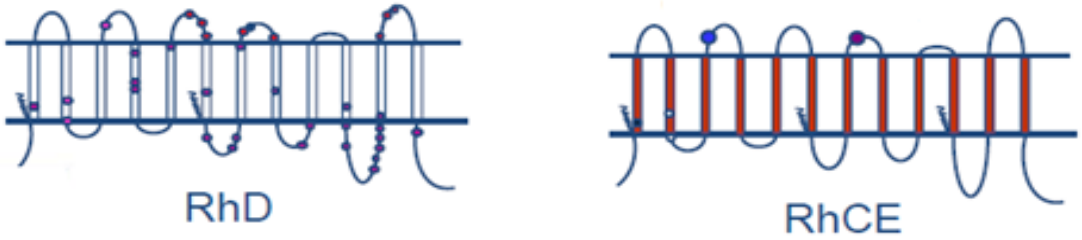
Refs: Wagner FF, Flegel WA Blood 2000; 95: 3662-8.

- A third ancestral homologous gene (RHAG), on chromosome 6, encodes the Rh-associated glycoprotein (RhAG) (Rh antigen expression).

RH Locus



D Positive



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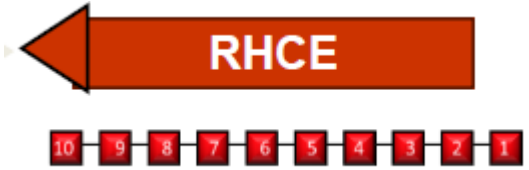
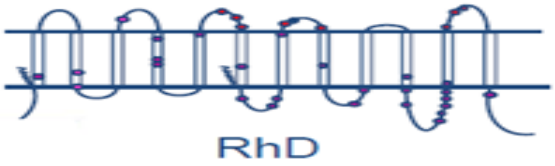
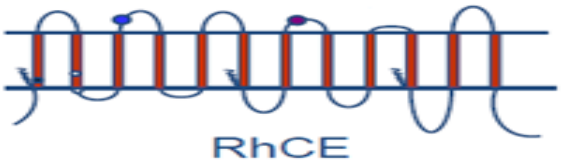
RH Locus

X Deleted RHD X



D Negative



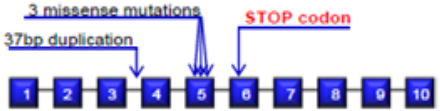


Common ways to be D negative

- Deletion of the RHD gene

-No D protein
-D negative

- Inactive RHD pseudogene (Dψ) – common in Blacks



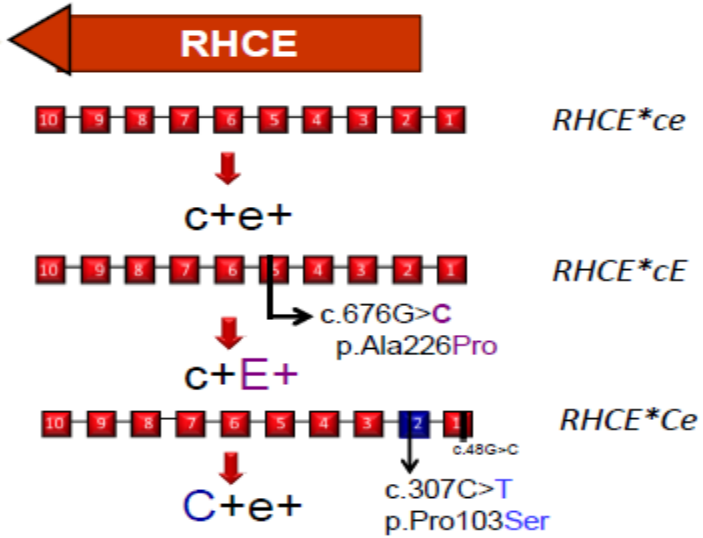
-Premature stop codon
-No D protein
-D negative

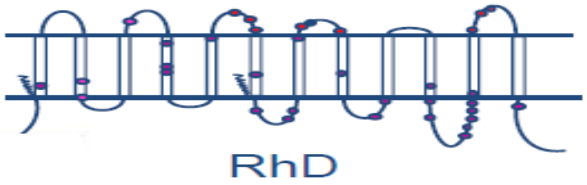
- Hybrid allele
  - Specifically Hybrid *RHD*DIIIa-CE(4-7)-D*

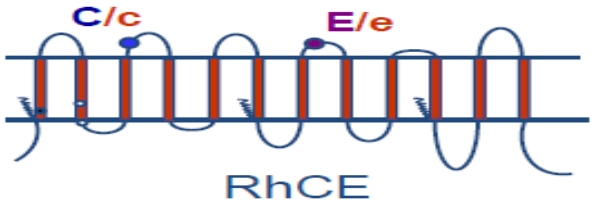
[C+(partial)]

-Protein made does not express D antigen
-D negative

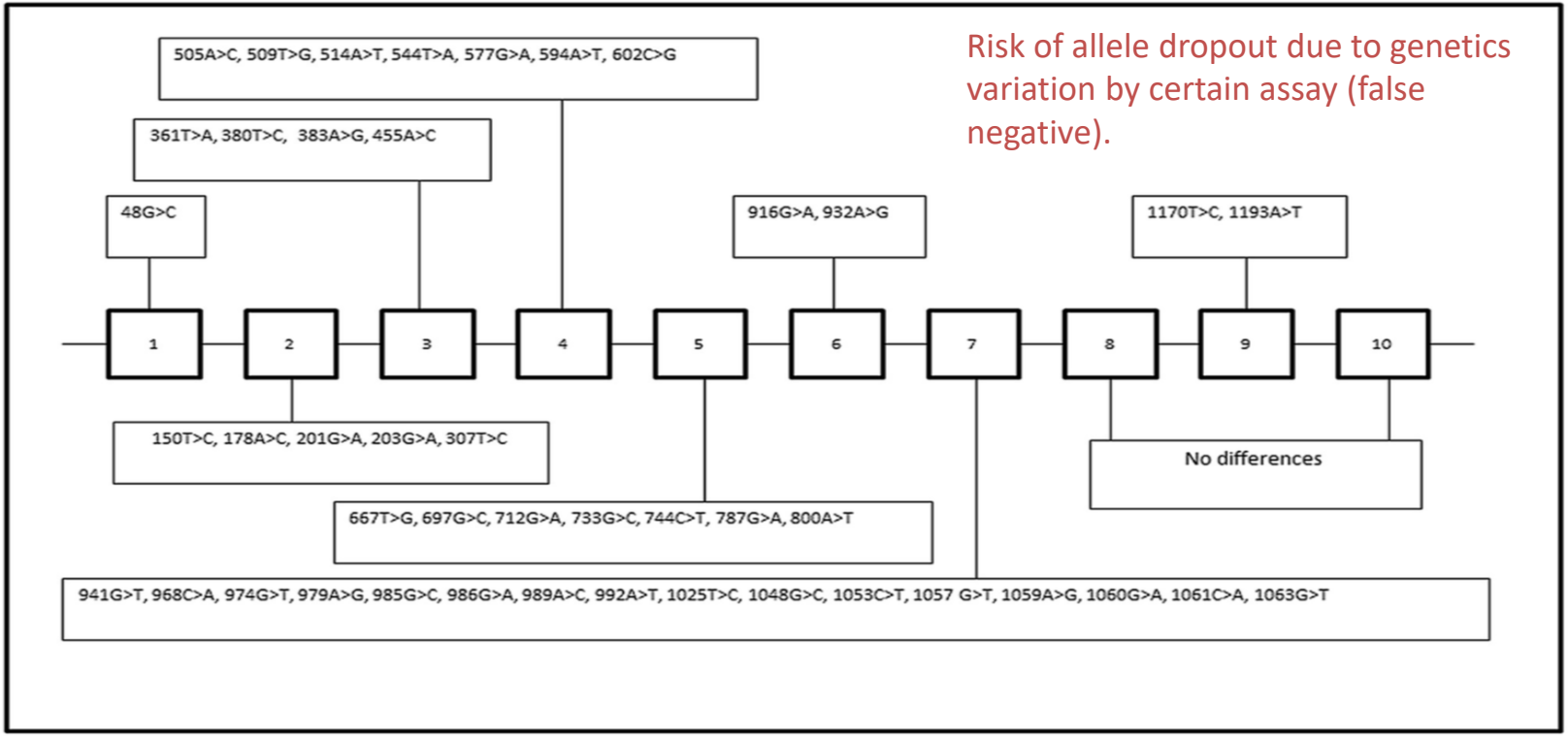
RH CE Locus







Chou et al 2017



Risk of allele dropout due to genetics variation by certain assay (false negative).

Fig. 1. Nucleotide differences between *RHD* and *RHCE* genes. In the figure, boxes in bold represent the exons. The nucleotide differences between *RHD* and *RHCE*ce* are signed. The high homology between *RHD* and *RHCE* exons 1, 2, 6, 8, 9 and 10 are important to highlight and justify why NGS-based assays designed for the molecular Rh typing have to be adapted so that *RHD* and *RHCE* specific amplicons are individually sequenced or algorithms must be designed for result interpretation.

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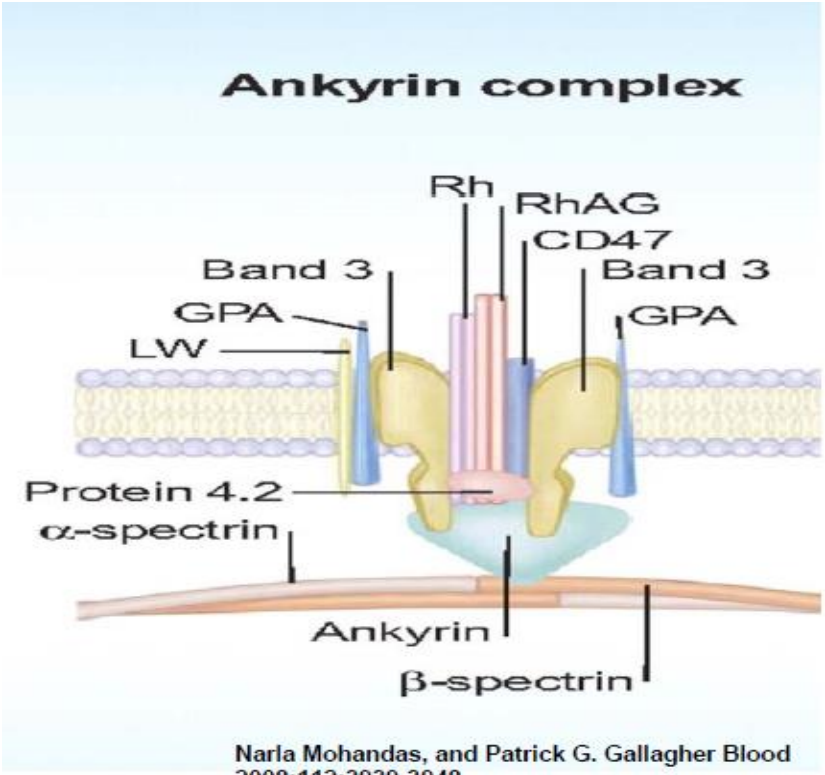
Marcia et al.,2017. RHD and RHCE genotyping by next-generation sequencing is an effective strategy to identify molecular variants within sickle cell disease patients. Blood Cells, Molecules and Diseases 65 (2017) 8–15

Red Blood Cell Membrane



Lack of Rh and RhAg
affect the red cell membrane complex
Leads in Rh null phenotype

Impacts expression of other antigens
Such as:
1. LW antigens not expressed
2. Expression of S/s and U antigens is reduced
3. Impacts RBC membrane integrity and function.

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Rh_{null} and Rh_{mod} Phenotypes

Regulator type Rh_{null}
Various inactivating mutations in *RHAG*

Amorph type Rh_{null}
Deleted *RHD* with homozygosity for *RHCE* containing inactivating mutations

Rh_{mod}
Mutations in *RHAG*
Low level of Rh antigens-needs adsorption elution studies

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Why there are variety Rh antigens and phenotypes?

