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APPENDICES

APPENDIX A

Reagent for agarose gel electrophoresis

1. 50x Tris-acetate buffer (TAE)

Tris base	424.0	g
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	100	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

2. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the solution and store in the dark at 4°C.

3. 1.5% Agarose gel

Agarose	0.3	g
1x TAE	20	ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

4. 5x Loging buffer 100 ml

Tris HCL	0.6	g
EDTA	1.68	g
SDS	0.5	g
Bromphenol Blue	0.1	g

Sucrose	40	g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5 microtube and store at 4°C.

APPENDIX B

Reagent for DNA extraction

1. Red Cell Lysis Buffer (RCLB)

NH_4Cl	1.875	g
Tris-HCl	0.25	g

Dissolve NH_4Cl and Tris-HCL in 500 ml of distilled water. Adjust pH to 7.2. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated. Shelf life is approximately 6 months.

2. Nuclei Lysis Buffer (NLB)

1 M Tris (pH 8.0)	10	ml
5 M NaCl	0.5	ml
0.5 M EDTA (pH 8)	0.4	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.

3. 1 M Tris

Tris base	12.11	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

4. 5 M NaCl

NaCl	29.22	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

5. EDTA

EDTA	37.22	g
Distilled water	200	ml

Adjust volume to 200 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated.

6. 5.3 M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

7. Proteinase K 10 mg/ml

Proteinase K	100	mg
Distilled water	10	ml

Mix the solution and store at -20°C.

8. 10% SDS

SDS	10	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

APPENDIX C

Haplotype analysis by PHASE

PHASE program is produced by the Mathematical Genetics Group, University of Oxford, Oxford, UK. The software is available online at http://www.stats.ox.ac.uk/mathgen/ (Stephens and Donnelly 2003).

Input file format

The input file is supplied by the user to specify how many individuals there are to be analysed, how many loci/sites each individual has been typed at, what sort of loci/sites these are (SNP or microsatellite), and the genotypes for each individual. The default format input file, as illustrated in the accompanying file test.inp. The default structure for the input file can be represented as follows:

NumberOfIndividuals
NumberOfLoci
P Position(1) Position(2) Position(NumberOfLoci)
LocusType(1) LocusType(2) ... LocusType(NumberOfLoci)
ID(1)
Genotype(1)
ID(2)
Genotype(2)
:
:
:
ID(NumberOfIndividuals)]
Genotype(NumberOfIndividuals)

Where the quantities above are as follows:

- Number Of Individuals An integer specifying the number of individuals who have been genotyped.

- Number Of Loci An integer specifying the number of loci or sites at which each individual has been typed.

- P The character 'P' (upper case, without quotation marks).

- Position(i) A number indicating the position of locus i, relative to some arbitray reference point (typically in units of base pairs, but any units can be used: if you use a unit other than base pairs, see the documentation on the -R option). The loci must be in their physical order along the chromosome (ie these Positions must be increasing).

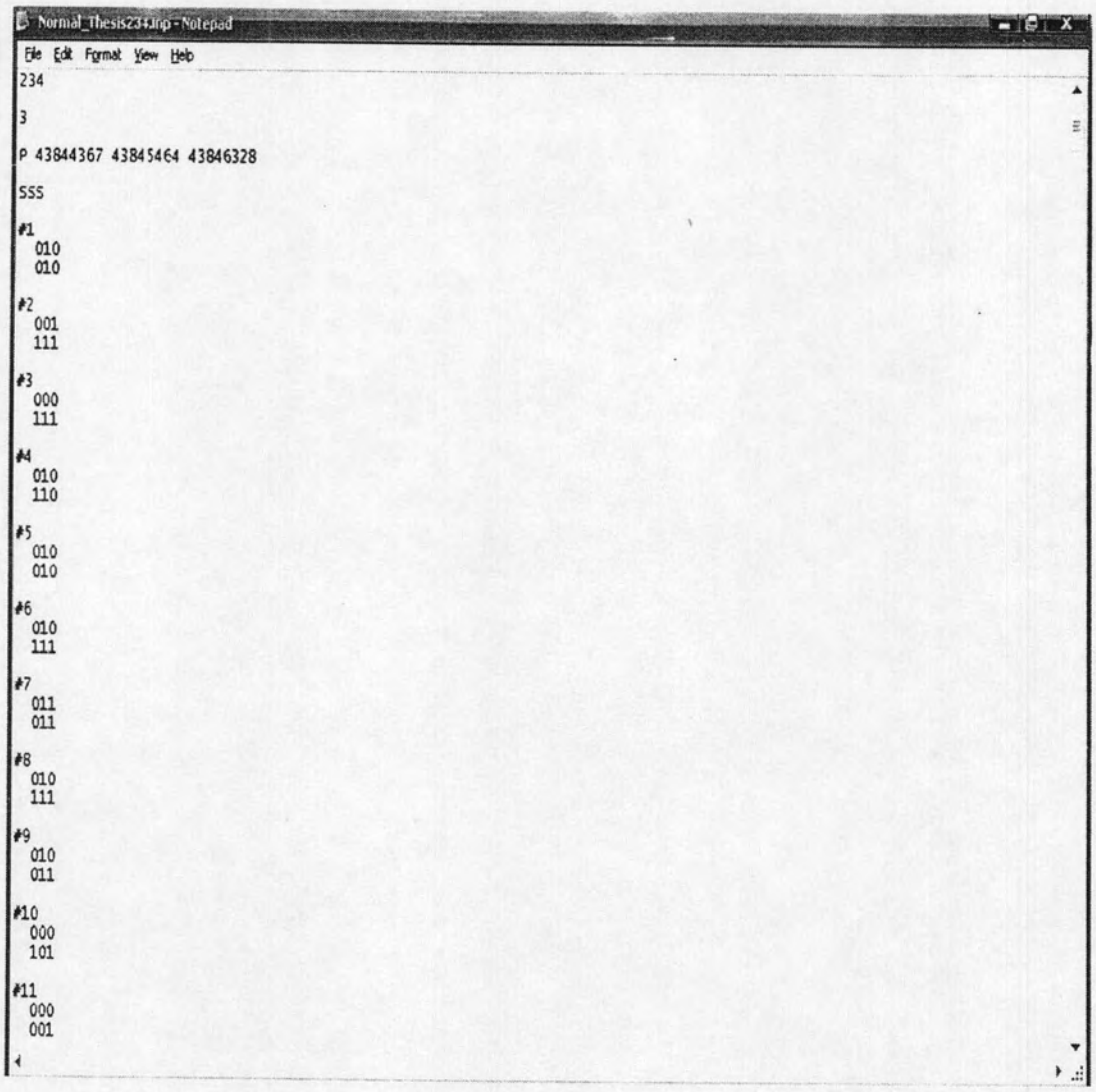
- LocusType(i) A letter indicating the type of locus i. The options are (a) S for a biallelic (SNP) locus, or biallelic site in sequence data. (b) M for microsatellite, or other multi-allelic locus (eg triallelic SNP, or HLA allele). The default assumption is that this denotes a microsatellite locus with stepwise mutation mechanism.

