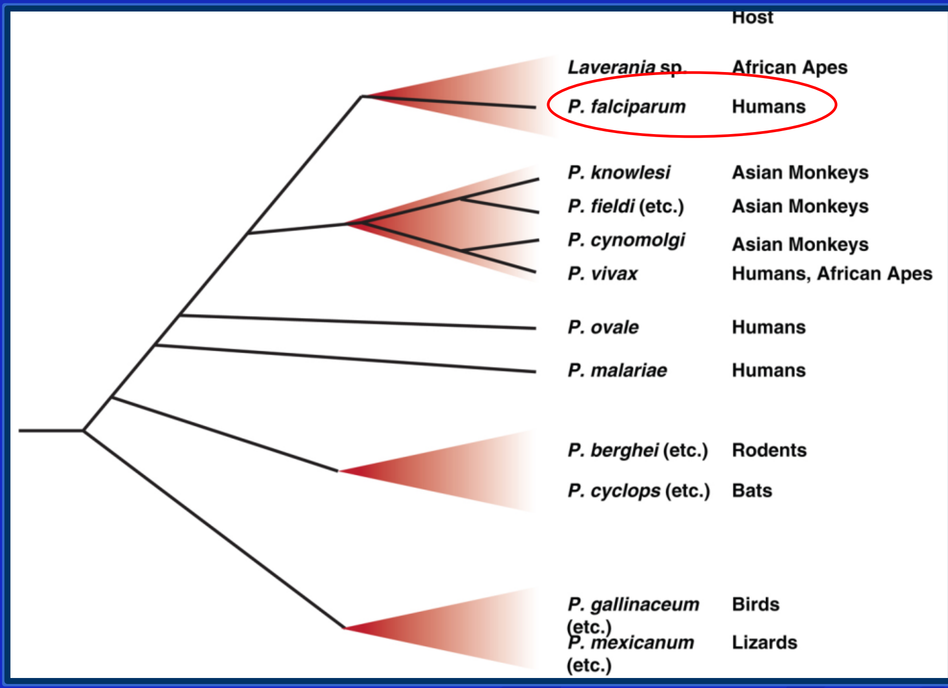
Scratching away at Plasmodium falciparum in Papua New Guinea.

Phylomadness in Tassie 2014
Abby Harrison, WEHI

Who, what, and where…?

Malaria

- Numerous malaria species
  - infecting a range of hosts
  - *Primates, Rodents, Birds, Lizards …*



- 5 species infect Humans
  - *P. falciparum* and *P. vivax*

    - cause majority of deaths
  - *P. malariae*, *P. ovale*

    – generally mild infection, rarely fatal
  - *P. knowlesi* – zoonotic

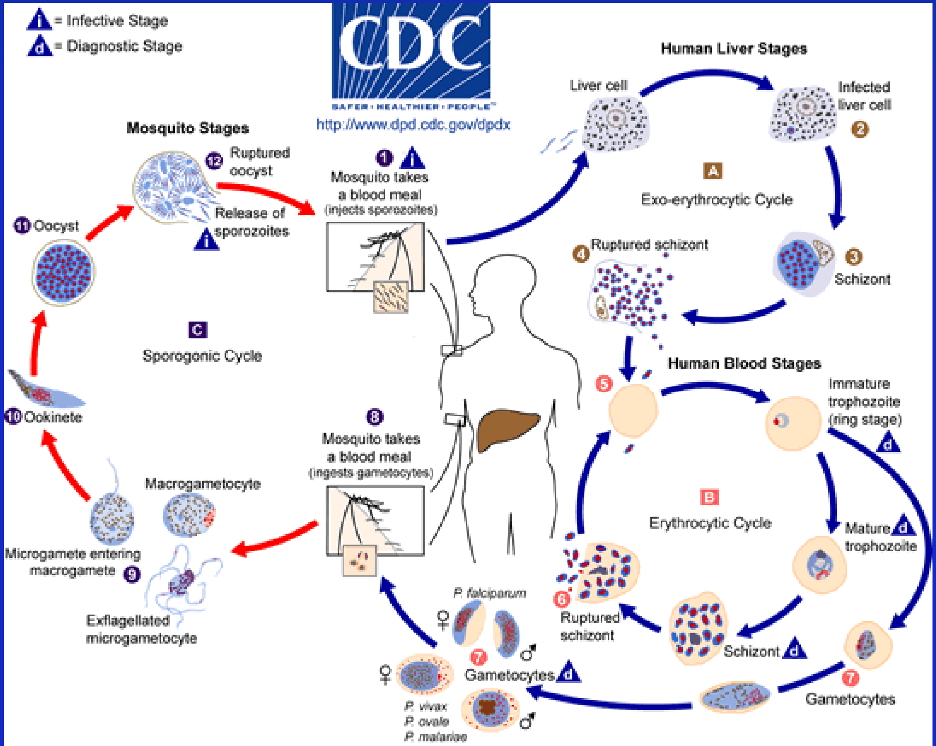
- Multiple cross species transfer events

