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## APPENDICES

## APPENDIX A

### BUFFERS AND REAGENT

#### 1. Lysis Buffer I

Sucrose	109.54	g
1.0 M Tris – HCl (pH 7.5)	10	ml
1.0 M MgCl <sub>2</sub>	5	ml
Triton X – 100 (pure)	10	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store in a refrigerator (at 4<sup>0</sup>C).

#### 2. Lysis Buffer II

5.0 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

#### 3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to	100	ml

Mix the solution and store at room temperature.

#### 4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to	1	ml



Mix the solution and store in a refrigerator (at  $-20^{\circ}\text{C}$ ).

5. 1.0 M Tris – HCl

Tris base 12.11 g

Dissolve in distilled water and adjusted pH to 7.5 with HCl

Distilled water to 100 ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.2H<sub>2</sub>O 186.6 g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to 1,000 ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M MgCl<sub>2</sub> solution

Magnesium chloride.6H<sub>2</sub>O 20.33 g

Distilled water to 100 ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

Sodium chloride 29.25 g

Distilled water to 100 ml

Dispense the solution into aliquot and sterilize by autoclaving.

## 9. 10X Tris borate buffer (10X TBE buffer)

Tris – base	100	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and store at room temperature.

## 10. 6X loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Glycerol	50	ml
1M Tris (pH 8.0)	1	ml
Distilled water until	100	ml

Mixed and stored at 4<sup>0</sup>C

11. 7.5 M Ammonium acetate (CH<sub>3</sub>COONH<sub>4</sub>)

Ammonium acetate	57.81	g
Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

## 12. 25:24:1 (v/v) Phenol-chloroform-isoamyl alcohol

Phenol	25	volume
Chloroform	24	volume
Isoamyl alcohol	1	volume

Mix the reagent and store in a sterile bottle kept in a refrigerator.

13. 2% Agarose gel (w/v)

Agarose 1.6 g

1X TBE 80 ml

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

14. Ethidium bromide

Ethidium bromide 10 mg

Distilled water 1 ml

Mix the solution and store at 4°C



## APPENDIX B

### Estimating Haplotype (EH)

File in this Window package.

1.EH.PAS : Source code of EH program.

2.EH.EXE : Executable code of EH program, which is compiled with a maximum of 30 alleles per locus. Loci, 1000 haplotypes, and 3600 genotypes (product of numbers of genotypes at each locus).

EH.DAT, EH.OUT Sample input and output files.

Protocol for using EH consisted of 2 steps.

1.Create the data file (.dat)

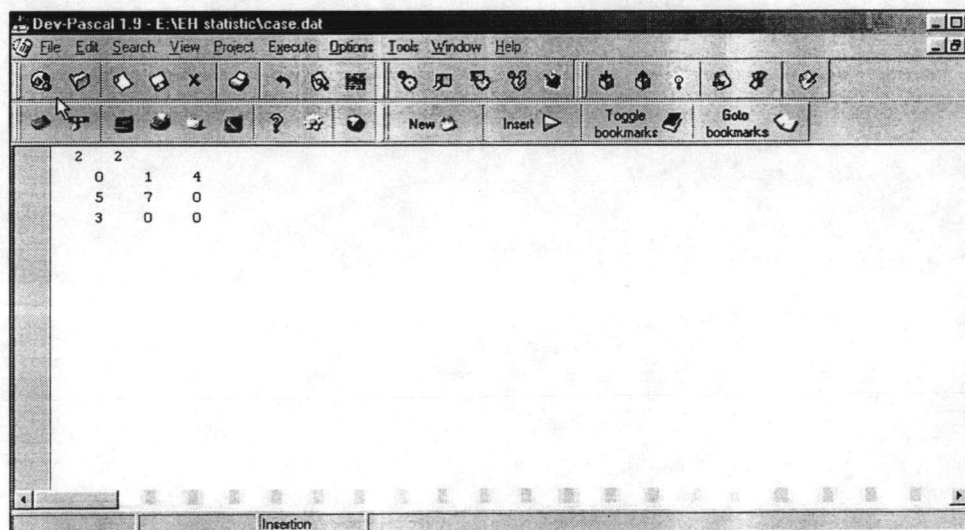
We created the data file in the Pascal program. The first line was the number of alleles at the first locus, number of alleles at the second locus, and so on. Assuming you have 2 loci, each locus has two alleles A & B and C & D, respectively.

The possible haplotypes.