- ID(i) A string, giving a label for individual i.

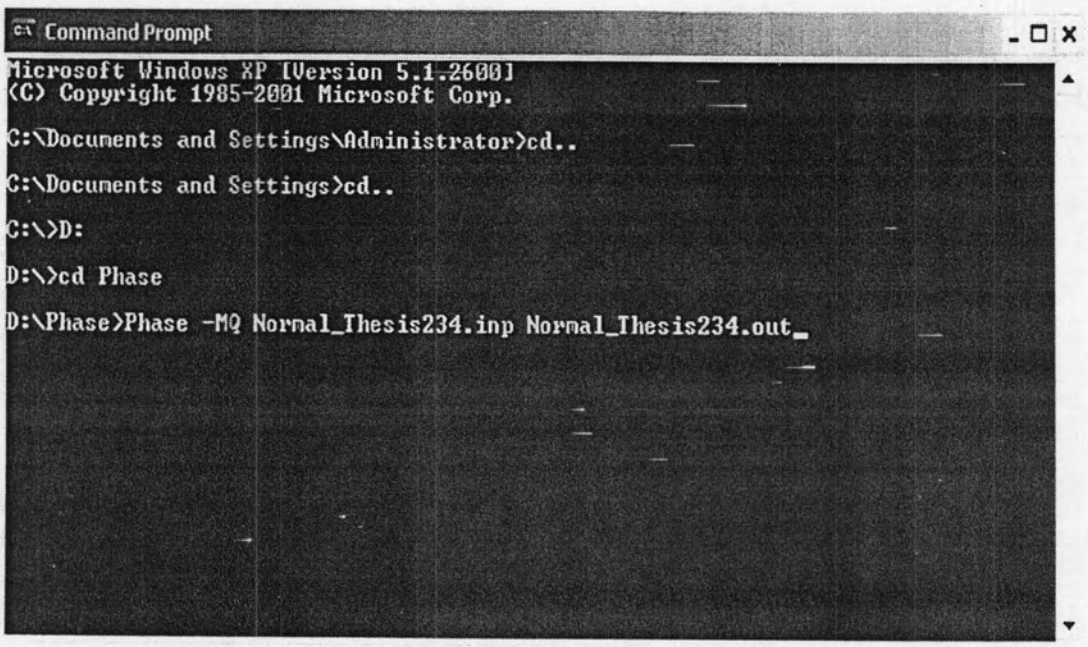
- Genotype(i) The genotypes for the ith individual. This is given on two consecutive rows. At each locus, one allele is entered on the first row, and one on the second row. It does not matter which allele is entered on each row. For biallelic loci, any two characters (e.g. A/C, G/T, 0/1) can be used to represent the two alleles, and they do not need to be separated by a space. Missing alleles at SNP loci should be entered as ?. For multiallelic loci a positive integer must be used for each allele (representing the number of repeats at microsatellite loci), and data for each locus should be separated by aspace. Missing alleles at multiallelic loci should be represented by -1.

This study, consider the example input file, Normal_Thesis234.inp, which is as follows:

The example of input file (Normal_Thesis234.inp) was shown below.



Running of PHASE program was shown below.



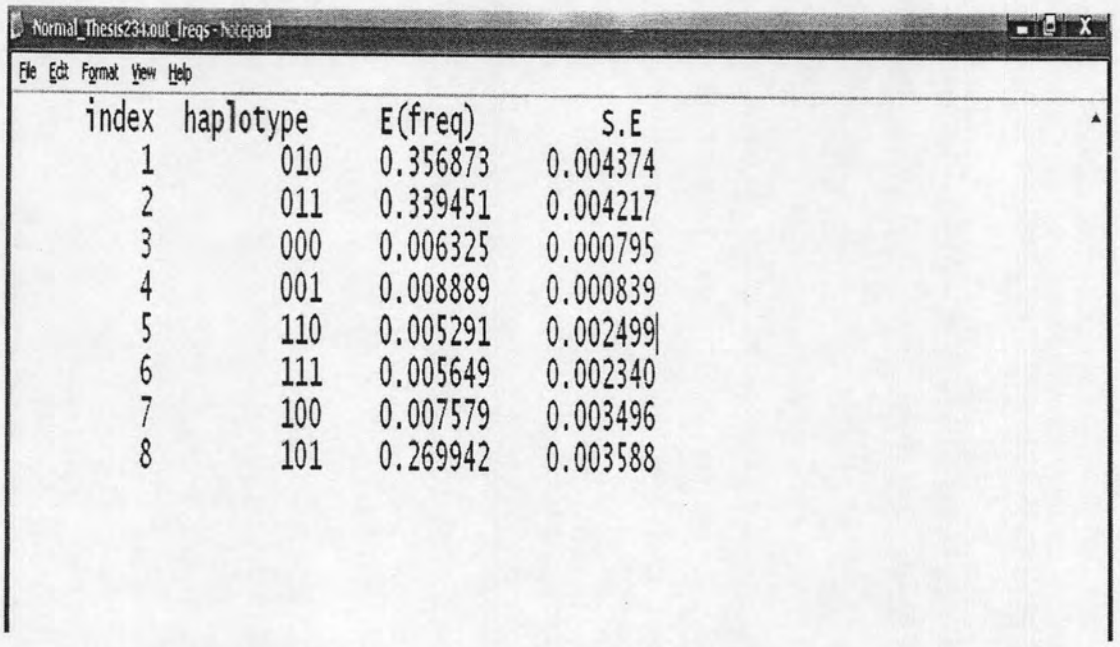
Output file

When run, the program initially outputs the data it has read from the input file. The program produces a number of output files. The first, which has the user-specified name, and a similar format to previous versions of PHASE, contains a summary of the individual haplotype estimates for each individual.

Type of output file, which were used in this study, consists of two types.