Malaria

Plasmodia

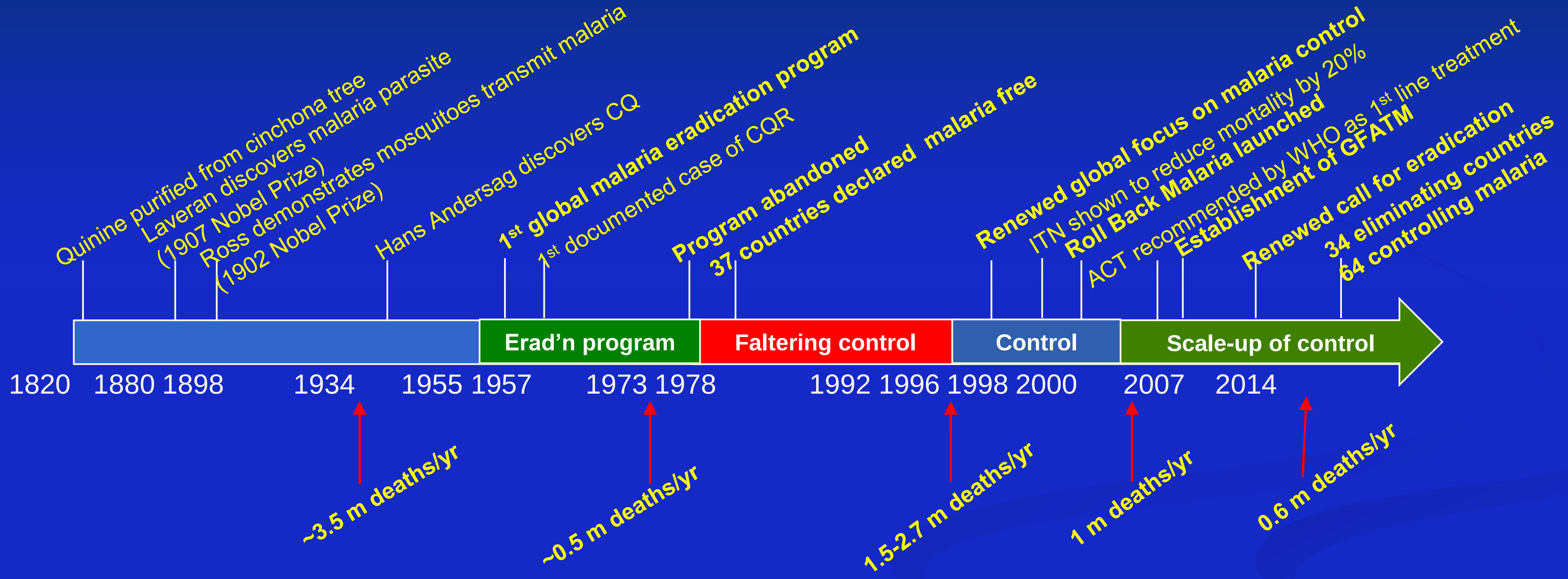
- Protozoan – unicellular eukaryotic organism
- Genome size 20-30MB
  - 16 chromosomes, including ~6Kb Mitochondrion, and 30-40Kb Apicoplast
  - Exosome-like vesicles (DNA content unknown)
- Mosquito vector
  - Many vector species
- Parasite life cycle involves two hosts
- Multiple concurrent species and clone infections
- Has **haploid** and diploid phases
  - Haploid in humans
  - Diploid in mosquito

Malaria life cycle: delightfully complex



- Multiple phases and stages of infection
- Multiple concurrent clonal infections
- Genome replicates in Liver and Red blood cells
- Haploid in human host
- Fertilization of female gamete occurs in the mid gut of the mosquito (Recombination occurs)

The continuing fight against malaria



Quinine purified from cinchona tree

Laveran discovers malaria parasite (1907 Nobel Prize)

Ross demonstrates mosquitoes transmit malaria (1902 Nobel Prize)

Hans Andersag discovers CQ

1st global malaria eradication program

1st documented case of CQR

Program abandoned

37 countries declared malaria free

Renewed global focus on malaria control

ITN shown to reduce mortality by 20%

Roll Back Malaria launched

ACT recommended by WHO as 1st line treatment

Establishment of GFATM

Renewed call for eradication

34 eliminating countries

64 controlling malaria

Erad'n program | **Faltering control** | **Control** | **Scale-up of control**

1820 1880 1898 1934 1955 1957 1973 1978 1992 1996 1998 2000 2007 2014

~3.5 m deaths/yr

~0.5 m deaths/yr

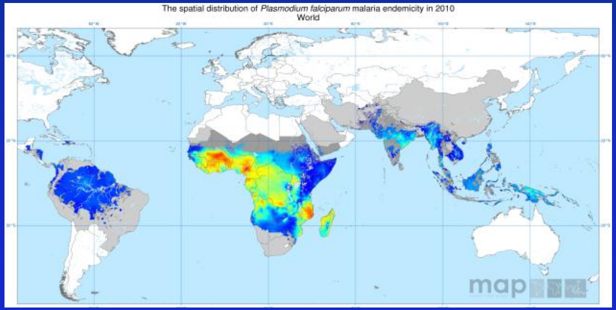
1.5-2.7 m deaths/yr

1 m deaths/yr

0.6 m deaths/yr

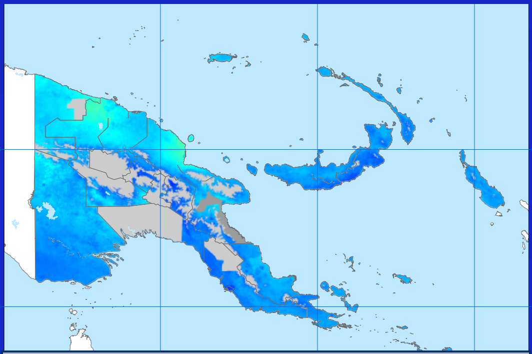
Malaria and Papua New Guinea

Over the last decade there has been a heightened push for control and elimination of Malaria, thanks in part to the Global Fund to combat AIDs, TB, and Malaria.



World wide prevalence of *Plasmodium falciparum.*
Taken from the Oxford Malaria Atlas project
(http://www.map.ox.ac.uk/browse-resources/)

Papua New Guinea (PNG) has the highest burden of malaria outside Africa ranging from holo-endemic, to endemic to absent.



P. falciparum prevalence 2-10 year olds PNG 2010
Oxford Malaria Atlas project
(http://www.map.ox.ac.uk/browse-resources/)

Bednets (LLIN)s and drugs(RDTs)

Study objectives

Using population genetic models and tools we aim to:

1. Map the population structure of *P. falciparum* in PNG.

- Develop tools for molecular surveillance (Barcodes).
- Apply these tools to understand the dynamics of the population structure
  - eg, identify "hot spots" (sources of outbreaks).

2. Investigate genetic signatures in the genome.

- How have external forces, such as drugs and the introduction of bed nets, effected change in the genome and population structure :
  - Genetic diversity
  - Linked genetic characters
  - Selection landscape

- Bottle necks
- Expansions

PNG: a diversity hotspot

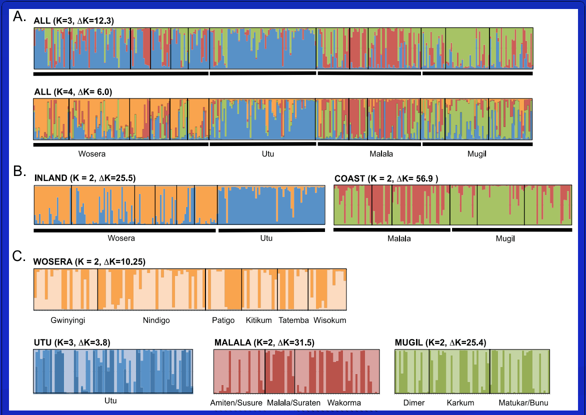
The diverse Geography, Biogeography, Host, Mosquito spp., and malaria control conditions throughout PNG produces diverse micro-epidemiologies.