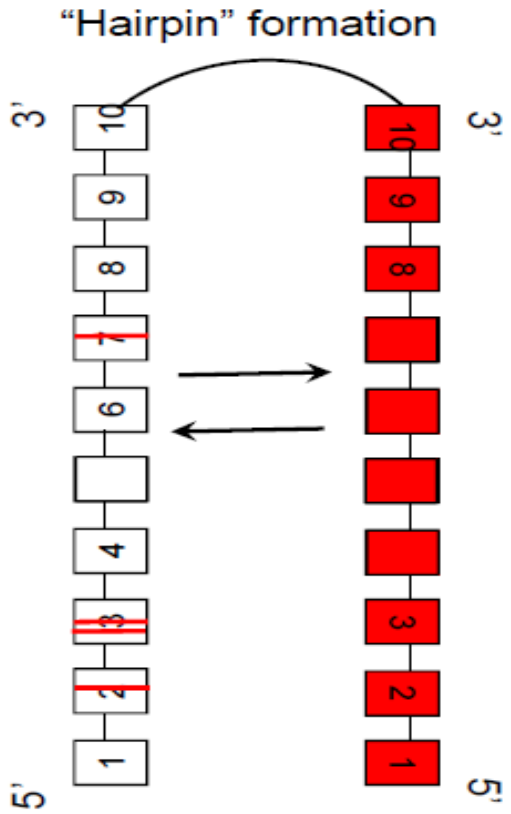
- Point mutations
- Gene conversion:
  - Portions of *RHCE* into *RHD*
  - Portions of *RHD* into *RHCE*

– *RHD*: >495 alleles

Weak D, Partial D, Del

- *RHCE*: >100 alleles

including hrB–, hrS –



Three Main Categories of D Antigen Expression

Weak D

Definition: react weaker (<2+ tube may require IAT for detection)
Decrease antigen expression level because of amino acid changes that affect the insertion of the protein in the red cell membrane.
Not at risk for developing anti-D (rare exceptions)
Many different mutations cause weak expression of D.

Partial D

Changes or missing epitopes
Type as D+ but can make anti-D when exposed to D antigen.
Results from hybrid genes in which portion of *RHD* are replaced by the corresponding portions of *RHCE.*
Gives positive reaction: not serologically distinguishable between partial D type weakly D+.
At risk for clinically significant anti-D.

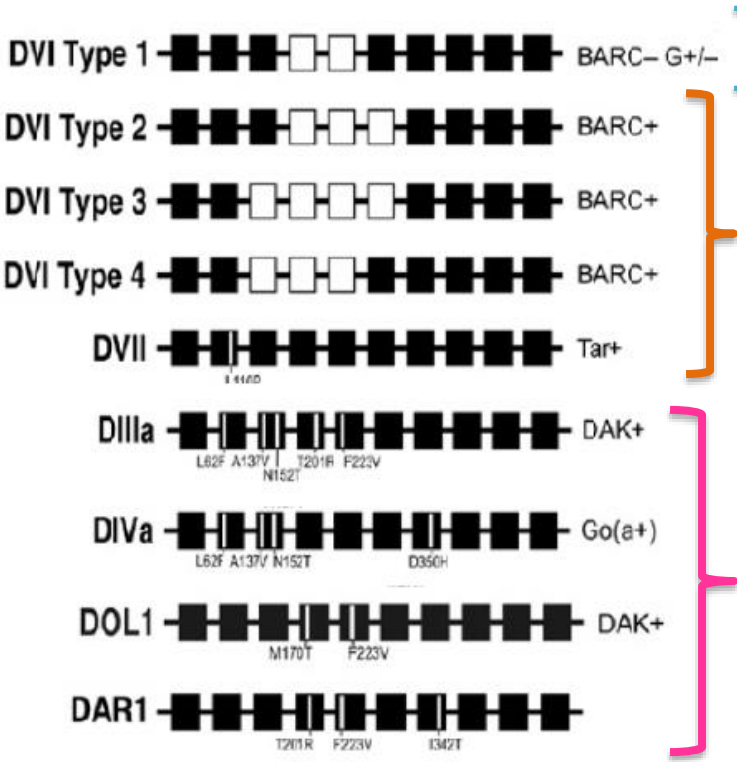
Del

Type D-negative even by IAT; adsorb and elute anti-D, express very low level of D antigen
Point mutations affect insertion.
Common in Asians (10-30%)
Important to Identify in donor population for labelling the units.

Examples of Altered RHD Alleles

Partial D

Risk of allo anti-D



Linked to cE

Linked to Ce

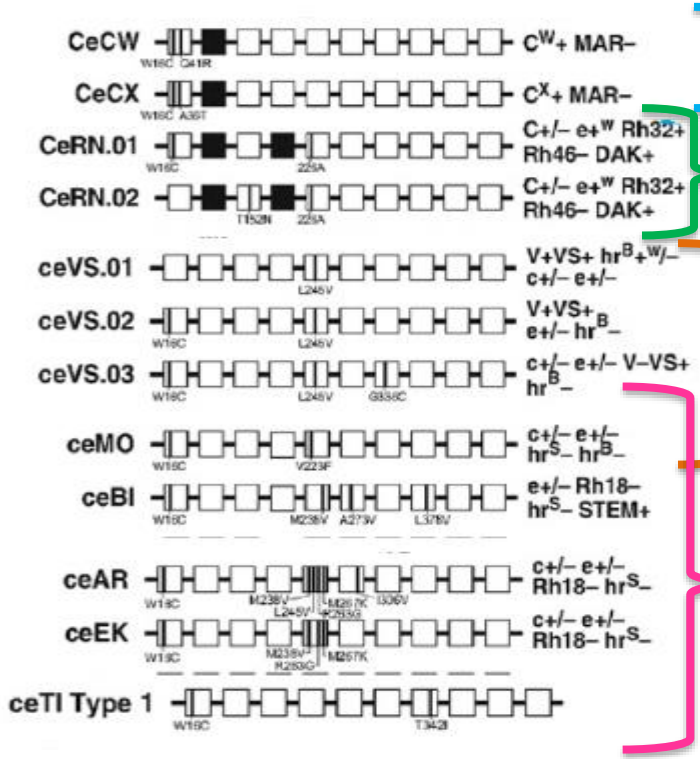
Caucasian

African Blacks

Often linked to RHCE*ce variants

Marion E. Reid et al.,2012. The Blood Group Antigen FactsBook

Some altered RHCE alleles



Caucasian
Risk of allo ant-C, -e, and -Ce

Partial C and e
Risk of allo anti-C, -e, -Ce

hrB–

Partial e and c

Risk of allo anti-e, -c, -ce(f)

hrS–

Risk of allo anti-hrB and/or -hrS

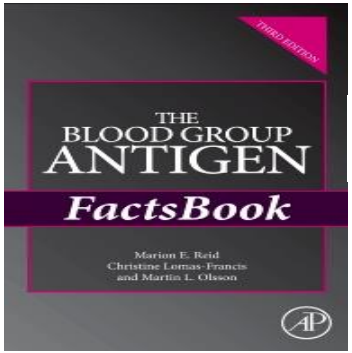
Most linked to D variants

African Blacks/ Hispanics

Marion E. Reid et al.,2012. The Blood Group Antigen FactsBook

Resources for cataloguing RBC antigen alleles variants

ISBT



BGMUT

Human RhesusBase

These resource provide a tool to validate and design single nucleotides assays to predict phenotypes.

Ethnicity of RHD/RHCE variants

- Rh genes have a massive diversity among different ethnic populations (Avent & Reid, 2000)
- Arab people represent the large population for Rh gene polymorphisms (Flegel, 2011)
- Several populations have been extensively investigated, but limited data are available regarding Arab residents (Ouchari *et al*., 2013)

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What are the Associated Rh Variants?

Ethnicity/Nationality	RHCE Allele name	RHD Allele name	Reference
African ancestry **African ancestry** **African ancestry** **Brazaville and Teke groups**	RHCE*ceTI	RHD*DIVa	Westhoff et al., 2013 Vege et al., 2007 von Zabern et al., 2013 Touinssi et al., 2009
African ancestry	RHCE*ceTI (Dex2)	RHD*DIVa	Ba et al., 2015
Finland, Uk and Sydney **Germany**	RHCE*cE	DVI type 1	Avent et al., 1997 Wagner et al., 1998
France **Germany**	RHCE*Ce	DVI type 2	Mouro et al., 1994 Wagner et al., 1998
Han Chinese **Chinese** **Germany**	RHCE*Ce	DVI type 3	Yan et al., 2007 Ye et al., 2012 Wagner et al., 1998
Spain **China**	RHCE*Ce	DVI type 4	Esteban et al., 2006 Li et al., 2009 Aug
China	RHCE*cE	DCS-3	Ye et al., 2012
USA	RHCE*ce1025T	DIIIa	Westhoff et al., 2010
Sub-Saharan Africa	RHCE*ceAR	RHD*DAR	Granier et al., 2013
France	RHCE*ceEK	RHD*DAR	Silvy et al., 2012
African Mali **South-Western Germany**	RHCE*ce48C, 667T RHCE*ceMO	RHD*DAU0	Wagner et al., 2003 Wagner et al., 2002
Afro-Caribbean blacks France	RHCE*ceMO.02	RHD*DAU0	Noizat-Pirenne et al., 2002
African descent	RHCE*ceBI	RHD*DOL	Roussel et al., 2012
USA	RHCE*ce48C, 733G	RHD*DIIIa	Westhoff et al., 2010
USA	RHCE*ceS	Often with RHD*DIIIa	Westhoff et al., 2010

Rh Antibodies

- Most occur from immunization due to transfusion or pregnancy.
- Associated with HTR and HDFN.
- Characteristics IgG but may have minor IgM component. May be detected at 37C but most frequently detected by IAT.
- Order of immunogenicity: D > c > E > C > e
- Do not bind complement, extravascular destruction.
- Detectable antibody persists for many years and sometimes for life.

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What is the clinical impact of Rh complexity?

- Complexity in RH create clinical challenges and significant rate of alloimmunization.
- Higher rate of alloimmunization persist even when the patient receive transfusion from serologically matched. (e.g. African American SCD)suggesting the need for higher resolution typing

Chou ST Blood 2013, 2018, Sippert 2015

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- Alloimmunization incidence rates of up to 36% have been reported in patients with SCD, compared to 5% in transfusion-dependent patients with thalassemia, and approximately 2% in other regularly transfused patient groups.

The Scope of the Problem

- Sickle Cell Disease widely recognized and medically critical Hemoglobinopathies in Arabian Peninsula in general and in Kuwait in particular.
- Due to International migration have spread through the world and occur in high frequency in Mediterranean area, Asia and Africa (Williams and Weatherall 2012) FINDBASE (HTTP://WWW.FINDBASE.ORG).
- The most common inherited disease in Kuwait and Saudi Arabia due to the high prevalence of consanguineous marriages (Adekile 2001, 2017).
- The rate of alloimmunization is high in Sickle Cell Disease patients due to :

  Disparity in blood group antigen expressed on donor versus patient red blood cell

  Number of red blood cell units exposure

  Genetic makeup most likely due to complicated Rh variants and other factors

Country	Rate of Alloimmunization (%)	Alloantibodies	References
USA	17.6%–42.9%	Anti-E, C, K, and Jk^b	Luban et al., 1989 Godfrey et al., 2010; Luban et al., 1989; Rosse et al., 1990; Tahhan et al., 1994
France	8.2%-42%	Anti-C, c , D, E, K and Fy^a	Norol et al., 1994 Silvy et al., 2014 Allali et al., 2017
Brazil	12.7%	Anti-Rh and K	Pinto et al., 2011;
Egypt	9.7-16.7%	Anti-K, E , C, Rh	Aly et al., 2012; Hussein et al., 2014
Saudi Arabia	13.7-22%	Anti-E, K, C, c	Bashawri, 2007 Gader et al., 2008 Adam & Badawi, 2017
Kuwait	43.3%	Anti-C, c, E, e, K	Ameen et al., 2009
Oman	20-32%	Anti-Rh and K	Alkindi et al., 2017
Tunisia	7-10%	Anti-Rh and K	Guirat-Dhouib et al., 2011

Laboratory Testing (Current Practice in Clinical Setting)

- **Serology**

- Molecular

Rh Phenotyping (Serological Testing)

- Indications
  - Parentage testing
  - Predicting hemolytic disease of the fetus and newborn (HDFN)
  - Confirmation of Rh antibody specificity
  - Finding compatible blood for recipients with Rh antibodies.
- Laboratory Procedure
  - Mix unknown RBCs with Rh antisera
  - Agglutination indicates presence of antigen on cell and determines phenotype.