	AA	AB	BB	→ first locus
CC				
CD				
DD				

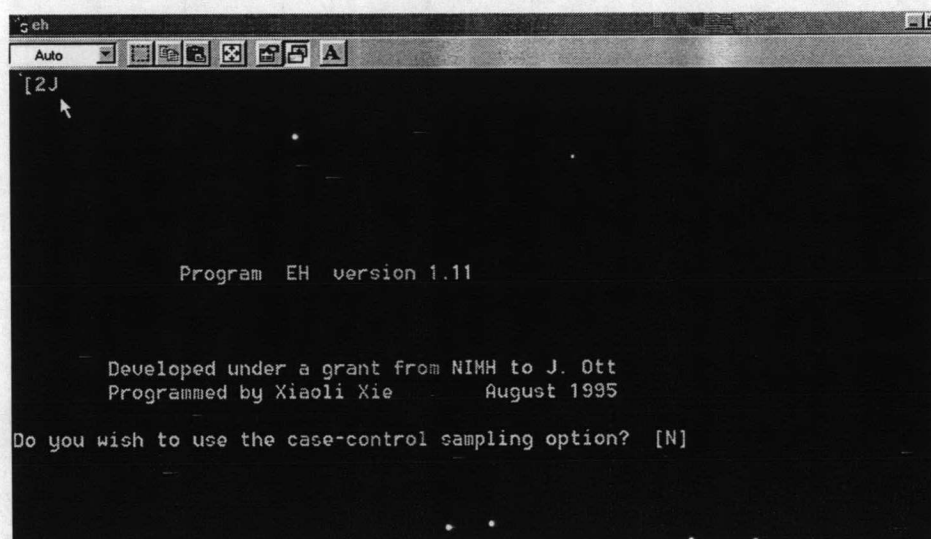
second locus

The number of haplotype in the box was filled in Pascal program and save unit as a .dat file.



## 2. Running the EH program

-Running EH program showed the window as below.



```

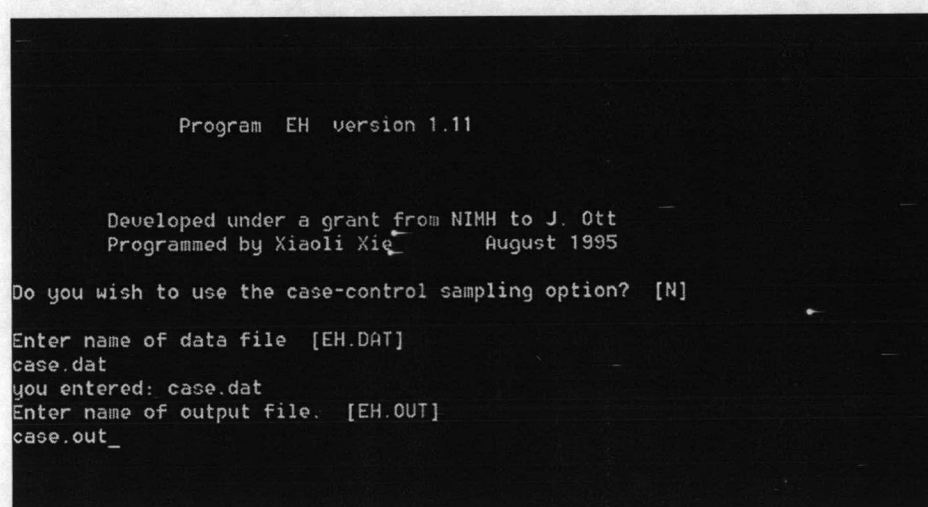
eh
Auto
[2J
Program EH version 1.11

Developed under a grant from NIMH to J. Ott
Programmed by Xiaoli Xie August 1995

Do you wish to use the case-control sampling option? [N]

```

-Type your data filename and output filename.



```

Program EH version 1.11

Developed under a grant from NIMH to J. Ott
Programmed by Xiaoli Xie August 1995

Do you wish to use the case-control sampling option? [N]

Enter name of data file [EH.DAT]
case.dat
you entered: case.dat
Enter name of output file. [EH.OUT]
case.out_

```

-The output file presented the haplotype frequencies in two kind. "Independent" these are obtained from the allele frequencies at the individual loci. That is, these haplotype frequencies are not estimated but calculated from allele frequencies under the assumption of no association. "w/Association" these are estimated from the data, allowing for association (linkage disequilibrium), assuming Hardy Weinberg equilibrium.

Dev-Pascal 1.9 - E:\EH statistic\case.out

File Edit Search View Project Execute Options Tools Window Help

Estimates of Gene Frequencies (Assuming Independence)

locus \ allele	1	2
1	0.5500	0.4500
2	0.6000	0.4000

# of Typed Individuals: 20

There are 4 Possible Haplotypes of These 2 Loci.  
They are Listed Below, with their Estimated Frequencies:

Allele at Locus 1	Allele at Locus 2	Independent Haplotype Frequency	w/Association
1	1	0.330000	0.150002
1	2	0.220000	0.399998
2	1	0.270000	0.449998
2	2	0.180000	0.000002

# of Iterations = 7

	df	Ln(L)	Chi-square
H0: No Association	2	-40.58	0.00
H1: Allelic Associations Allowed	3	-31.41	18.35

Case-control data.