- Historical separation & isolation of human populations
  - Genetic heritage, cultural behaviour, and movement
- Mosquito
  - 11 anophelene species
- Biogeography
  - Diverse fauna and flora (5% of worldwide diversity)
- Geography
- Control
  - Past and present

Population structure of P. falciparum

Microsatellite studies have found

- Moderate to high population structure,
  - suggesting fragmented demes
- High levels of genetic diversity across all populations
- Variable allelic (microsatellite) richness
  - lower richness in inland populations



Structure analysis of *P falciparum* microsatellite haplotypes from PNG.

Prevalence of infection

- No significant difference between catchment areas,
- High variation between villages ranging from 1.7% to 43.5%
- Pre bed-net distribution the prevalence was more uniform,
- Post bed-net distribution prevalence was more variable with “hot spots” interspersed with relative low prevalence.

Mapping the population structure of Plasmodium falciparum

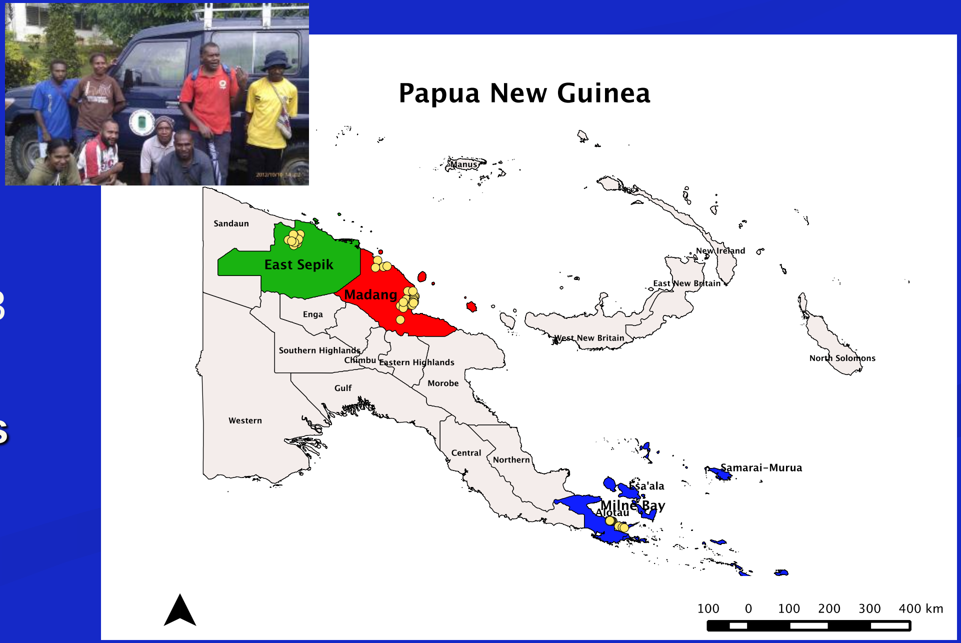
These initial works are excellent and informative but…

- Microsatellites have limitations
  - Do not know inherited history
  - There are issues with quality control of data
- Whole genome sequencing (WGS) and Single Nucleotide Polymorphisms (SNPs) provide a more robust data set to draw inferences from.
  - SNPs give a more detailed picture of evolutionary relationships between individuals
  - Can identify specific SNP markers for different biological questions and applications

Plasmodium whole genome sequencing & SNPs

- MalariaGen consortium (Sanger, Hinxton, UK)
- 100s of researchers over many countries
- 1000s genomes sequenced from across the globe
- To date identified 680 000 *quality* SNPs in *P. falciparum*

- PNG *P. falciparum*
- 132 whole genomes sequenced
- ~ 90 single clone infections
- 54 Madang sequences, collected '08
- 50 East Sepik sequences, collected ’13
- 29 Milne Bay sequences, collected ’13
- **Each sample typed for 680 000 SNPs**



WANTED: Informative SNPS

SNP selection

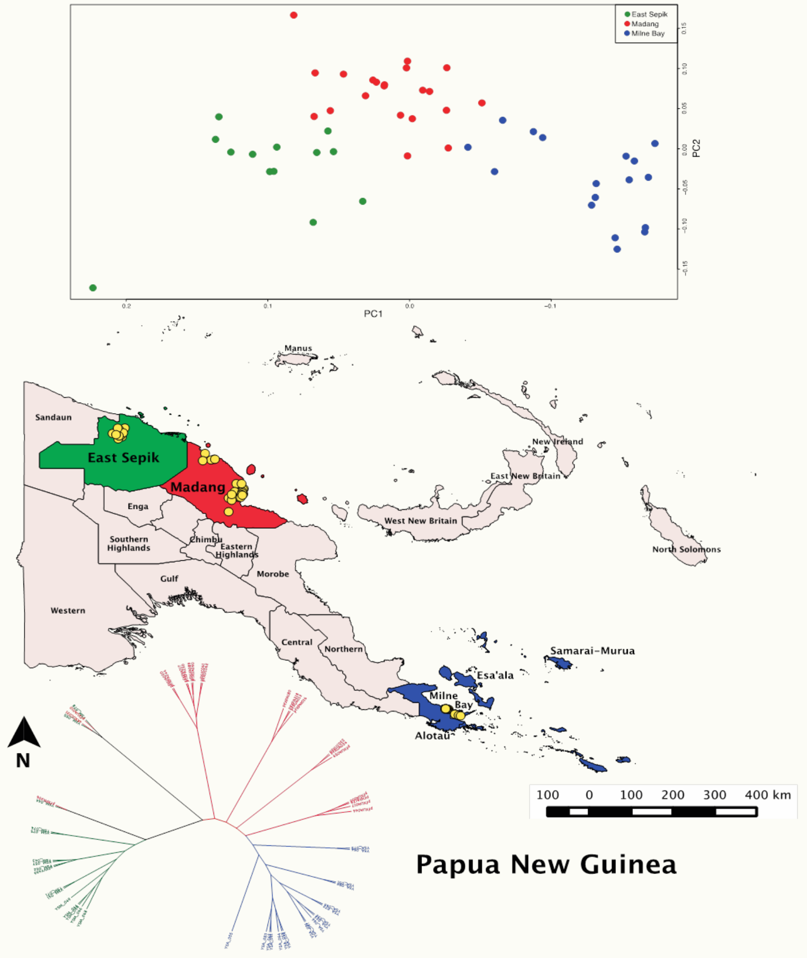
~ 15000 variant SNPs identified in PNG
6025 Minor Allele Frequency (MAF) > 0.1
5786 with high read coverage