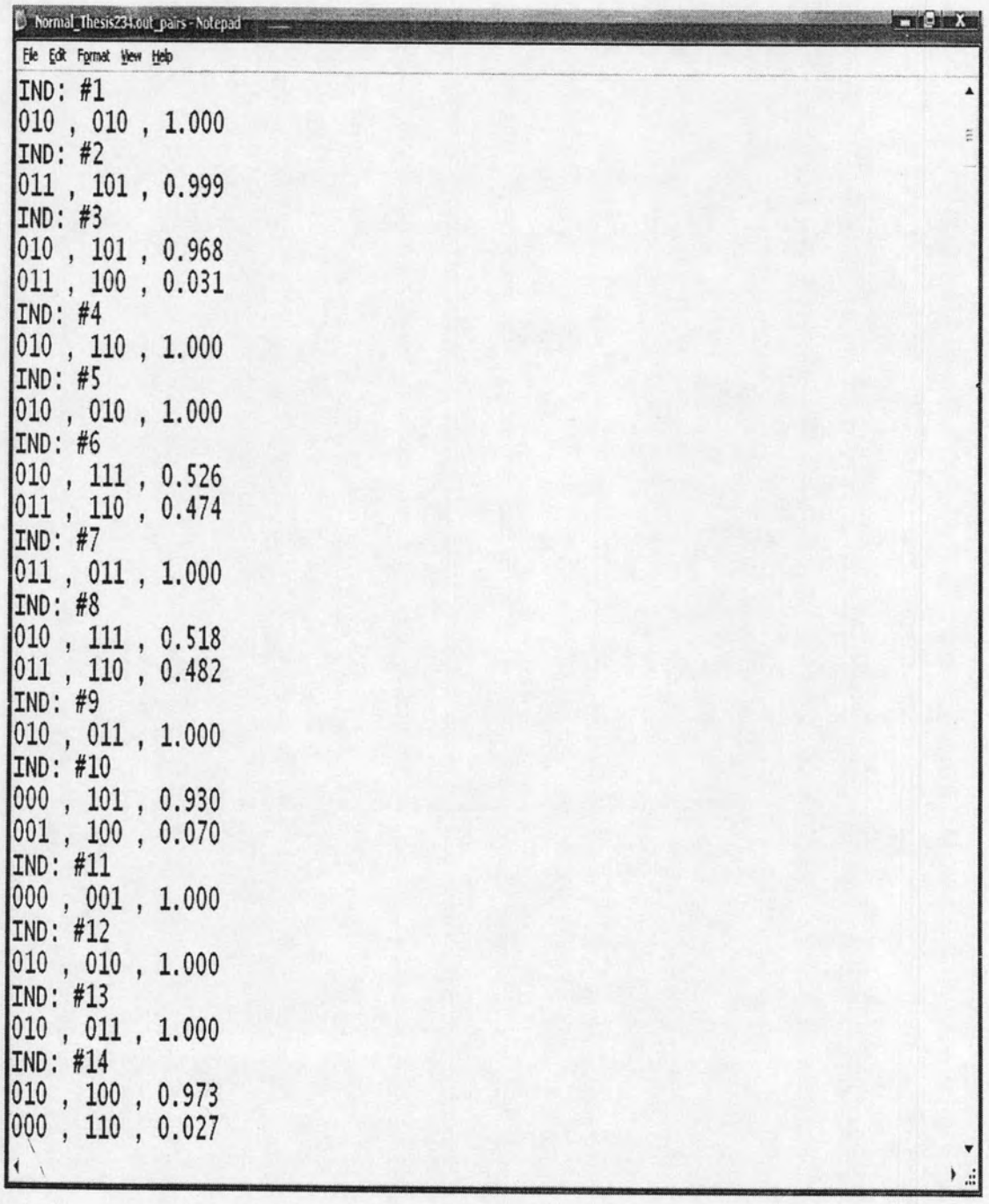
1. Output frequencies : defined as how many haplotype in this study group and used the number (frequencies) to estimate how many the people, who have these haplotypes.

The example of output file (output frequencies) was shown below.



2. Output pairs : defined as the individuals haplotype

The example of output file (outputpairs) was shown below.



APPENDIX D

Linkage Disequilibrium Analysis

The LDPlotter Tool allows conversion of a Nickerson Lab prettybase format file into a plot showing pairwise LD of various type (r^2 , r, D and abs(D)). r^2 values are calculated using an iterative EM algorithm taken from " Estimation of LD in randomly mating populations", WG Hill, Heredity: 33(2), 229-239 (1974).

The software is available online at http://innateimmunity.net/IIPGA2/Bioinformatics/