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Detection of D Antigens

Types of anti-D reagents

- **High Protein:** (20-40% protein +macromolecular additives). Rapid tube or microplate, high chance of false positives; requires use of Rh control tube.
- **Low protein reagents:**
  - **IgM** monoclonal antibodies (Low protein); (fewer false positives). Negative results from a test performed at the same time serve as a control.
  - Most FDA approved anti-D regents combined monoclonal IgM (direct agglutination RT + monoclonal /polyclonal IgG that is reactive by IAT for determination of weak expression of D.
  - FDA licensed reagents to detect weak D, partial D or D like epitopes.

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Weak Expression of D

- Not all D positive cells react equally well with anti-D.
- RBCs not immediately agglutinated by anti-D must be tested for weak D.
- Policies needs to be developed of blood components for transfusion should be based on the patient population, risk of alloimmuniztion and supply of D negative.

Limitation of Serological Testing

- Standard serological testing does not detect all altered Rh antigen expression whether D weak or partial D.
- Does not detect variation commonly expressed on RBCs (e.g. black population).
- Licensed anti-D reagents cannot distinguish individual with partial D from those expressing a normal D antigens.
- Serological testing for extended typing are often costly and labor intensive as antisera may be expensive or unavailable (e.g., to detect V/VS, Goa, and DAK antigens).
- The use of multiple antigen identification panel may be necessary.
- Cannot provide precision in complex cases.
- Cannot use anti-sera on recently transfused individuals, molecular testing can differentiate.
- Monoclonal reagents from different manufacturers react differently with variant D antigens, molecular test specific.
- Many Rh variants can be defined by a panel of monoclonal anti-D or anti-e, however, these reagents are not fool-proof because they can give different reactions depending on the formulation of the reagent, the condition of the RBCs, and haplotypes *in trans*.

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Laboratory Testing (Current Practice in Clinical Setting)

- Serology
- **Molecular**

Molecular Typing Methods Used

- **Low Resolution:** Gel- Based Methods SSP-PCR and PCR-RFLP for for documented SNPs. High melt resolution analysis.

- **Medium Resolution:** Arrays (Immuore BeadChip and Grifols ID core), Real time PCR based genotyping. Single base extension (SBE) using MALDI-TOF.

- **High Resolution:** DNA Sequencing analysis, Exon Scanning, cDNA analysis, NGS

HLA typing is rapidly moving to targeted NGS (commercial investment). Limited commercial interest available for RBC and PLT type.

Limitations of SBT

- Limitation to the resolution of cis/trans linkage of polymorphisms.
- Cannot resolve sequences of heterozygous samples in diploid genomes leading to ambiguous typing results.
- In RHD and RhCE sometimes it is difficult to interpret, especially in cases of inclusions, deletions and hybrid alleles,

Application of DNA Based Molecular Testing

- Molecular testing being developed for almost all blood group genes.
- D zygosity can be determined.
- Fetal genotyping for D can be done on fetal DNA present in maternal plasma.
- Identify donors with rare phenotypes.
- Permits automated blood group.
- Phenotypes prediction based on genotype.
- Evaluate common polymorphism to predict protein based antigens.
- Theses assays incorporated SNV for the most clinically significant blood groups which allow more extensive characterization of patients and donor.
- RH genotyping guide transfusion support for SCA patient and selection of the units selection.
- Molecular techniques may be useful for identification of donor RBCs that are predicted to lack certain combinations of antigens and/or a high-prevalence antigen and thereby increase the inventory of antigen-negative blood.

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Limitations of the Currently used Molecular Methods

- Cannot detect all RH variation and it is based on certain population
- Develop algorithm based on populations (i.e. integrating multiple assays for more complex genes.
- Limited in the number of blood group system covered and range of SNV targeted within any blood group.
- RHD and RHCE homologous genes creating gene-rearrangement events.
- Need for special testing instruments, reagents, and workflow.
- Target selective gene regions without evaluating all potentially contributing genetics changes.
- Do not include all clinically significant changes.

Marcia et al.,2017. RHD and RHCE genotyping by next-generation sequencing is an effective strategy to identify molecular variants within sickle cell disease patients. Blood Cells, Molecules and Diseases 65 (2017) 8–15

One June 26, 2000 President Clinton, with J. Craig Venter, left, and Francis Collins, announces completion of "the first survey of the entire human genome."

$ 3 Billion (2000)

10 years (1993-2003)

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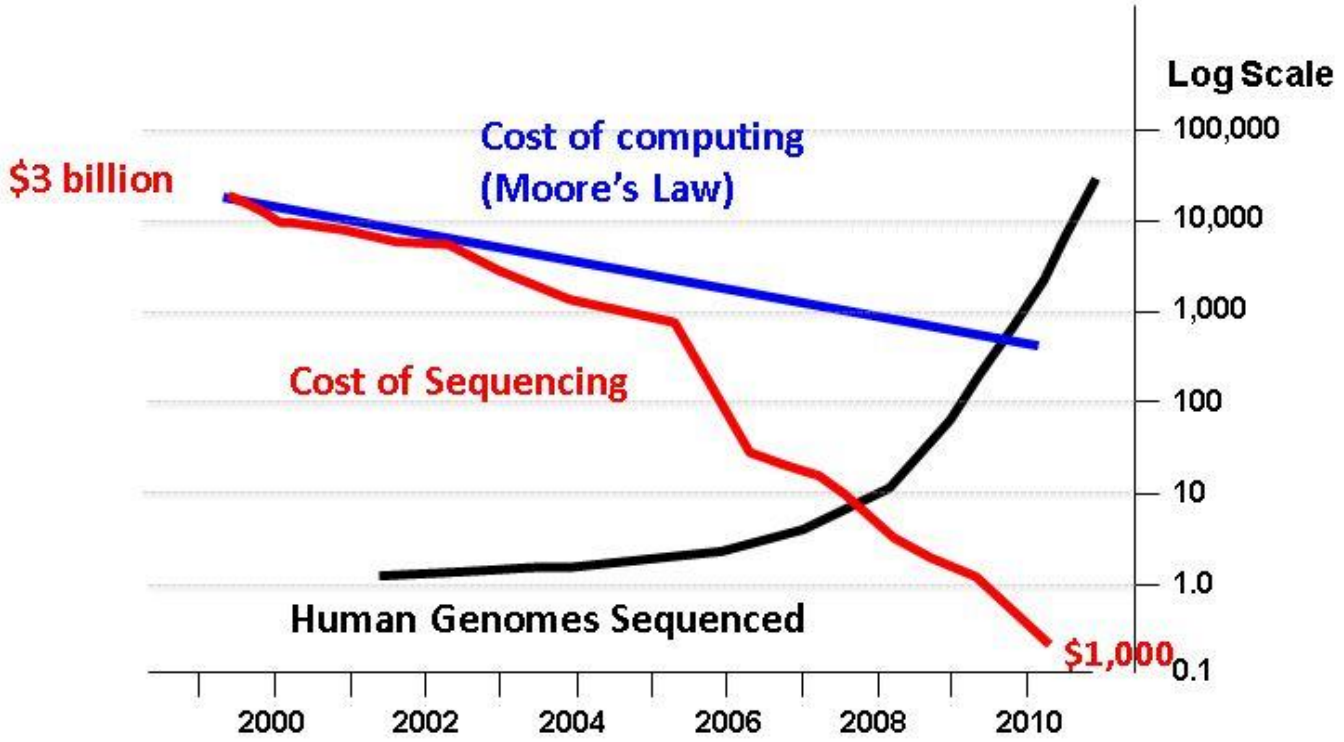
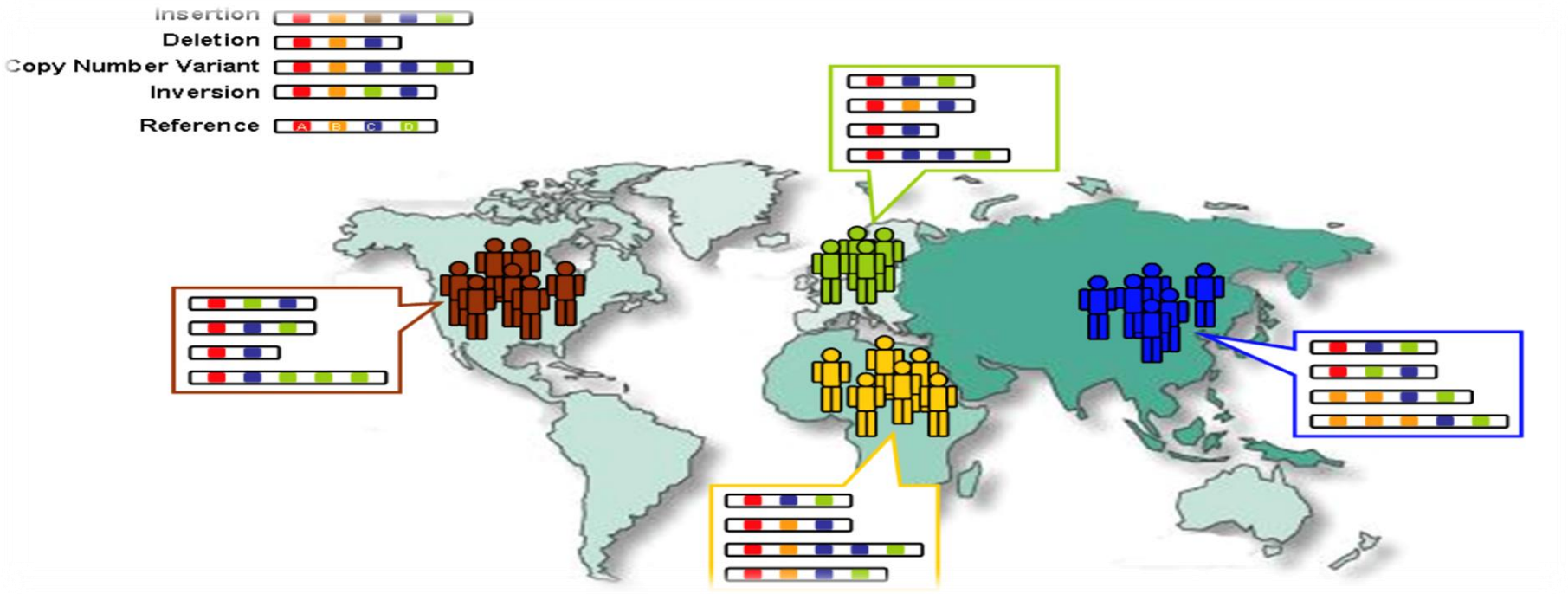
Schuster, Nature Methods 2008