Discard SNPs under selection pressure or in linkage disequilibrium
(*BayeScan, and other* F_{ST} *estimates, Tajima's D*)
Rank SNPs by F_{ST} values
Select top 96 SNPs evenly distributed across the genome
Select 96 neutral SNPs randomly distributed

Validate results
PCA and phylogenetic analyses

Design Fluidigm assay

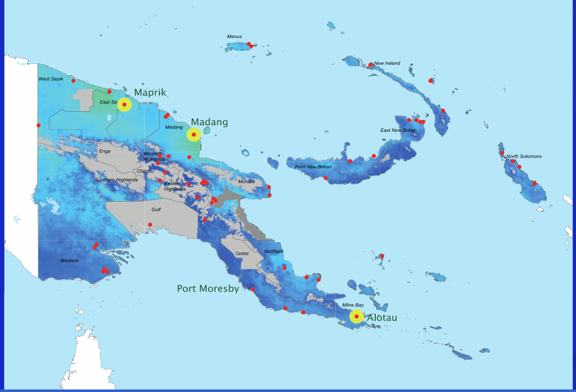
PCA & Phylogenetic analyses from our 192 iSNPs



Mapping P. falciparum across PNG

LLIN and RDTs Malaria Control assessment

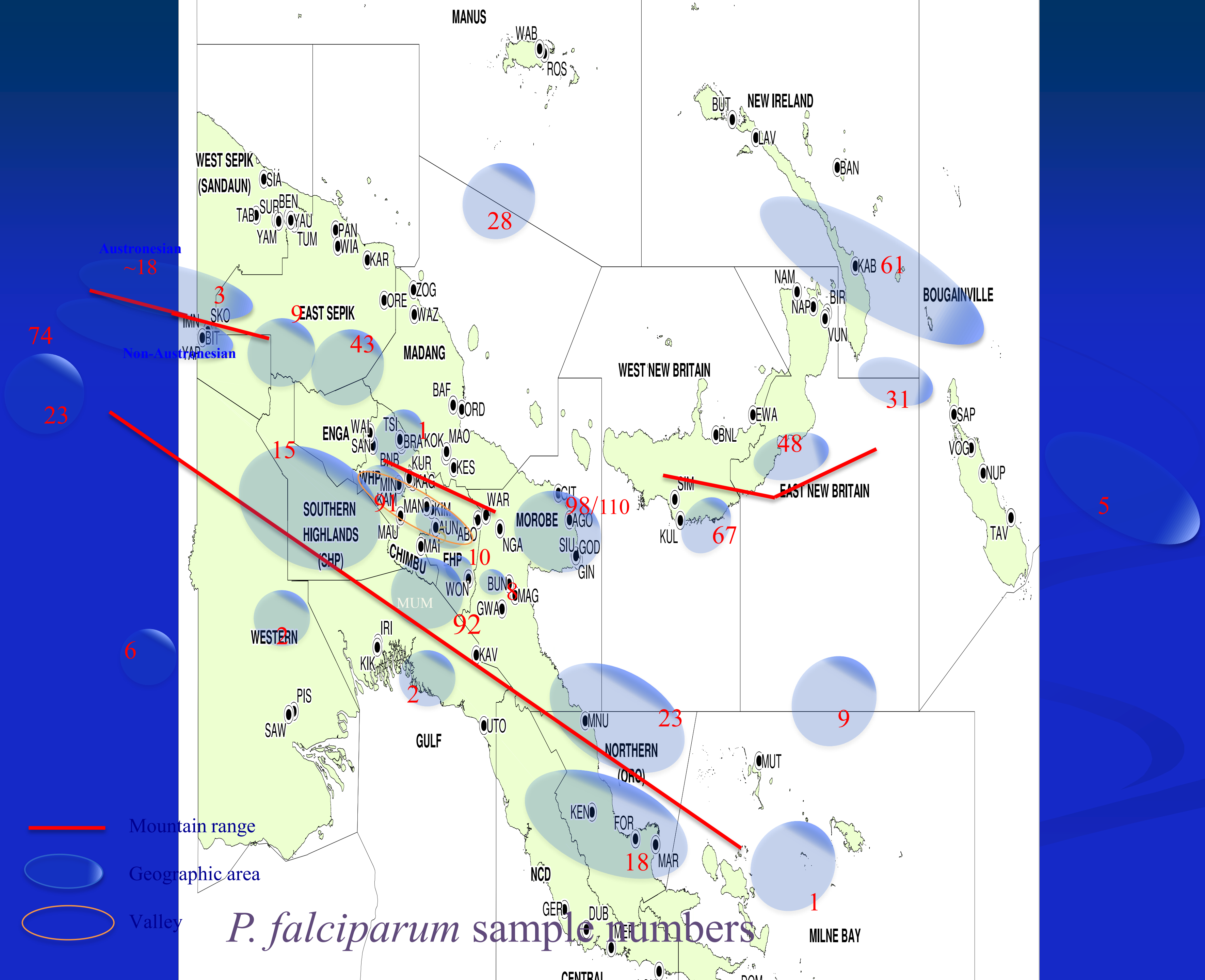
- 10,156 blood spots collected 2008
- 81 villages across 17 Provinces
- 3784 *P. falciparum* infections
- 2349 typed for number of clones per infection
- 585 single clone infections
- 549 dual clone infections



Malaria Control assessment sample collection sites

Province	Samples screened	Pf positive	MSP2 positive	Single infections
Bougainville	470	34	7	3
Central	393	41	3	2
Chimbu	14	0	0	0
East New Britian	366	73	29	17
East Sepik	844	329	260	76
EHP	446	73	4	4
Gulf	330	59	13	4
Madang	1480	367	301	133
Manus	277	98	30	16
Milne Bay	55	11	8	5
Morobe	1938	466	271	148
New Ireland	411	157	76	43
Northern (Oro)	712	190	26	14
West New Britian	626	198	140	86
West Sepik	756	138	46	22
Western	616	51	6	4
WHP	422	64	13	8
Totals	10156	2349	1233	585