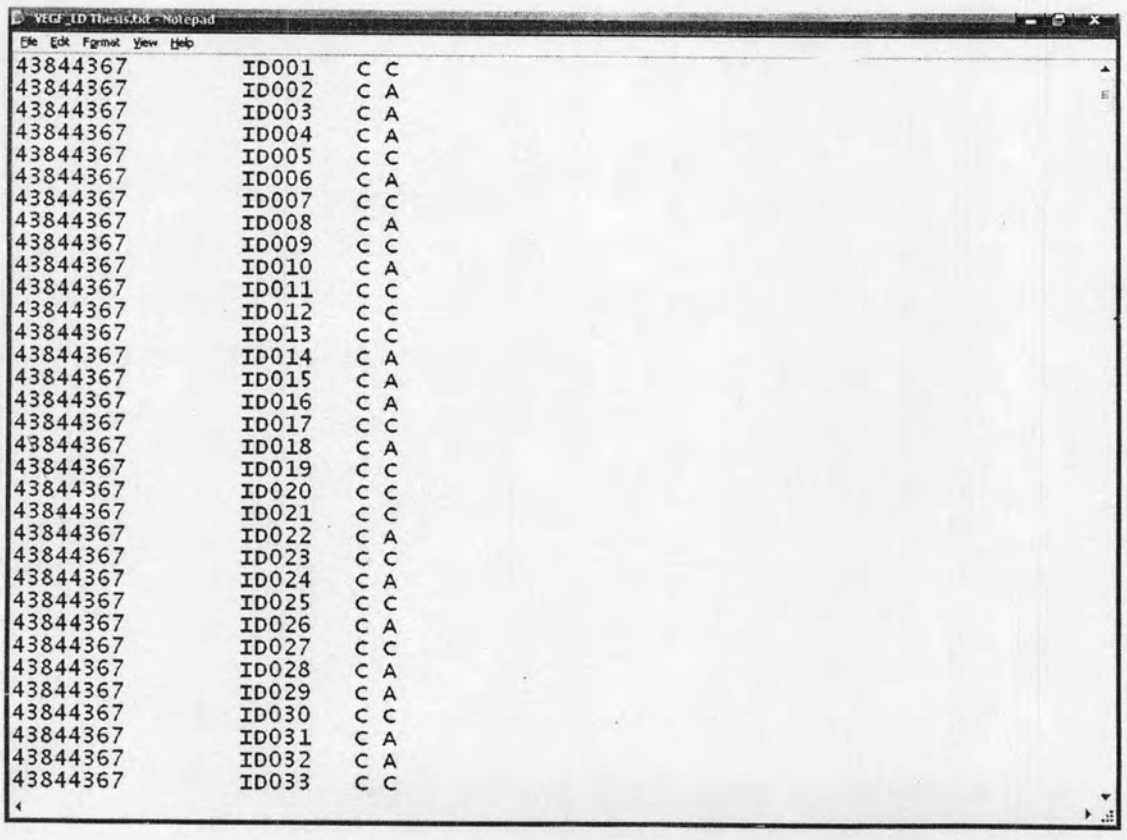
I Genename

II Prettybase

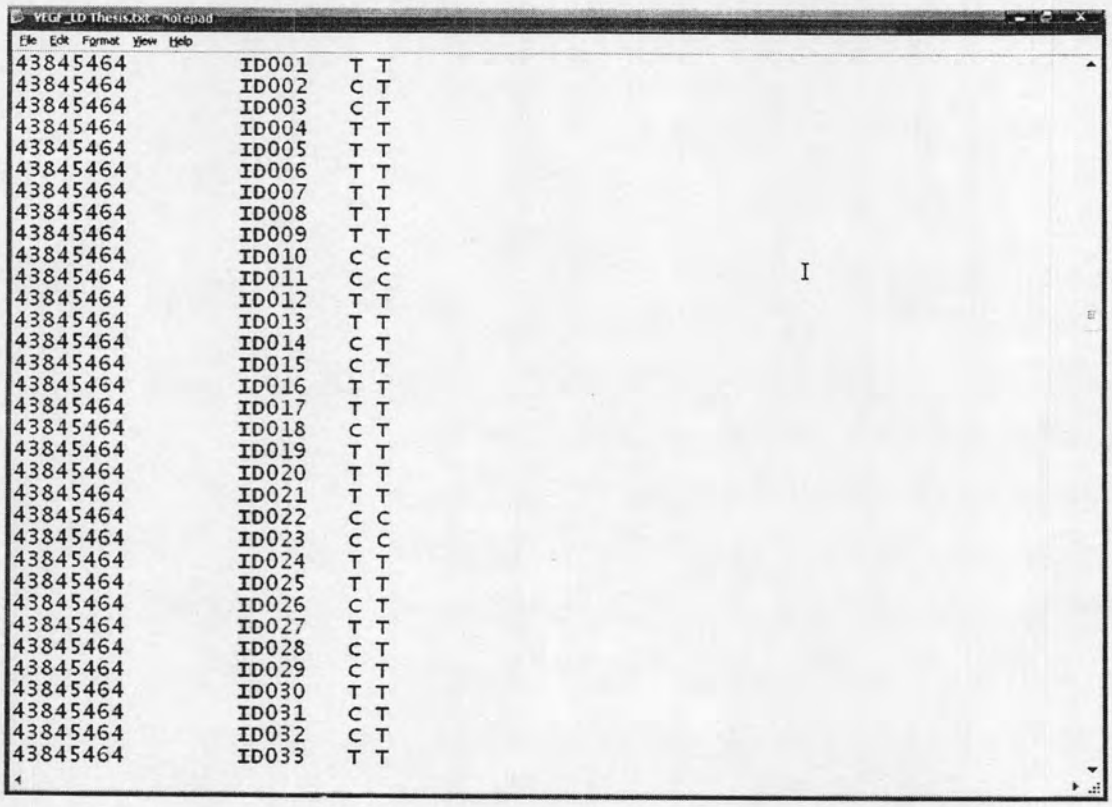
Input is a standard prettybase file. This study, consider the example input file, TNF.LD.txt, which is as follows:

The example of input file (VEGF_LD Thesis.txt) was shown below.

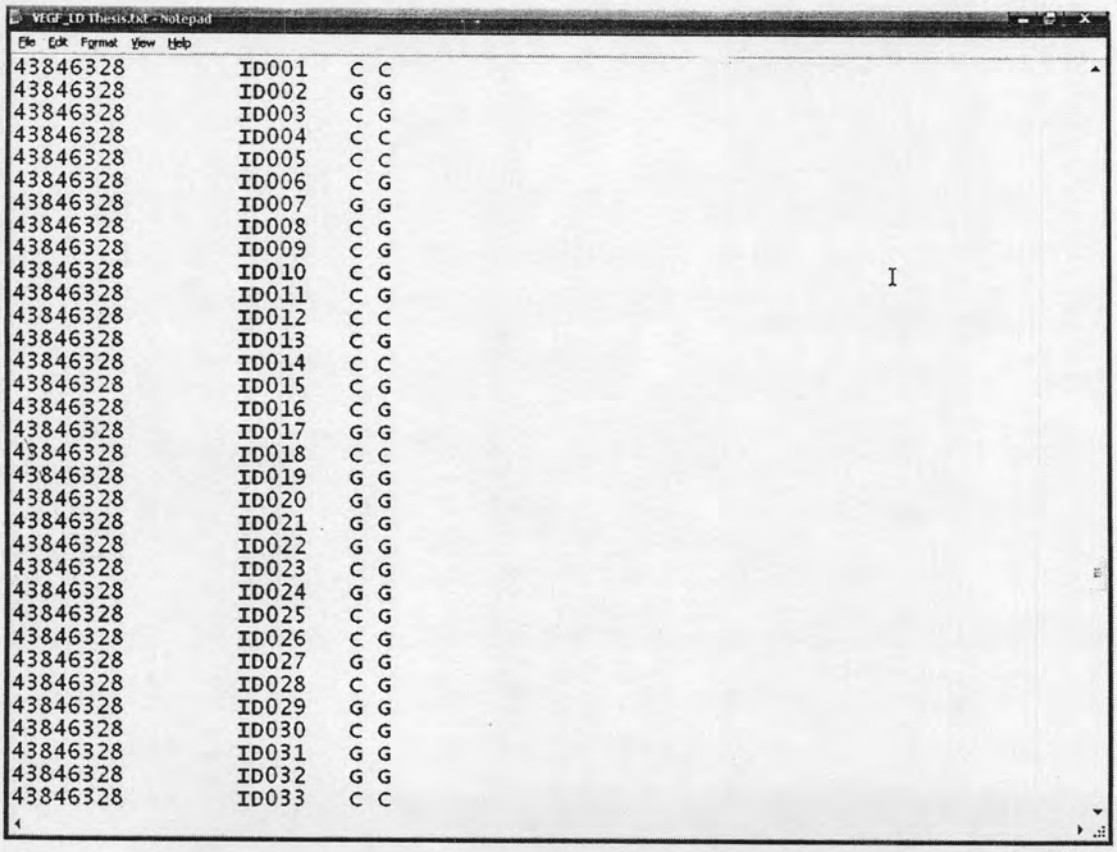
VEGF (-1557C/A)



VEGF (-460C/T)



VEGF (+405C/G)



III. LD Type

Indicates which measure of LD would like to plot

- ☐ $r^2 = D^2/P_A*P_B*(1- P_A)*(1- P_B)$
- ☐ $r=sqrt(r^2)$
- ☐ $D' = D/D_{max}$
- ☐ $Abs(D)= \left| P_{AB} - (P_A*P_B) \right|$

IV. Configure Populations

This text area allows you to configure how the LD Plotter will split the sample in the field should have one line for each population represented in dataset. Each line should be a population identifier, followed by a colon, followed by a description of the population. Leaving this text area empty will indicate that not wish to partition sample set, but instead, would like to consider all of the samples to be part of a single population.