Adapted from The Economist

The Sequencing Explosion

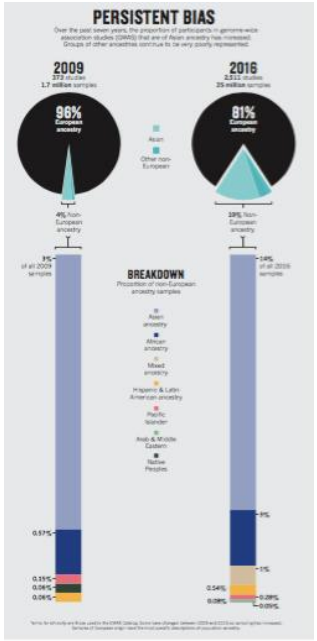


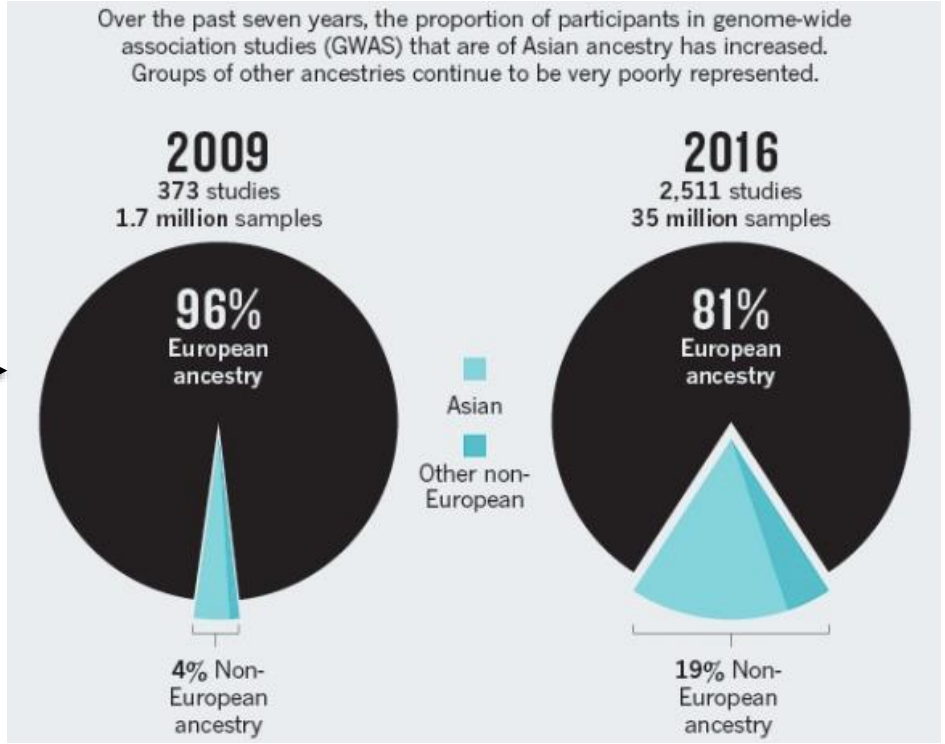
Population Genetics (Out of the African Theory)



Changes in the number and order of genes create genetic diversity within and between populations, Random sampling of gemates, structural variation (insertion/deletion (indels) copy number variations (CNV), and SNPS understand the pattern within and between species.

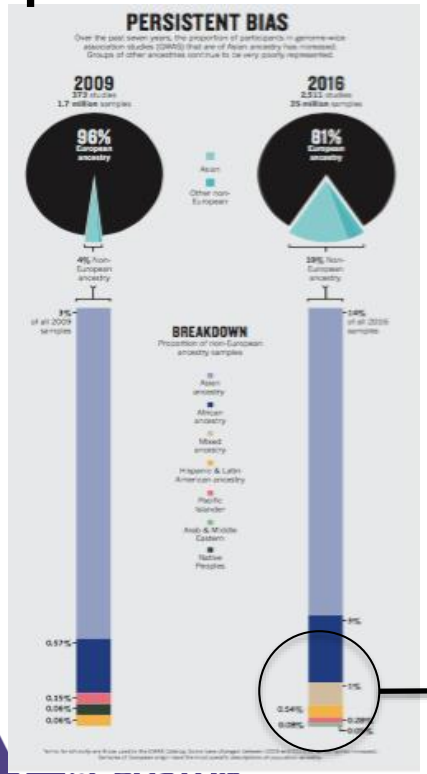
Genomics is Failing Diversity

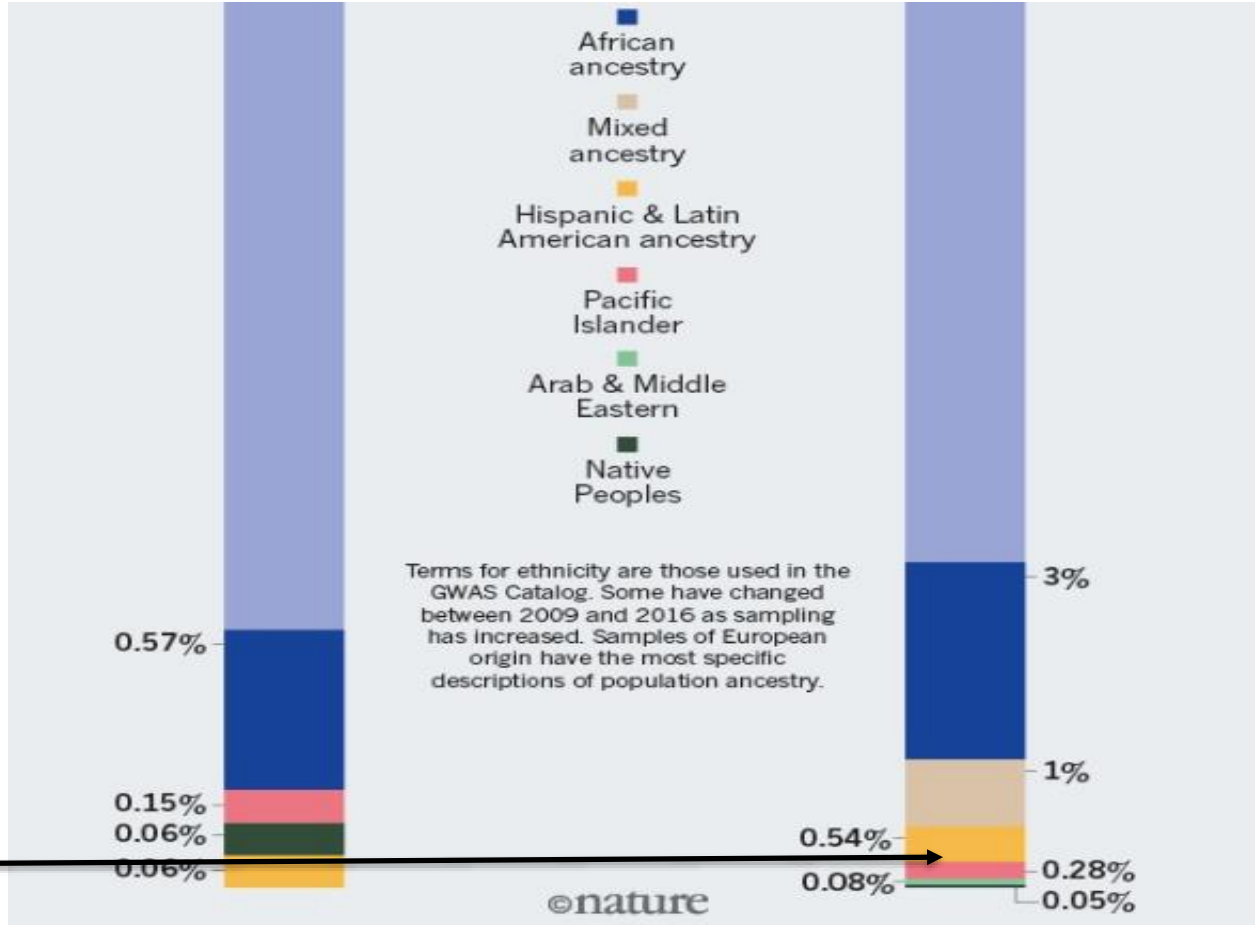




Popejoy & Fullerton (2016) Genomics is Failing on Diversity. Nature; 568:161-164

Lack of Genome data on Arab & ME populations





Popejoy & Fullerton (2016) Genomics is Failing on Diversity. Nature; 568:161-164

Human Journey

An understanding of the interrelationship between different human populations allows researchers to understand the genetic basis of disease. The Arabian Peninsula is at the cross road of human migration giving rise to the diversity in the Arab population.
https://genographic.nationalgeographic.com/human-journey

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Kuwait
Satellite
Google
Map data ©2018 Google, INEGI
Kuwait
Terms
Send feedback
2000 km

Map of Middle East

What's missed

- Under representation of population of mixed ancestry or not European.
- Information about the major ancestral population across the world is missing.
- Sequence the whole genome and whole exomes (the complete set of protein-codes genes) currently being used more widely for discovery.
- Population that have rare variations that might be clinically relevant.
- It is important to study under represented population to ensure the benefits of research are distributed fairly.
- There is a genomic bias as a results of logistic and systemic factors.

Genomics Data 3 (2015) 116–127

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journal homepage: http://www.journals.elsevier.com/genomics-data/

Kuwaiti population subgroup of nomadic Bedouin ancestry—Whole genome sequence and analysis

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"Tent-dwelling" Bedouins

ABSTRACT

Kuwaiti native population comprises three distinct genetic subgroups of Persian, "city-dwelling" Saudi Arabian tribe, and nomadic "tent-dwelling" Bedouin ancestry. Bedouin subgroup is characterized by presence of 17% African ancestry; it owes it origin to nomadic tribes of the deserts of Arabian Peninsula and North Africa. By sequencing whole genome of a Kuwaiti male from this subgroup at 41X coverage, we report 3,752,878 SNPs, 411,839 indels, and 8451 structural variations. Neighbor-joining tree, based on shared variant positions carrying disease-risk alleles between the Bedouin and other continental genomes, places Bedouin genome at the nexus of African, Asian, and European genomes in concordance with geographical location of Kuwait and Peninsula. In congruence with participant's medical history for morbid obesity and bronchial asthma, risk alleles are seen at deleterious SNPs associated with obesity and asthma. Many of the observed deleterious 'novel' variants lie in genes associated with autosomal recessive disorders characteristic of the region.

Bedouin subgroup (who roamed the deserts of Middle East; they epitomize the best adaptation of human life to desert conditions). Is characterized by presence of 17% African ancestry; the origin to nomadic tribes of the deserts of Arabian Peninsula and North Africa.

Thareja et al. BMC Genomics (2015) 16:92
DOI 10.1186/s12864-015-1233-x

BMC Genomics

RESEARCH ARTICLE Open Access

Sequence and analysis of a whole genome from Kuwaiti population subgroup of Persian ancestry

Gaurav Thareja†, Sumi Elsa John†, Prashantha Hebbar, Kazem Behbehani, Thangavel Alphonse Thanaraj* and Osama Alsmadi*

Abstract

Background: The 1000 Genome project paved the way for sequencing diverse human populations. New genome projects are being established to sequence underrepresented populations helping in understanding human genetic diversity. The Kuwait Genome Project an initiative to sequence individual genomes from the three subgroups of Kuwaiti population namely, Saudi Arabian tribe; "tent-dwelling" Bedouin; and Persian, attributing their ancestry to different regions in Arabian Peninsula and to modern-day Iran (West Asia). These subgroups were in line with settlement history and are confirmed by genetic studies. In this work, we report whole genome sequence of a Kuwaiti native from Persian subgroup at >37X coverage.

Results: We document 3,573,824 SNPs, 404,090 insertions/deletions, and 11,138 structural variations. Out of the reported SNPs and indels, 85,939 are novel. We identify 295 'loss-of-function' and 2,314 'deleterious' coding variants, some of which carry homozygous genotypes in the sequenced genome; the associated phenotypes include pharmacogenomic traits such as greater triglyceride lowering ability with fenofibrate treatment, and requirement of high warfarin dosage to elicit anticoagulation response. 6,328 non-coding SNPs associate with 811 phenotype traits: in congruence with medical history of the participant for Type 2 diabetes and β-Thalassemia, and of participant's family for migraine, 72 (of 159 known) Type 2 diabetes, 3 (of 4) β-Thalassemia, and 76 (of 169) migraine variants are seen in the genome. Intergenome comparisons based on shared disease-causing variants, positions the sequenced genome between Asian and European genomes in congruence with geographical location of the region. On comparison, bead arrays perform better than sequencing platforms in correctly calling genotypes in low-coverage sequenced genome regions however in the event of novel SNP or indel near genotype calling position can lead to false calls using bead arrays.

Conclusions: We report, for the first time, reference genome resource for the population of Persian ancestry. The resource provides a starting point for designing large-scale genetic studies in Peninsula including Kuwait, and Persian population. Such efforts on populations under-represented in global genome variation surveys help augment current knowledge on human genome diversity.

Keywords: Persian genome, Personal genome, Whole genome sequencing, Kuwaiti population, Arabian Peninsula

Arabian ancestry is seen more in the Saudi Arabian tribe ancestry subgroup (69%) than in the Bedouin group (40%); and West Asian ancestry is seen more in the Persian subgroup (56%) than in any of the other two groups.