P. falciparum sample numbers



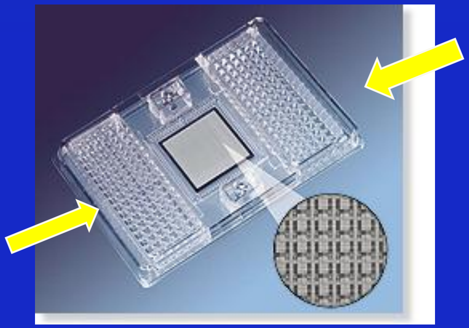
Mountain range

Geographic area

Valley

Mapping P. falciparum population structure across PNG

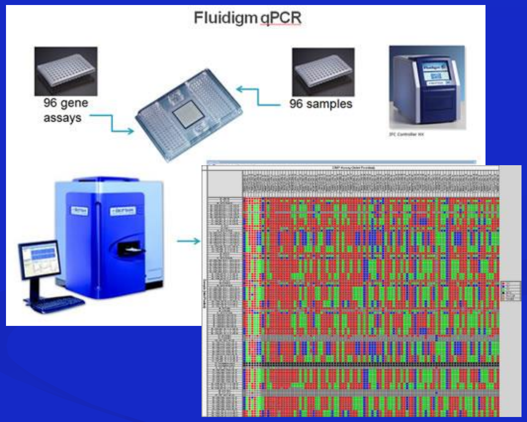
- Fluidigm 96.96 Dynamic ArrayTM IFC genotyping
- 792 samples, approx. 900 clones, will each be typed for 192 iSNPs



Sample inlets
Samples (DNA)

Assay inlets
SNP assays
(primers)

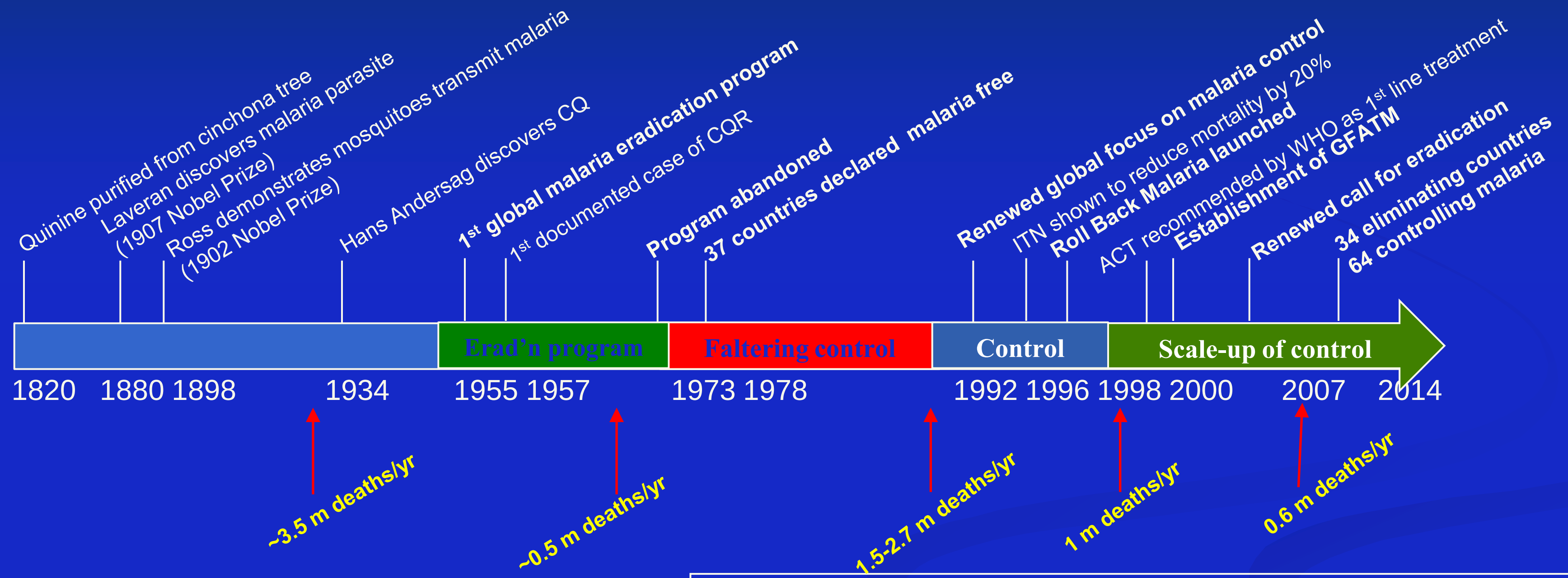
Work flow



Using our genotype barcode the plan is to map *P falciparum* population structure and then investigate dynamics

- *eg., flow between mainland and different Islands areas*
- *eg., the origins of strains involved in epidemic outbreaks in highlands areas.*
- *eg., origins of imported or locally acquired cases among the workforce of resource companies (mining companies)*

The global fight against malaria



Quinine purified from cinchona tree

Laveran discovers malaria parasite (1907 Nobel Prize)

Ross demonstrates mosquitoes transmit malaria (1902 Nobel Prize)

Hans Andersag discovers CQ

1st global malaria eradication program

1st documented case of CQR

Program abandoned

37 countries declared malaria free

Renewed global focus on malaria control

ITN shown to reduce mortality by 20%

Roll Back Malaria launched

ACT recommended by WHO as 1st line treatment

Establishment of GFATM

Renewed call for eradication

34 eliminating countries
64 controlling malaria

Erad'n program | **Faltering control** | **Control** | **Scale-up of control**

1820 1880 1898 1934 1955 1957 1973 1978 1992 1996 1998 2000 2007 2014

~3.5 m deaths/yr

~0.5 m deaths/yr

1.5-2.7 m deaths/yr

1 m deaths/yr

0.6 m deaths/yr

> *"We shall defend our island, whatever the cost may be, we shall fight on the beaches, … we shall never surrender."*
>
> *Churchill*

Global mortality has been reduced by 40%
3.3 million lives saved

52 countries are on track to reduce their malaria case incidence rates by 75%

But the effect of these efforts need to be monitored and maintained…

P. vivax, P. falciparum and acquired immunity

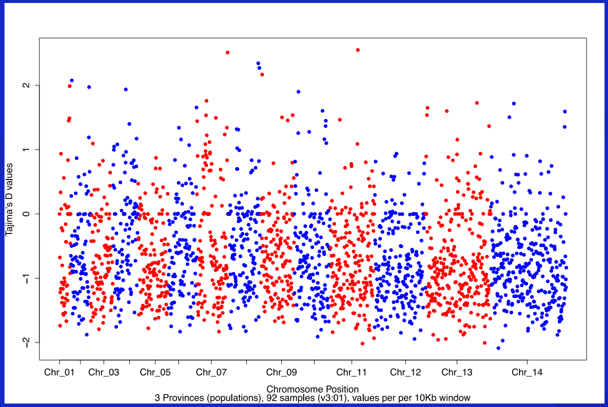
Accurate knowledge and understanding of a pathogen’s epidemiology and population structure is vital in any effort to control and eventually eliminate it.