V. Plot Title

The plot title has two modes of operation, one for simple use, and the other for more advanced purposes for users who are familiar with Phython format strings, and would to specify exactly what the plot title should look like.

- **Simple**: With a simple plot title, we begin with an arbitrary string such as: Pairwise LD and then add information about the plot to the heading:
  1. ☐ Append Gene name to plotTitle: The gene name entered above

     will to the title of the plot.
  2. ☐ Append Population to plotTitle: Each plot will be labeled with the population. If you are splitting your dataset by populations using the **Population Configuration** above, it is highly recommend that you keep this option checked,

otherwise you will not know which plot belongs to which population.

3. ☐ Append LD type to plotTitle: The type of LD measure calculated will be displayed in the plot title.
4. ☐ Appened minfaf to plotTitle: The minraf used for the run will be displayed in the plot title.

- **Advanced**: The advanced title option allows you to input the plot title as a legal python format string. This format string is evaluated against a dictionary of variables/values (plotTitle% variables). At the current time, the dictionary is populated with the variables *miniraf, population, and ldtype*. These are the same variables which are available in the "Simple" option above, but you have the flexibility of arranging the items however you would link in the title.

Errors in the title will be displayed in the title itself if at all possible.

VI. Miscellaneous

- **Miniraf**: You can set an arbitrary threshold for minimum allele frequence. The default value of 0.0 will not exclude SNPs based on rare frequency. If you specify a higher value, SNPs with frequency for the rare allele below this threshold in any for the rare population will NOT appear in the plot.
- **Color Scheme**: Several color schemes can be used to indicate the extend of LD between two loci.

☐ **Circle**: The extend of LD between two loci is draw by default as a colored square at the intersection point of the two loci.

☐ **SNP Map**: The SNPs map is a representation of the gene running along the diagonal of the half matrix plot which show the relative position of each SNP locus in the gene. It is only useful if you are using SNP IDs which directly correlate the position of the SNP in the gene.

☐ **Full-matrix**:

☐ **Numerical:** This option allowed you to download a spreadsheet of LD *values*

instead of a graphical plot. This is useful if you would like to process the infprmation using another program, or if you have another plotting program.

☐ **Download:** The download option will indicate that instead of displaying the result in the browser window, you would like to be prompted for a location where the fill will be saved on your computer.

The output file: The output file was shown below.

VEGF-LD.txt - Notepad

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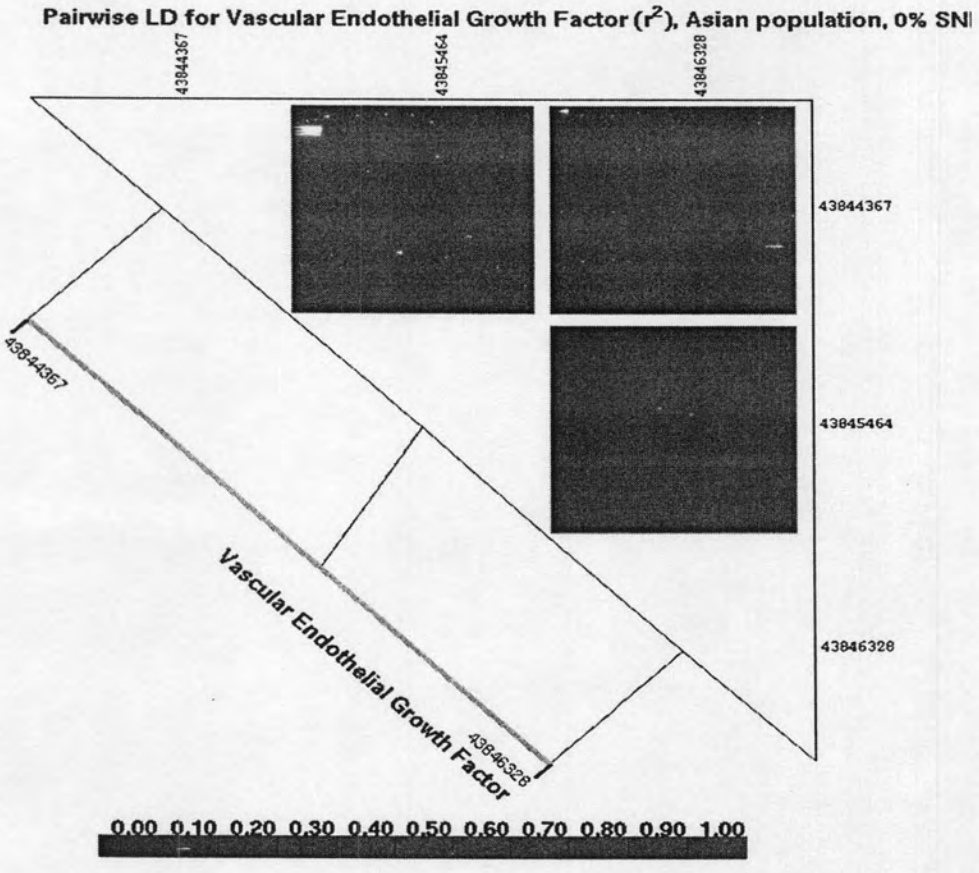
```
Pairwise LD values for sample = ID
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    r2
2  1  2  2  234   232   231   0.0   0.0   0.8780   0.8780
3  1  2  2  234   232   231   0.0   0.0   0.1963   0.1963
3  2  2  2  234   232   231   0.0   0.0   0.1746   0.1746


Pairwise LD values for sample = ID
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    r
2  1  2  2  234   232   231   0.0   0.0   0.8780   0.9370
3  1  2  2  234   232   231   0.0   0.0   0.1963   0.4431
3  2  2  2  234   232   231   0.0   0.0   0.1746   0.4178


Pairwise LD values for sample = ID
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    D'
2  1  2  2  234   232   231   0.0   0.0   0.8780   0.9468
3  1  2  2  234   232   231   0.0   0.0   0.1963   0.8963
3  2  2  2  234   232   231   0.0   0.0   0.1746   0.8365


Pairwise LD values for sample = ID
M1   M2 al1 al2     N df(0) df(1)   ChiSq  pvalue delta2    |D|
2  1  2  2  234   232   231   0.0   0.0   0.8780   0.1932
3  1  2  2  234   232   231   0.0   0.0   0.1963   0.0972
3  2  2  2  234   232   231   0.0   0.0   0.1746   0.0921
```

Linkage disequilibrium coefficients ($|D'|$ and r^2) among VEGF SNPs

		$\|D'\|$		
		-1557	-460	+405
r^2	-1557	-	0.9468	0.8963
	-460	0.8780	-	0.8365
	+405	0.1963	0.1746	-

In this study, linkage disequilibrium coefficients (ID'I and r^2) was not found among -1557(C/A), -460(C/T) and +405(C/G) of VEGF promoter and Exon polymorphisms.