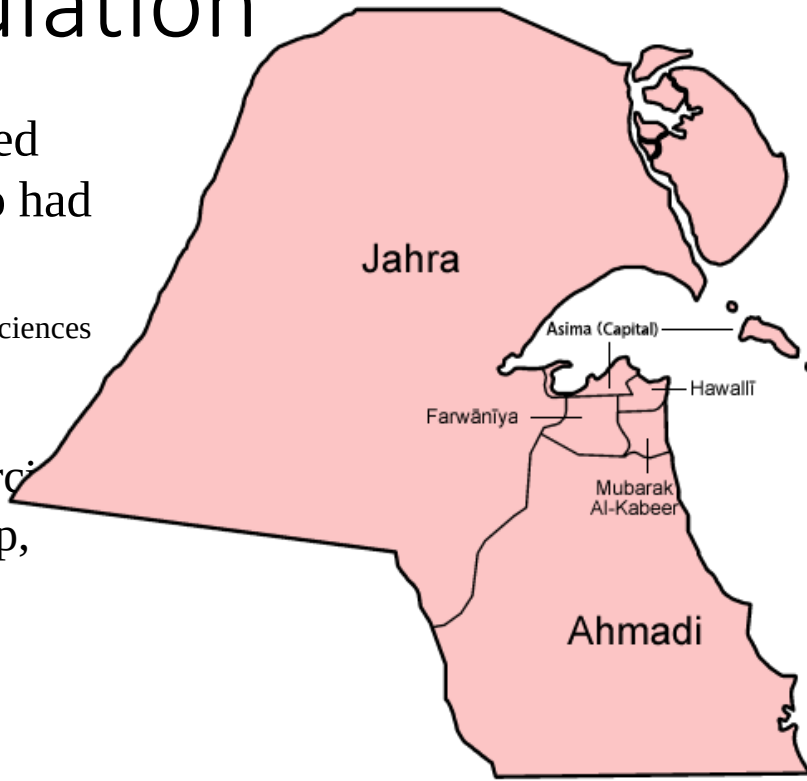
The Persian subgroup is composed of people of West Asian (particularly Iranian) descent. Iran, centrally located in the Asian continent, has served for centuries as gateway for movement of human population across diverse spheres of Asia and Europe.

Data of the Kuwaiti Population

- Blood samples were collected from 917 unrelated bone marrow donors above 18 years of age who had consented to be a part of Kuwait Bone Marrow National Registry. Ethical Approval was obtained from Health Sciences Center and Ministry of Health

- Red cell genotyping was performed using commerci available kits (RHD Beadchip kit, RHCE Beadchip, BioArray Solutions, Immucor, Warren, NJ, USA).

- Kuwaiti residence 1.394.945 (30.22%).
- Non Kuwaiti residence 3.220.829 (69.78%).



Initial Findings

Table 1: RHD Variants and Frequency

Variant Allele(s)	Number	Genotype results
*RHD*DIIIa* (hemizygous or homozygous) -or- *RHD*DIIIa/03N.01*	2	186G>T(L62F) 410C>T(A137V) 455A>C(N152T) 602C>G(T201R) 667T>G(F223V)
*RHD*DFR1* -or - *RHD*DFR3*	1	509T>G(M170T) 514A>T(I172T)
*RHD*DIIIa*	1	186G>T(L62F) 410C>T(A137V) 455A>C(N152T) 602C>G(T201R)
*RHD*03N.01*	1	186G>T(L62F) 410C>T(A137V) 455A>C(N152T)
*RHD*DIVa*	1	missing 410C>T change
*RHD*08N.01*	3	37bp duplication (psi D) 667T>G(F223V) 807T>G(Y269X)
*RHD*DAR3 -or - RHD*DAR5*	3	602C>G(T201R) 667T>G(F223V)
RHD-CE(3-7)-D (hemizygous or homozygous)	2	697G>C(E233Q) 733G>C(V245L) 787G>A(G263R)
RHD01N.04 (hemizygous or homozygous)	1	697G>C(E233Q) 835G>A(V279M)
Total (%)	**15 (1.63%)**	

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Legend: 15 (1.63%) from 917 samples had an *RHD* variant allele detected, with their *RHD* possible mutation were found in a homozygous or heterozygous state and were associated with *RHCE* alleles,.Manuscript in Preparation

Initial Findings

Table 2. RHCE Variants and Frequency

Variant Allele(s)	Predicted Phenotype	Number	Genotype Results
*RHCE*ce.01/cE.04,* (homozygous)	cEe	1	48G>C (W16C) 602C>G (T201R) 916A>G (I306V)
*RHCE*ce.01/ce.02.01,* (homozygous)	ce	1	48G>C (W16C) 1025C>T (T342I)
*RHCE*ce.VS.03/cE.04,* (homozygous)	cEe	1	48C>G (W16C) 733C>G (L245V) 1006G>T (G336C) 602C>G (T201R)
*RHCE*ce.VS.01/cE.04,* (homozygous)	cEe	1	733C>G (L245V) 602C>G (T201R)
*RHCE*ce.01/cE.08.01,* (homozygous)	Cce	2	48G>C (W16C) 122A>G (Q41R)
*RHCE*ce.01*	*cE*	3	48G>C (W16C)
*RHCE*ce.01*	*ce*	19	48G>C (W16C)
*RHCE*ce.01*	*Ce*	43	48G>C (W16C)
*RHCE*ce.01*	*CE*	1	48G>C (W16C)

Initial Findings

Table 2. RHCE Variants and Frequency (Contd.)

Variant Allele(s)	Predicted Phenotype	Number	Genotype Results
*RHCE*ce.VS.02*	Ce	2	48C>G (W16C) 733C>G (L245V)
*RHCE*ce.VS.02 -or- RHCE*ce.01/ceVS.01*	ce	2	48C>G (W16C) 733C>G (L245V)
*RHCE*ce.VS.03*	Ce	2	48C>G (W16C) 733C>G (L245V) 1006G>T (G336C)
*RHCE*ce.VS.03 -or- RHCE*ce.01/ceVS.02*	ce	2	48C>G (W16C) 733C>G (L245V) 1006G>T (G336C)
*RHCE*ce48C, 733G, 744C*	Ce	2	48C>G (W16C) 733C>G (L245V) 744T>C (S248S)
*RHCE*cE.04*	ce	3	602C>G (T201R)
*RHCE*cE.04*	Ce	12	602C>G (T201R)
*RHCE*cE.04*	cE	0	602C>G (T201R)
*RHCE*ce.VS.01*	Ce	18	733C>G (L245V)
*RHCE*ce.VS.01*	cE	3	733C>G (L245V)

Initial Findings

Table 2. RHCE Variants and Frequency (Contd.)

Variant Allele(s)	Predicted Phenotype	Number	Genotype Results
*RHCE*ce.VS.01*	ce	13	733C>G (L245V)
*RHCE*ce.VS.05*	ce	1	733C>G (L245V) 1006G>T (G336C)
*RHCE*ce.04.01*	Ce	1	48C>G (W16C) 712A>G (M238V) 733C>G (L245V) 916A>G (I306V)
*RHCE*ce.04.01*	ce	1	48C>G (W16C) 712A>G (M238V) 733C>G (L245V) 916A>G (I306V)
*RHCE*Ce.08.01*	cE	1	122A>G (Q41R)
*RHCE*Ce.08.01*	Ce	3	122A>G (Q41R)
*RHCE*Ce.09*	ce	1	106G>A (A36T)
*RHCE*ce.05.01*	Ce	1	48C>G (W16C) 712A>G (M238V)
Total (%)		**140(15.26%)**	

Legend: 140 (15.26%) from 917 samples had an *RHCE* variant allele detected. The *RHCE* mutations were found in a heterozygous state and were associated with a conventional wild-type *RHCE* alleles in most cases

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Initial Findings

Table 3: Associated Variants and Frequency

RHD Variants	Number	RHCE Variants
possible D/DIVa type 2	1	ce(48C)/ceTI
possible D/RHD psi	2	WT/ce(48C)
RHD Deletion	1	WT/ce(48C)
RHD Deletion	1	WT/ce(733G)
possible D/weak D type 4.0 or 4.3	1	WT/ce(48C,733G) or ce(48C)/ce(733G)
DIIIa (hemizygous or homozygous) or DIIIa/DIIIa-CE(4-7)-D	1	WT/ce(48C,733G) or ce(48C)/ce(733G)
possible D/DIIIa	1	WT/ce(48C,733G,1006T)
possible D/DIIIa-CE(4-7)-D	1	WT/ce(48C,733G,1006T)
DIIIa (hemizygous or homozygous) or DIIIa/DIIIa-CE(4-7)-D	1	WT/ce(48C,733G,1006T) or ce(48C)/ce(733G,1006T)
possible D/DFR or DFR3	1	WT/ce(733G)
Total(%)	**11(1.2%)**	

Legend: 11(1.2%) from 917 samples were RHD/RHCE associated variants.
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Major Remarks of Initial Findings

- weak D type 1, 2, or 3 was not detected in these sample population.
- ce(48C)/ceTI and /DIVa type 2 in African ancestry (Westhoff et al., 2013)
- WT/ce(48C,733G,1006T) and possible D/DIIIa which is common in US (Westhoff et al., 2010).
- Understanding the prevalent RH alleles in a population is important for transfusion management of patients, especially those who are chronically transfused as well as their blood donors.
- This study provides important data about the blood group antigens in the Kuwaiti population to guide transfusion care.
- The SNPs used in the technique are designed based on European population, only by the use of NGS technology we will be able to find the SNPs in our population.

Conclusion

- Expression studies, and family studies, will be needed to substantiate novel findings and to confirm antibody/ antigen associations.
- The presence of novel changes in blood group genes has been a consistent finding in every study employing NGS for blood group genotyping. However, the clinical value of these novel alleles needs to be assessed within the transfusion context.
- Worldwide studies confirm that we have yet to uncover the full range of human genomic variation, particularly in the Arab Mediterranean population who are also frequently hemoglobinopathy carriers.
- It is important to characterize, classify, and catalog clinically relevant as well as benign blood group variation.
- To establish a central electronic database for the blood group with a process to streamline patient reporting and revision.
- Patient genome sequencing should be part of clinical care can be used for transfusion medicine decision making.

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RH diversity and whole genome sequencing of African American individuals

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Department of Genome Sciences
University of Washington

10/14/2018

Faculty Disclosure

(In compliance with ACCME policy, AABB requires the following disclosures to the session audience)

I have no relevant financial relationships to disclose.