Future direction: Chicken scratching

2. Investigate genetic signatures in the genome and changes in the population dynamics.

How have external forces, such as drugs and the introduction of bed nets, effected change in the genome and population structure:

- Bottle necks
- Expansions
- Genetic diversity
- Linked genetic characters
- Selection landscape



- Where? When? Why?

Chicken scratching's: Investigate signatures in the genome and changes in the population dynamics.

- Population dynamics and changes
  - Measure, map, and date population changes (Skyline plots)
  - 132 WGS, 3 sites
    - 15,000 nuclear SNPs - recombining
    - 6K Mitochondrion , 30-40KB Aplicoblast – non recombining
  - ~ 900 clones, nation wide
    - 192 Nuclear SNPs

- Multitude of programs ….
  - SNAPP , BEAST
  - Not all SNPs were created equal
    - *P. falciparum is* 70-90% AT rich
  - What's new toys are there in the play ground ?

Acknowledgements

PNG Institute of Medical Research
TO IMPROVE THE HEALTH AND QUALITY OF LIFE OF ALL PAPUA NEW GUINEANS

Madang team:

Livingstone Tavul,

Eline Kattenberg, Leanne Robinson

Inoni Betuela,

Goroka team:

Peter Siba, Jo Wapling, Justin Pulford

Sarah Javati, Lilah Tol, Elma Nate, Lincoln Timinao

Alotau team:

Albina Teliki, Tulaponi Setepano, Jennifer Gogomira, Karrol Sylvester, Thomas Mark, Gurney HC staff & CEO Billy Naidi of PHA Milne Bay Province;

Children & parents within the Gurney Catchment area,

Maprik Team:

Dorish Walsh, Nathan Yalabus, Robert Gitimbu, Lindy Markem, Lawrence Rare

FIAT LUX
Walter+Eliza Hall
Institute of Medical Research

Alyssa Barry,

Barry and Mueller group

Celine Barnadas, Mita Hapsari Hazairin,

Sofonias Tessema, Connie Li Wai Suen

Anita lerch

Melanie Bahlo

Bahlo group

Natacha Tessier

Systems Biology

Liz Miller

MalariaGEN
GENOMIC EPIDEMIOLOGY NETWORK

Dominic Kwiatkowski,

Magnus Manske

Olivo Miotto

Plasmodium population structure in PNG work flow

- Sequence whole genomes of samples collected across the north coast of Papua New Guinea (PNG)
- Identify 192 putatively biogeographically informative single nucleotide polymorphisms (SNPs)
- SNP type single clone *Plasmodium* infections collected from 17 provinces across PNG
  - Collected as part of Malaria control program (MalCon)
- Develop barcodes from most informative SNPs
- Investigate underlying biological questions from genomic data and apply to National data set (MalCon)

Population structure of P. falciparum

Identifying geographically informative SNPs

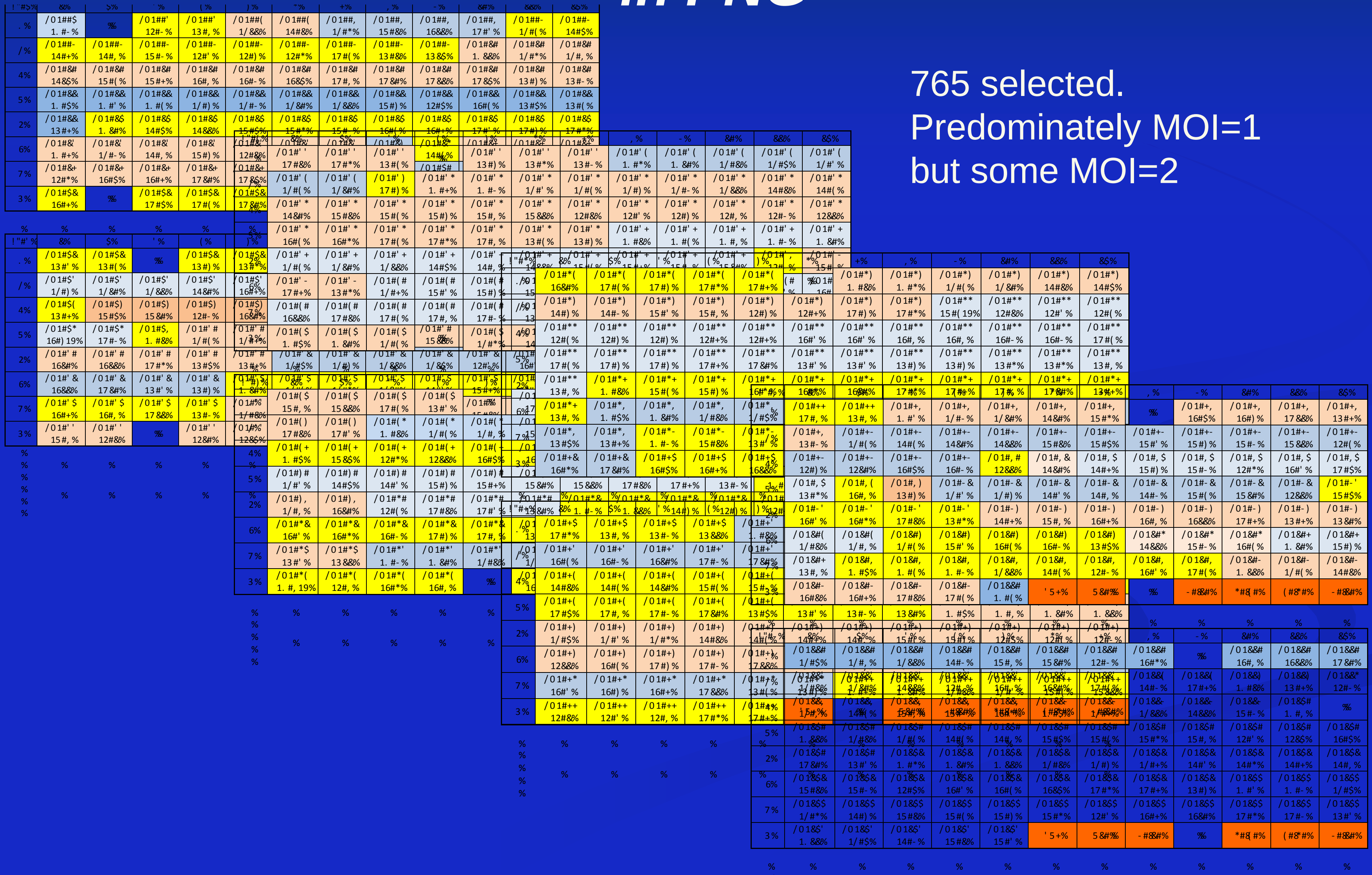
WANTED SMART SNPs

From the 700k high quality bialleic SNPs we will identify and select ~200 that are geographically informative to screen our population sample set with.