The output figure r^2 : The output figure r^2 (total 3 position.txt) was shown below.



APPENDIX E

Exact Hardy-Weinberg Equilibrium Test

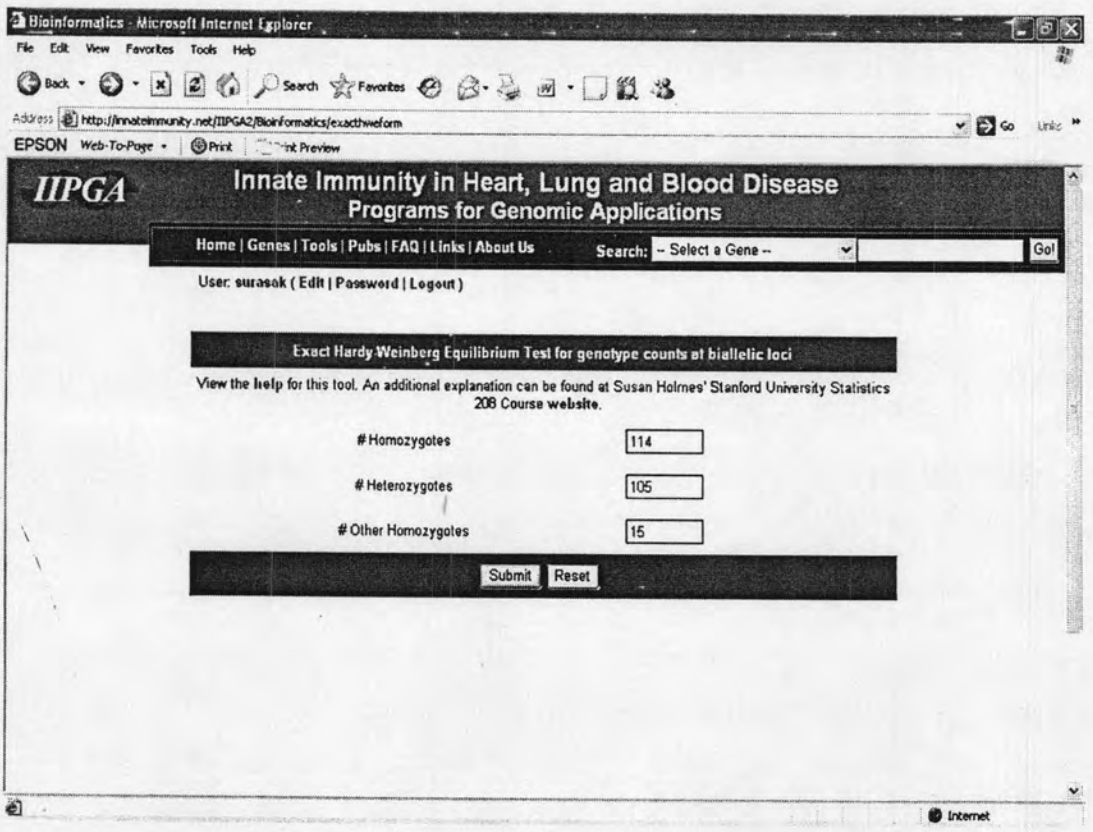
Exact Hardy-Weinberg Equilibrium Test for genotype counts, biallelic locus. This program will estimate the statistical significance of a test that the genotype counts are in Hardy-Weinberg equilibrium.

The software is available online at http://innateimmunity.net/IIPGA2/Bioinformatics/.

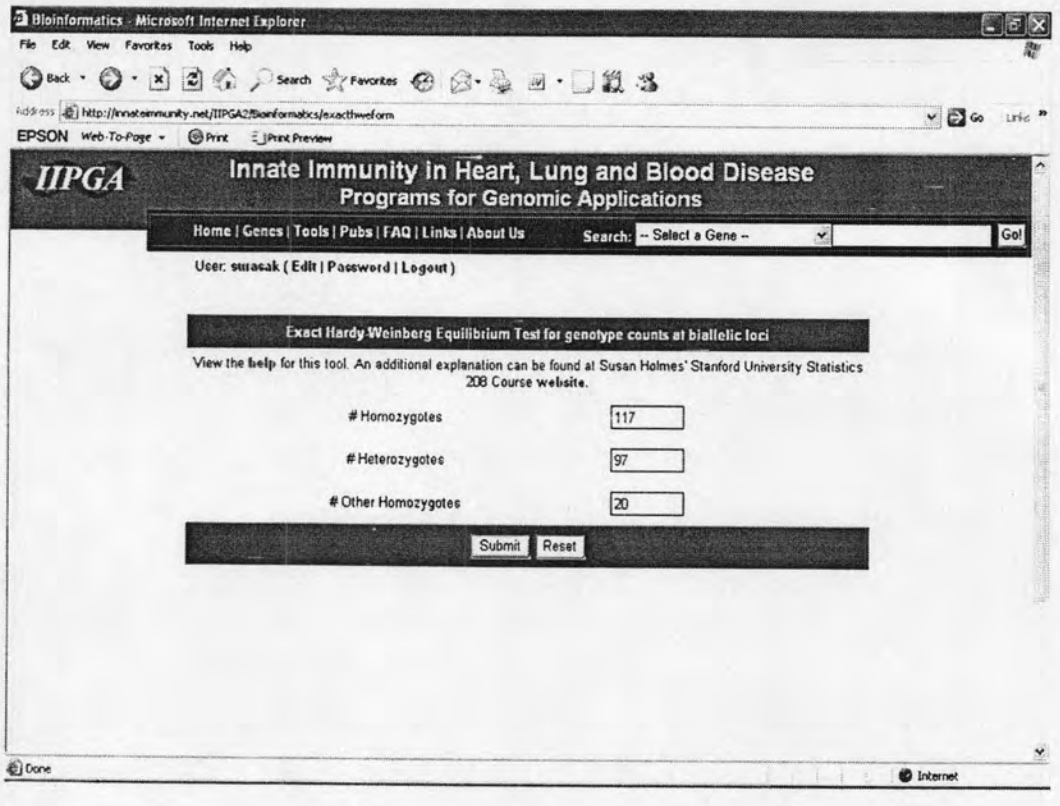
I Counts

The input to this tool is simply three interger counts representing the number of heterozygotes, common homozygotes, and rare homozygotes at a given locus in your data set.