Outline

- Overview of next generation sequencing
- Overview of the *RH* locus
- Discuss detection of *RH* genetic variation in African American whole genomes

Genetic variation

- On average humans are:
  - 99.9% identical
- On average humans have:
  - 4-5M variants

The 1000 Genomes Project Consortium, 2015

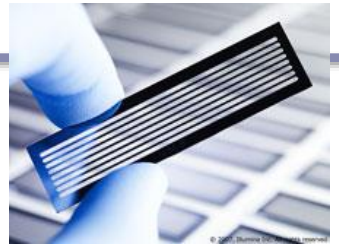
Methods for detecting genetic variation

1. SNP arrays
2. Sequencing:
   - Sanger sequencing
   - Next generation sequencing (NGS)
   - Third generation sequencing

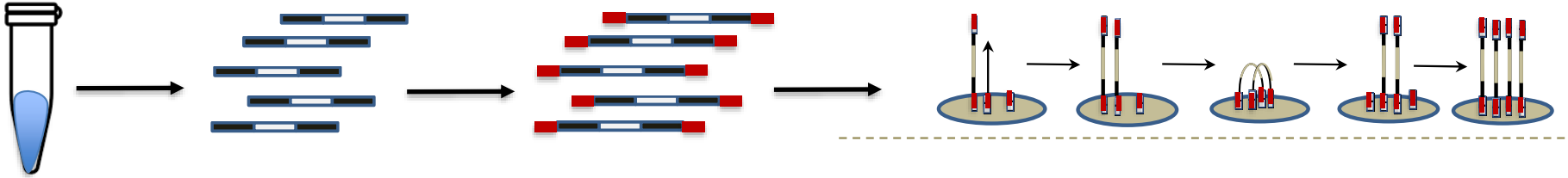
Next generation sequencing

- Generates hundreds of millions of reads
- 1000-fold less expensive than Sanger sequencing
- Short read lengths

Next generation sequencing



DNA library preparation:

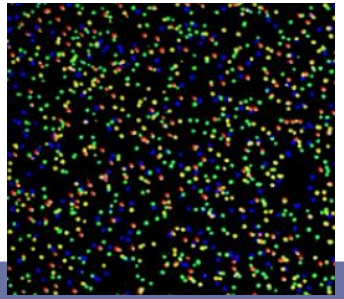


Genomic DNA

Fragmentation

Adaptor ligation

Amplification and sequencing



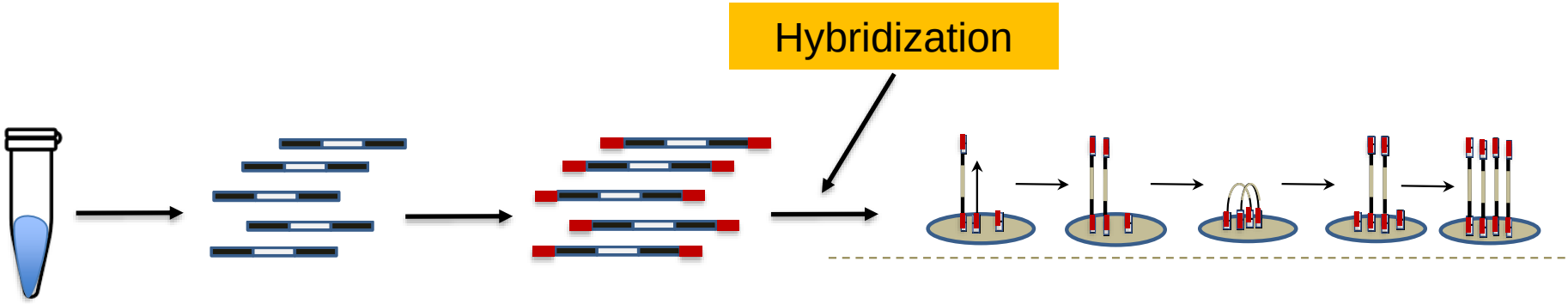
Align to a reference genome

Reference Genome

NGS short reads

Targeted and whole genome sequencing

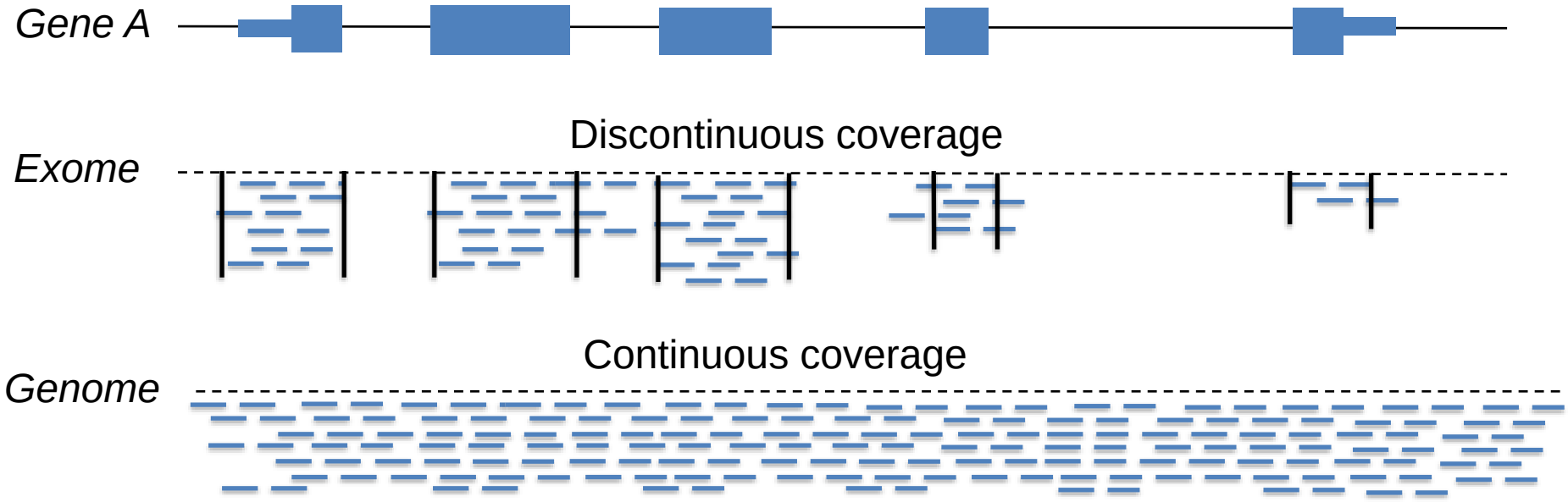
Targeted sequencing includes a ”selection” step such as hybridization



Targeted and whole genome sequencing

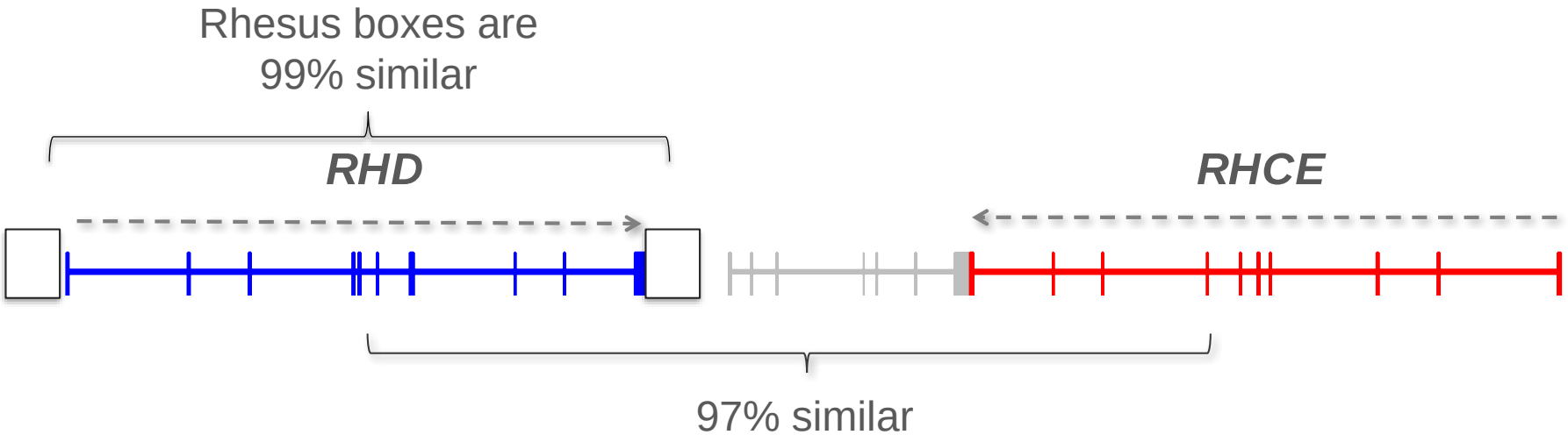
- Custom capture and whole exome
  - Includes genes/regions of interest
  - Exome focuses on coding regions of the genome
- Whole genome sequencing
  - Includes all regions in the genome

Targeted and whole genome sequencing



The *RH* locus

The *RH* locus is one of the most complex loci genome



Types of *RH* genetic variation

RH commonly exhibits single nucleotide variants (SNVs), small insertion and deletions (indels) and structural variation

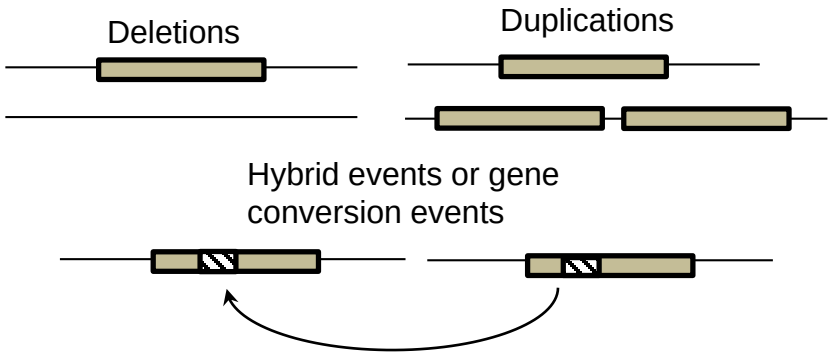
Single nucleotide variants (SNVs)

ATTCGAATCTCA
ATTCGATTCTCA

Insertion & deletions (indels)

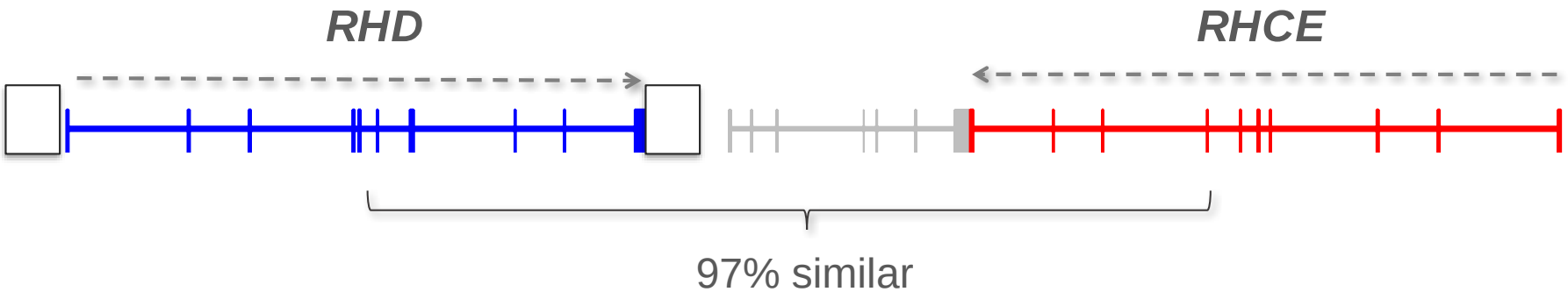
ATTCGAATCTCA
ATTCGA--TCTCA

Structural variants

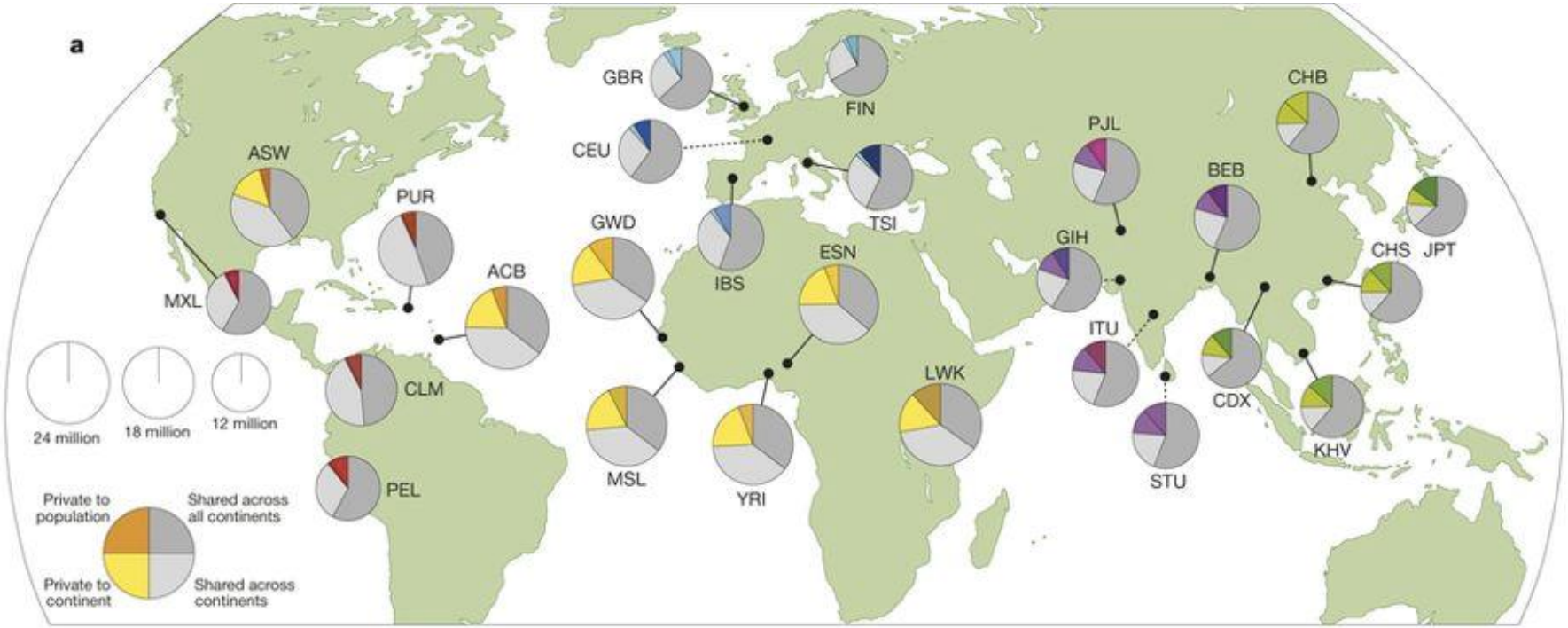


The Rh blood group

- > 280 *RH* alleles comprised of SNVs, indels or structural variants
- > 50 antigens including the polymorphic RhD and RhCE (C,c,E,e)