- Minor allele frequency (MAF) ≥0.1 in PNG
- SNPs evenly distributed through the genome i.e., not linked or hitchhiking
- SNPs not under selection pressure or balancing pressure
    - Evolving neutrally (synonymous)
    - Tajima's D test
- logistic regression
- High F_{ST} values
- SNPs demonstrating population structure

Pf Fluidigm samples plated and pre-amped in PNG

765 selected.
Predominately MOI=1
but some MOI=2



iii. to apply these tools to identify sources of outbreaks where previously unknown

Using our Genotype barcode we will investigate and characterize

- Samples collected at healthcare facilities in locations where imported or epidemic malaria is suspected

- Origin of imported or locally acquired cases among the workforce of resource companies

  - 200 odd samples from Exon

- Gene-flow between mainland and different Islands areas

- The origins of strains involved in epidemic outbreaks in highlands areas.

Depending on who you’re talking to merge the intro of Pf pop structure in PNG into one slide with multiple references and “pop outs” to describe each paper.

It’s not about “numbers” but it is about what their story tells.

NEED TO INCLUDE BACK GROUND OF POPULATION STRUCTURE OF other MALARIA IN PNG NOT JUST PF AND

POP STRUCTURE OF PF ELSE WHERE TO GIVE BACKGROUND ABOUT POSSIBLE STRUCUTRE

AND THE MANY PURPOSES OF WHY WE DO THIS – DRUG STUDY EVALUATION, POP STRUCURE, EVOLUTION, SELECTION PRESSURE, SELECTIVE SWEEPS,

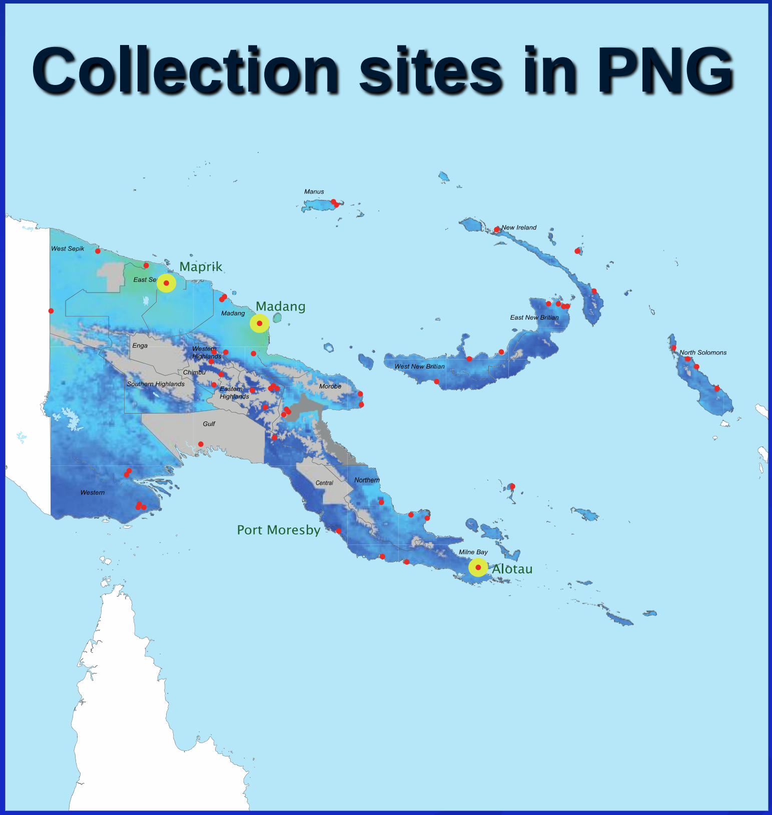
Other examples of pop strucutre – drug resistance marker and that 24 SNP barcode

ii. to develop tools for molecular surveillance

***BedNet* screening 2008-9**

Bill and Melinda, LLIN and RDTs
Malaria Control assessment

- 17 Provinces, 81 villages, 13,000 samples
~ 2500 P. falciparum infections
~ 1000 single (monoclonal) infections


Collection sites in PNG

● Genotyping (SNPs)

To apply these tools to identify sources of outbreaks

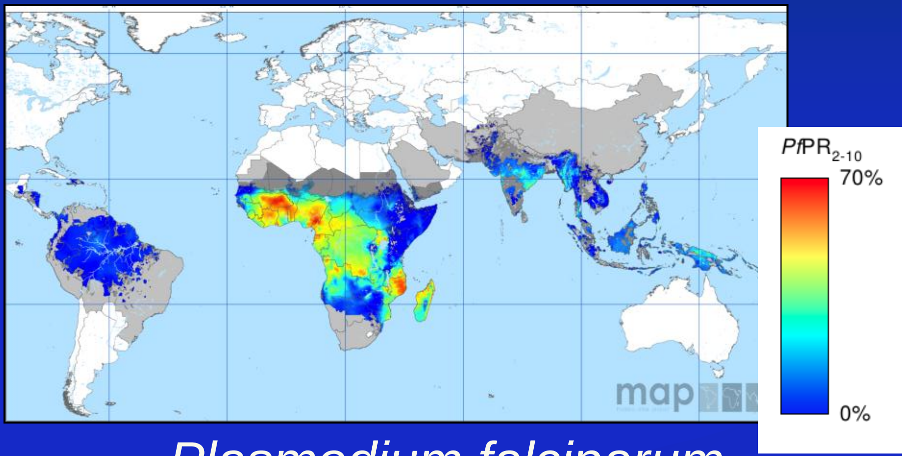
- iSNPs we will genotype a nation wide data set of P. falciparum samples and map the population structure of P falciparum across PNG
- The national geographic informative SNPs will be identified and a barcode produced.
- Our Genotype barcode will have the power to investigate and characterize:
    - Suspected imported malaria cases
    - P. falciparum migration and gene flow
    - The origins of strains involved in epidemic outbreaks in highlands areas.

The long term goal being to be able to genotype isolates from a few select SNPs and map outbreaks and local epidemics.

Malaria: Global burden

- Burden estimates
  - 219 million clinical cases
    - (CI 154 million to 289 million)
  - 660 000 deaths
    - (CI 490 000 to 836 000).