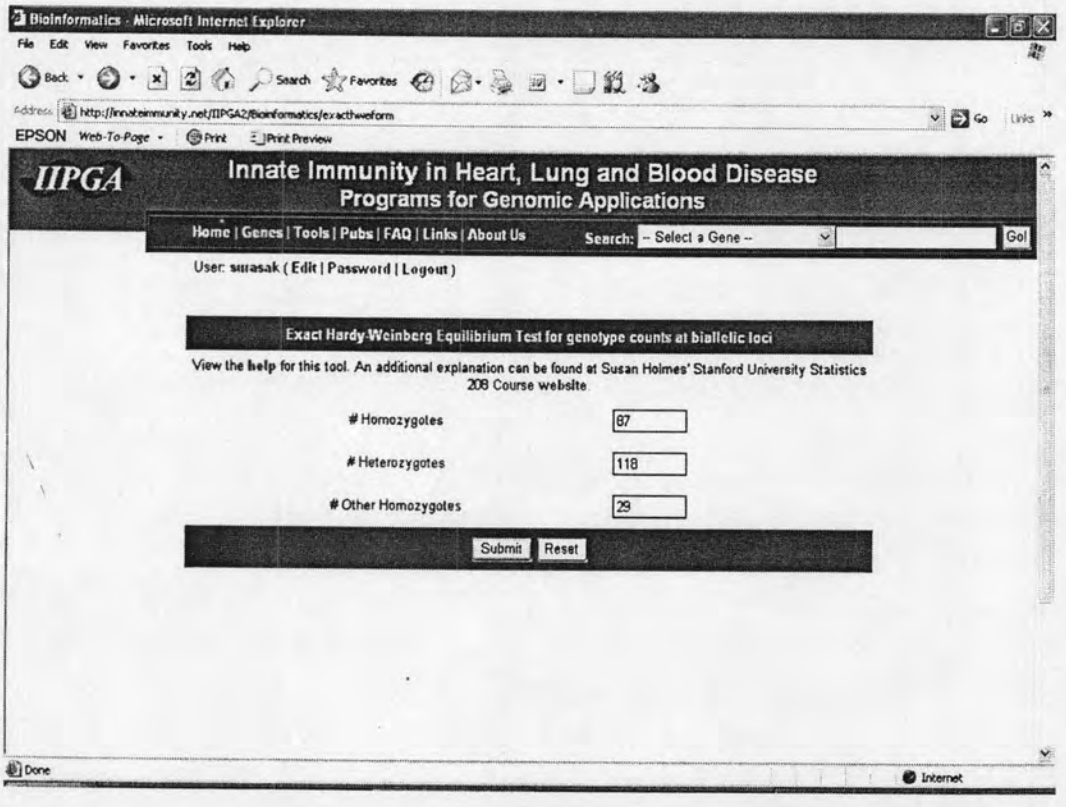
This study, the genotype counts of VEGF (-1557C/A) was shown below.



The genotype counts of VEGF (-460C/T) was shown below.

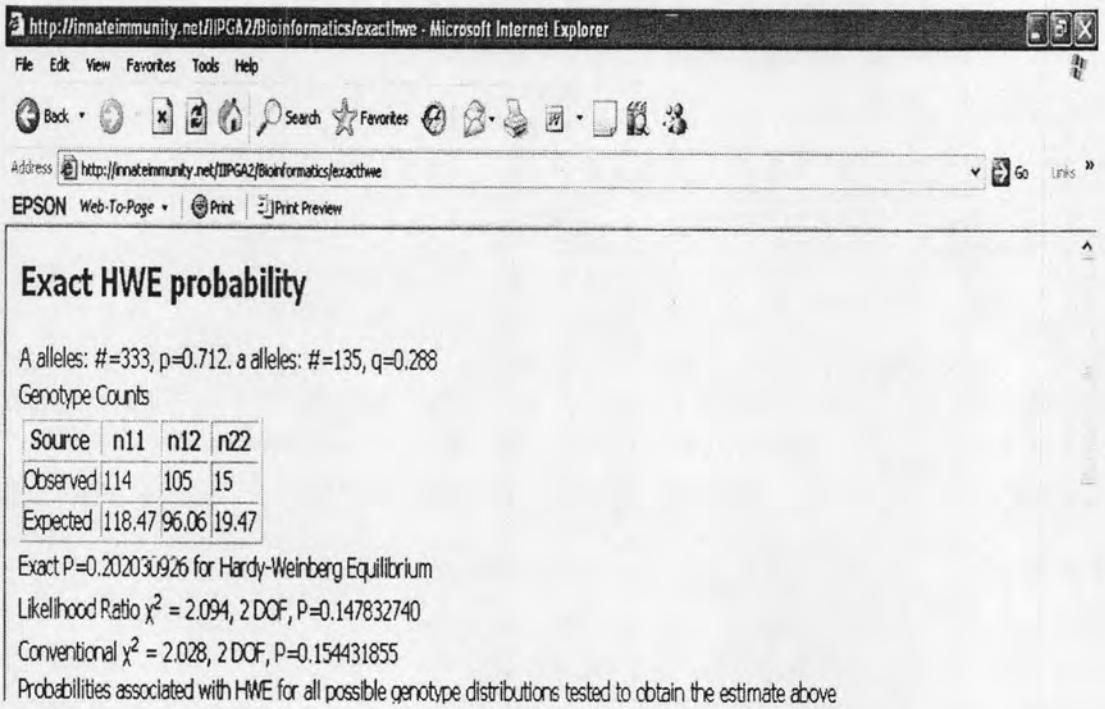


The genotype counts of VEGF (+405C/G) was shown below.



When run, the program will estimate the statistical significance of a test that the genotype counts are in Hardy-Weinberg equilibrium. The output file of VEGF (-1557C/A) was shown below.