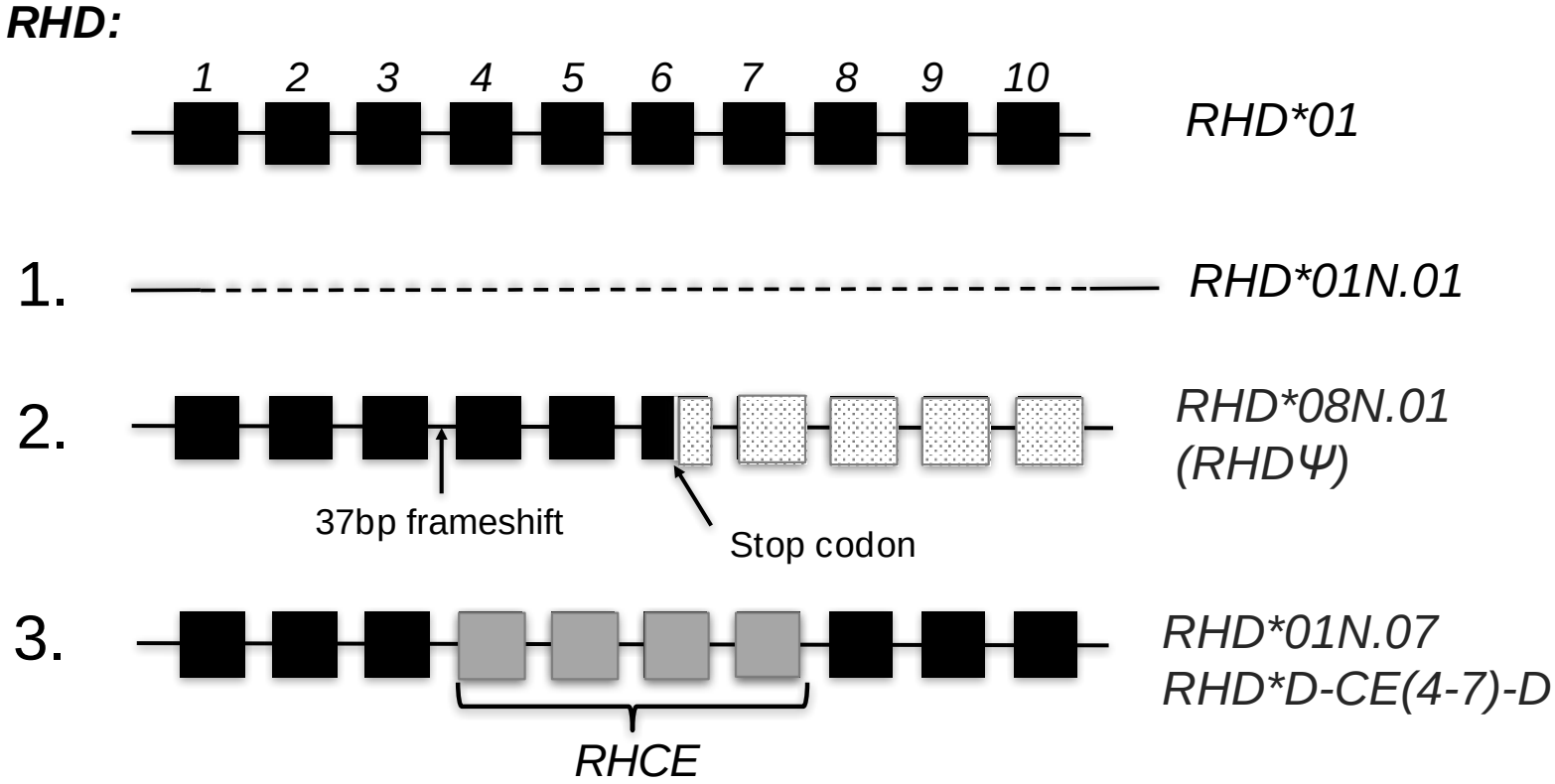


Genetic diversity in populations



The 1000 Genomes Project Consortium, 2015

Causes of RhD negative in African populations



Detecting *RH* variation in African American whole genomes

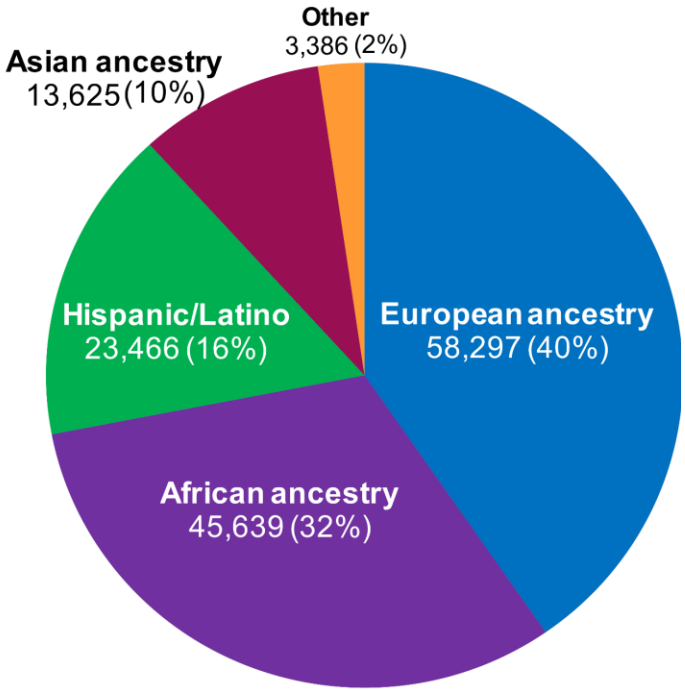
1. 1135 Asian and Native American samples

2. 1715 African American samples from the TOPMed Jackson Heart Study (JHS)
   – Randomly selected unrelated set of samples

Delaney et al. (2015); Wheeler et al. 2018

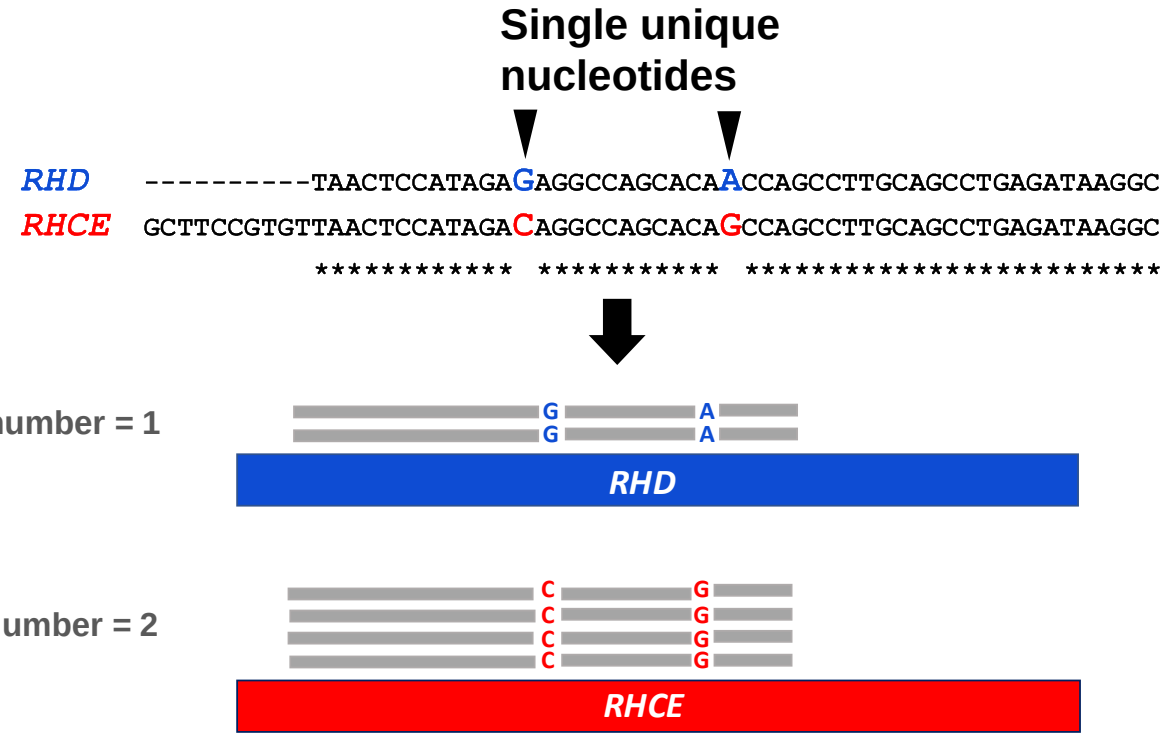
Trans-omics for precision medicine (TOPMed) Program

- >140,000 whole genomes
- 60% of individuals are of non-European ancestry



Detection of structural variation in *RH*

1. **Pairwise alignment**

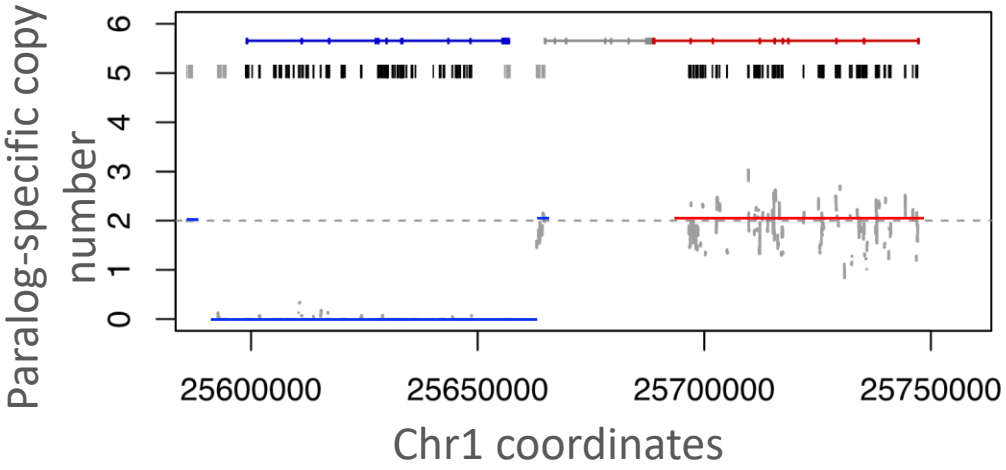


2. **Read depth derived paralog-specific copy number estimates**

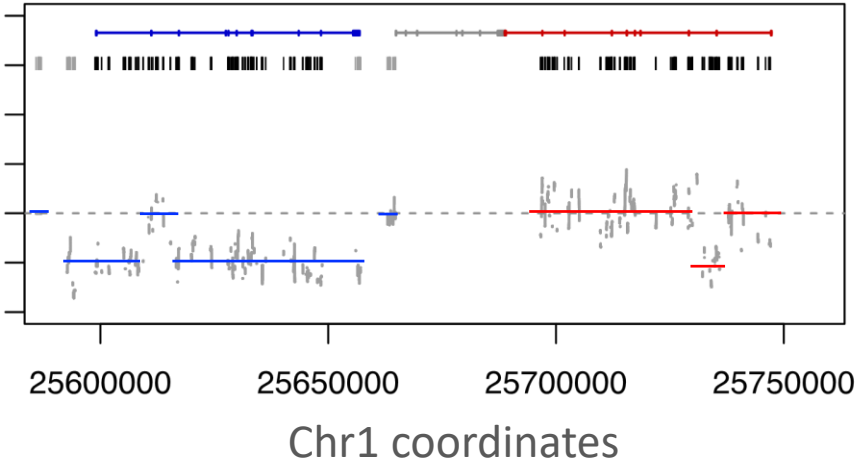
RH structural variation in African American samples