(accurate estimates of prevalence and deaths are difficult to establish due to limitations in reporting.)

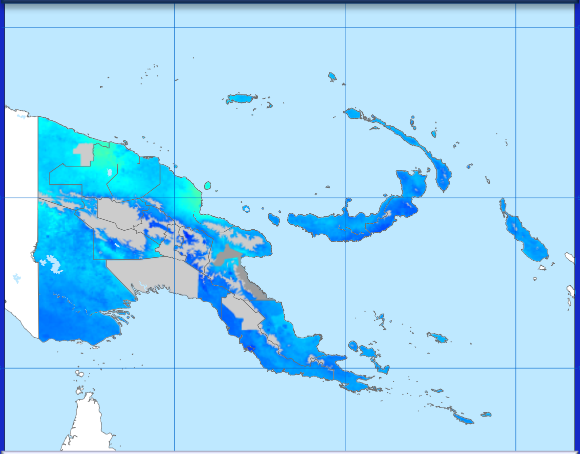


Plasmodium falciparum

- Has been eliminated from:
  - Australia, Europe, North America - well mostly
- DDT elimination attempts in the 1960s in developing countries not successful
- Over the last decade there has been a heightened push for control and elimination
  - bed nets, new drugs and vaccines

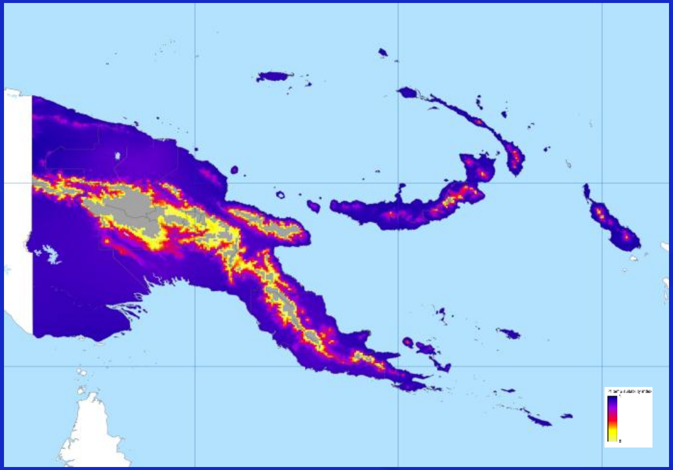
Malaria and PNG

Papua New Guinea (PNG) has the highest burden of malaria outside Africa ranging from holo-endemic, to endemic, to absent.



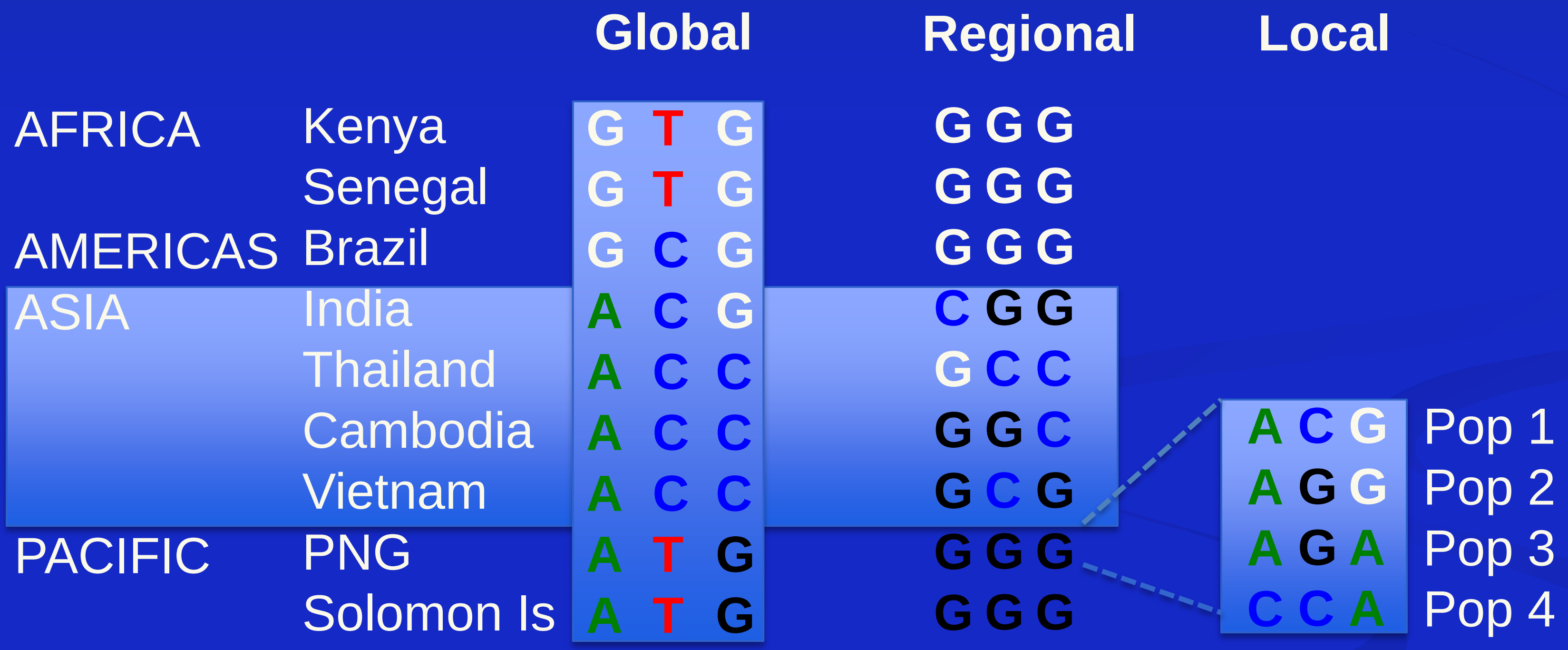
PNG P. falciparum prevalence in 2-10 year olds 2010.

Oxford Malaria Atlas project (http://www.map.ox.ac.uk/browse-resources/)



Temperature suitability index for P. falciparum transmission in PNG

Geographically informative SNPs



		Global	Regional
AFRICA	Kenya	G T G	G G G
	Senegal	G T G	G G G
AMERICAS	Brazil	G C G	G G G
ASIA	India	A C G	C G G
	Thailand	A C C	G C C
	Cambodia	A C C	G G C
	Vietnam	A C C	G C G
PACIFIC	PNG	A T G	G G G
	Solomon Is	A T G	G G G

Local	
A C G	Pop 1
A G G	Pop 2
A G A	Pop 3
C C A	Pop 4

Development of geographic “barcodes”