The output of the genotype counts of VEGF (-1557C/A) was shown below.



http://innateimmunity.net/IIPGA2/Bioinformatics/exacthwe - Microsoft Internet Explorer

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Exact HWE probability

A alleles: #=333, p=0.712. a alleles: #=135, q=0.288

Genotype Counts

Source	n11	n12	n22
Observed	114	105	15
Expected	118.47	96.06	19.47

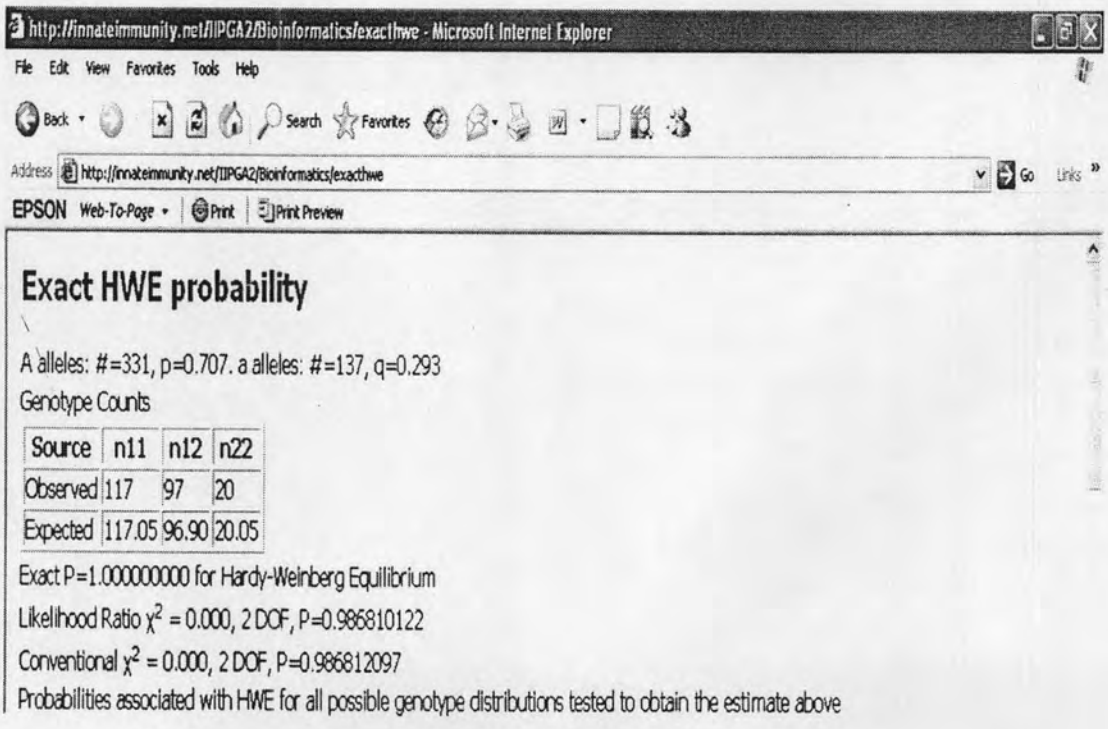
Exact P=0.202030926 for Hardy-Weinberg Equilibrium

Likelihood Ratio χ^2 = 2.094, 2 DOF, P=0.147832740

Conventional χ^2 = 2.028, 2 DOF, P=0.154431855

Probabilities associated with HWE for all possible genotype distributions tested to obtain the estimate above

The output of the genotype counts of VEGF (-460C/T) was shown below.



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Exact HWE probability

A alleles: #=331, p=0.707. a alleles: #=137, q=0.293

Genotype Counts

Source	n11	n12	n22
Observed	117	97	20
Expected	117.05	96.90	20.05

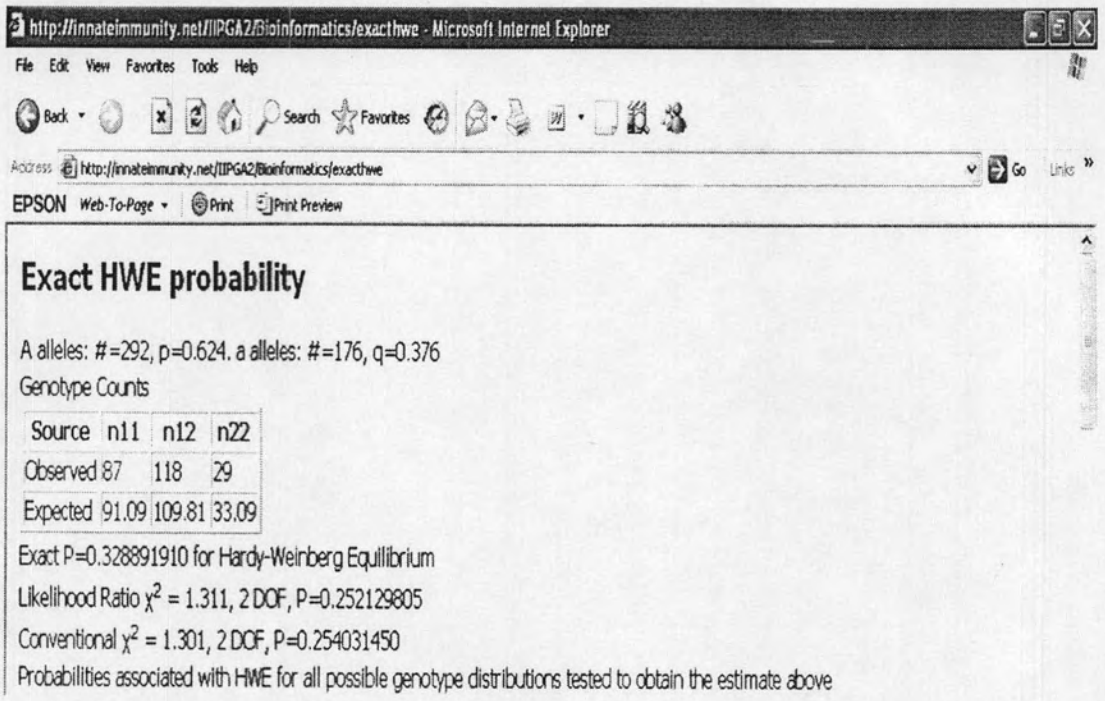
Exact P=1.000000000 for Hardy-Weinberg Equilibrium

Likelihood Ratio χ^2 = 0.000, 2 DOF, P=0.986810122

Conventional χ^2 = 0.000, 2 DOF, P=0.986812097

Probabilities associated with HWE for all possible genotype distributions tested to obtain the estimate above

The output of the genotype counts of VEGF (+405C/G) was shown below.



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Exact HWE probability

A alleles: #=292, p=0.624. a alleles: #=176, q=0.376

Genotype Counts

Source	n11	n12	n22
Observed	87	118	29
Expected	91.09	109.81	33.09

Exact P=0.328891910 for Hardy-Weinberg Equilibrium

Likelihood Ratio χ^2 = 1.311, 2 DOF, P=0.252129805

Conventional χ^2 = 1.301, 2 DOF, P=0.254031450

Probabilities associated with HWE for all possible genotype distributions tested to obtain the estimate above

BIOGRAPHY

Mr Surasak Yooyongsatit was born on March 20, 1982 in Kanchanaburi, Thailand. He graduated Bachelor of Science (Biology), Faculty of Science, Silpakorn University in 2003, and attended to particulate in Medical Microbiology program, Graduate School, Chulalongkorn University for his master degree.