61% of individuals exhibited structural variation

Homozygous *RHD* deletion
*RHD*01N.01*

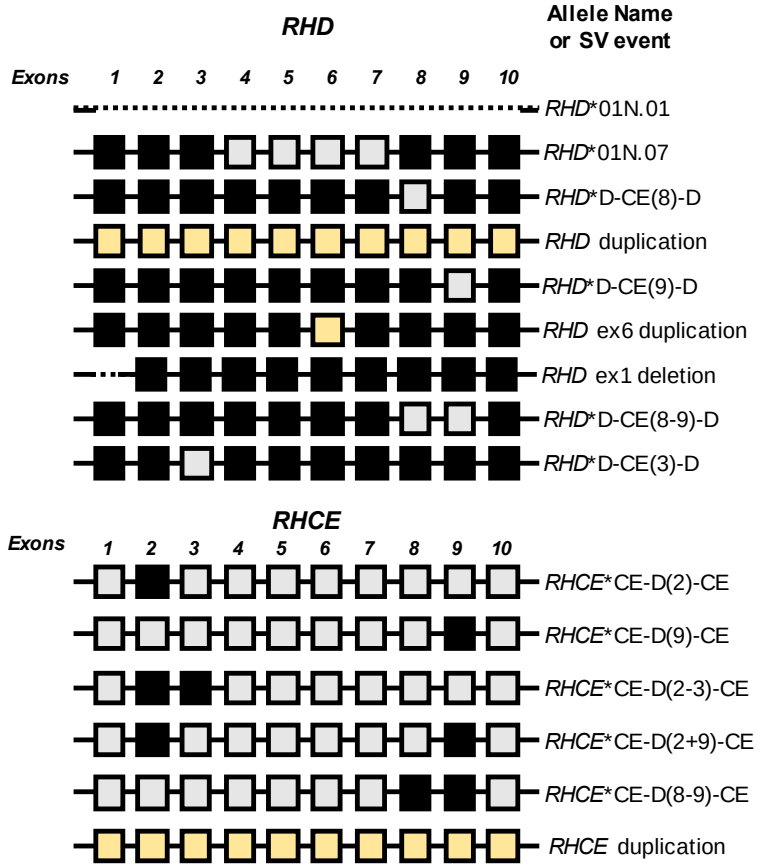


Heterozygous *RHD* deletion
*RHD*01N.01* and *RHCE*CE-D(2)-CE*



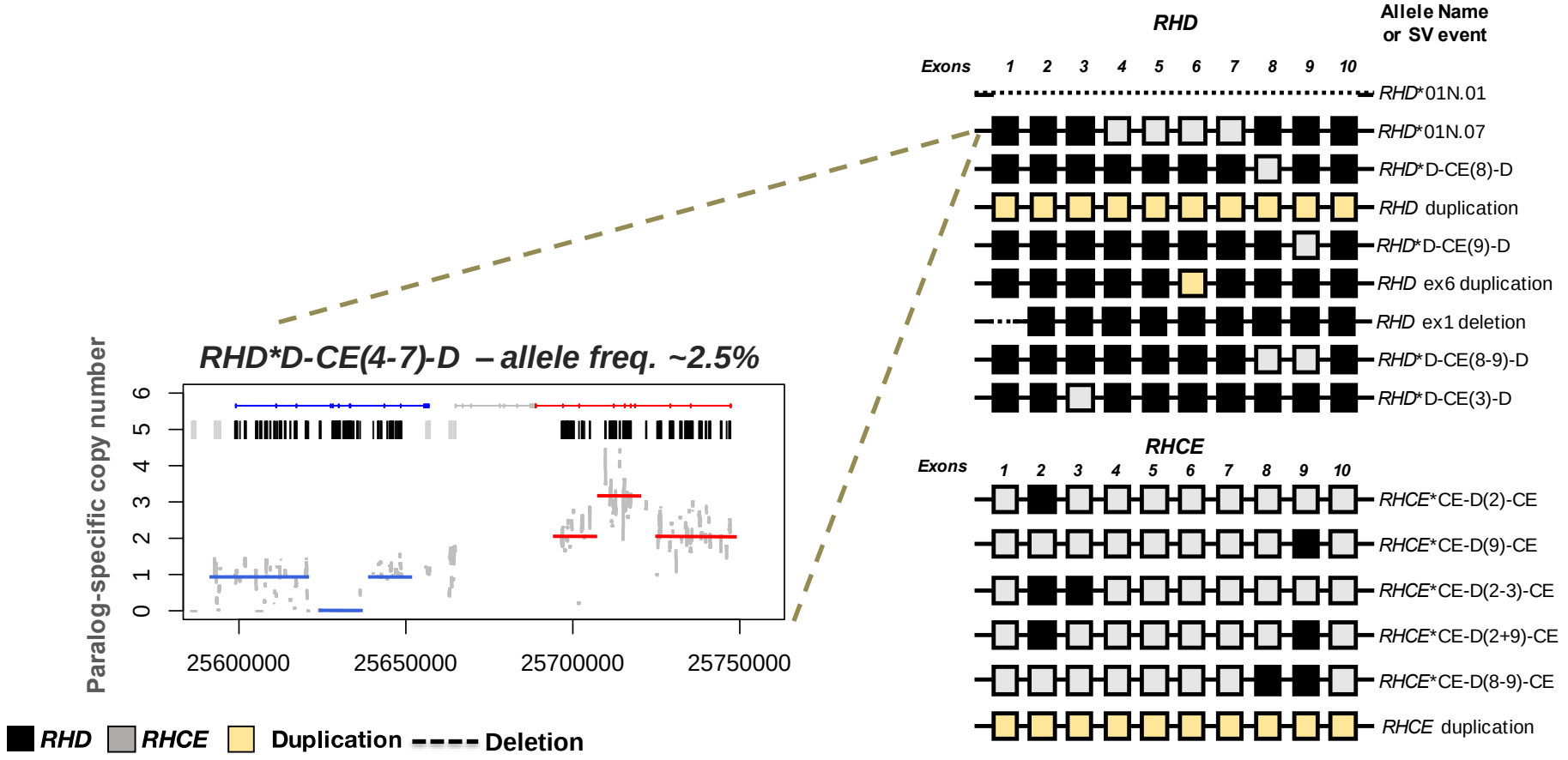
RH structural variation in African American samples

- 9 events in *RHD*
- 6 events in *RHCE*



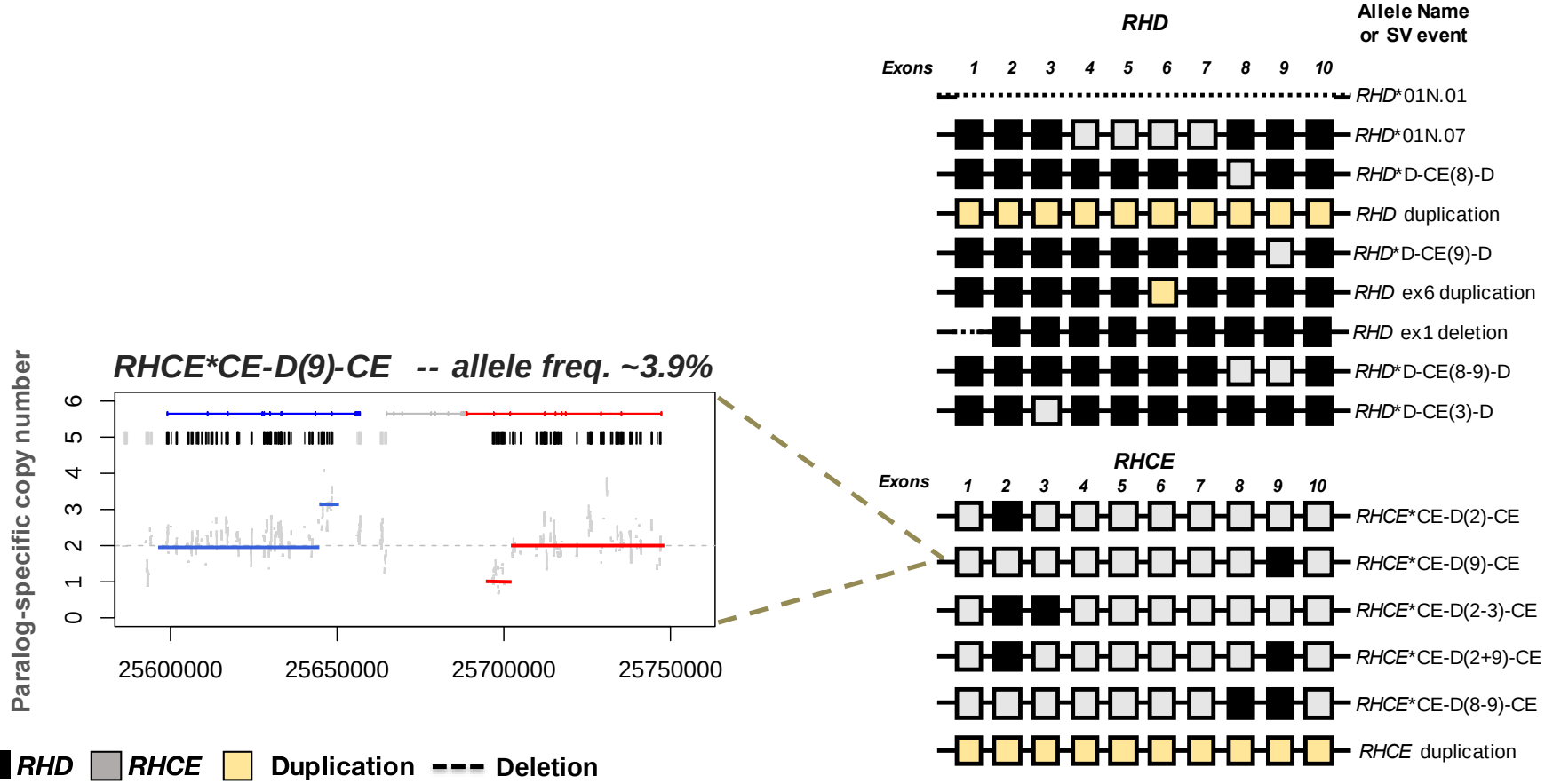
RHD RHCE Duplication ----- Deletion

RH structural variation in African American samples



Wheeler et al. 2018

RH structural variation in African American samples



Wheeler et al. 2018

RH SNVs and indels in African American samples

54 *RH* alleles (29 *RHD* and 25 *RHCE*) detected

Subset of *RHD* alleles:

Allele Name	AF (%)
*RHD*08N.01 (RHDΨ)*	3.178
*RHD*03.04 (RHD*DIII.04)*	2.245
*RHD*09.03 (RHD*DAR3)*	1.429
*RHD*10.00 (DAU0)*	22.24
*RHD*01N.18*	0.087

Subset of *RHCE* alleles:

Allele Name	AF (%)
*RHCE*01.01*	36.560
*RHCE*01.20.01 (V+VS+)*	13.790
*RHCE*01.20.07 (V/VS+w)*	0.087

Summary

- Genomics is a rapidly evolving field with several ongoing population resequencing projects
- Performed a systematic survey of *RH* variation in African American samples using whole genome data
- Technological advancements will continue to improve detection variation across populations

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JACKSON HEART STUDY

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