If you want to test whether haplotype frequencies are significantly different in case and controls, you run EH three times, 1) for cases, 2) for controls, and 3) for cases and controls combined. For a given data set (case.dat, control.dat, provided), results are shown below.

Case.out, control.out, mix.out

	df	Ln(L)	Chi-square
H0: No Association	2	-40.58	0.00
H1: Allelic Associations Allowed	3	-31.41	18.35

	df	Ln(L)	Chi-square
H0: No Association	2	-208.32	0.00
H1: Allelic Associations Allowed	3	-207.69	1.26

	df	Ln(L)	Chi-square
H0: No Association	2	-544.98	0.00
H1: Allelic Associations Allowed	3	-523.19	43.57



The relevant test statistic is  $T = \ln(L_{\text{cases}}) + \ln(L_{\text{controls}}) - \ln(L_{\text{cases}} + \text{controls together})$ . With a sufficient number of observation, when there is no difference between case and control haplotype frequencies, twice this value has an approximate chi-square distribution with a number of df equal to the number of haplotypes estimated. For the above data, one obtains  $(-31.41) + (-207.69) - (-523.19) = 284.09$   $\chi^2 = 2 * 284.09 = 568.18$  on 3 df is associated with an empirical significance level of  $< 0.005$

## Table: Chi-Square Probabilities

The areas given across the top are the areas to the right of the critical value. To look up an area on the left, subtract it from one, and then look it up (ie: 0.05 on the left is 0.95 on the right)

df	0.995	0.99	0.975	0.95	0.90	0.10	0.05	0.025	0.01	0.005
1	---	---	0.001	0.004	0.016	2.706	3.841	5.024	6.635	7.879
2	0.010	0.020	0.051	0.103	0.211	4.605	5.991	7.378	9.210	10.597
3	0.072	0.115	0.216	0.352	0.584	6.251	7.815	9.348	11.345	12.838
4	0.207	0.297	0.484	0.711	1.064	7.779	9.488	11.143	13.277	14.860
5	0.412	0.554	0.831	1.145	1.610	9.236	11.070	12.833	15.086	16.750
6	0.676	0.872	1.237	1.635	2.204	10.645	12.592	14.449	16.812	18.548
7	0.989	1.239	1.690	2.167	2.833	12.017	14.067	16.013	18.475	20.278
8	1.344	1.646	2.180	2.733	3.490	13.362	15.507	17.535	20.090	21.955
9	1.735	2.088	2.700	3.325	4.168	14.684	16.919	19.023	21.666	23.589
10	2.156	2.558	3.247	3.940	4.865	15.987	18.307	20.483	23.209	25.188



## APPENDIX C

### HARDY-WEINBERG EQUILIBRIUM

The Hardy-Weinberg model, named after the two scientists that derived it in the early part of this century, describes and predicts genotype and allele frequencies in a non-evolving population. The model has five basic assumptions: 1) the population is large (i.e., there is no genetic drift); 2) there is no gene flow between populations, from migration or transfer of gametes; 3) mutations are negligible; 4) individuals are mating randomly; and 5) natural selection is not operating on the population. Given these assumptions, a population's genotype and allele frequencies will remain unchanged over successive generations, and the population is said to be in Hardy-Weinberg equilibrium. The Hardy-Weinberg model can also be applied to the genotype frequency of a single gene.

#### Importance:

The Hardy-Weinberg model enables us to compare a population's actual genetic structure over time with the genetic structure we would expect if the population were in Hardy-Weinberg equilibrium (i.e., not evolving). If genotype frequencies differ from those we would expect under equilibrium, we can assume that one or more of the model's assumptions are being violated, and attempt to determine which one(s).

#### Question:

How do we use the Hardy-Weinberg model to predict genotype and allele frequencies? What does the model tell us about the genetic structure of a population?

#### Variables:

p	frequency of one of two alleles
q	frequency of the other of two alleles

#### Methods:

The Hardy-Weinberg model consists of two equations: one that calculates allele frequencies and one that calculates genotype frequencies. Because we are dealing with frequencies, both equations must add up to 1.

The equation

$$p + q = 1$$

describes allele frequencies for a gene with two alleles. (This is the simplest case, but the equation can also be modified and used in cases with three or more alleles.) If we know the frequency of one allele ( $p$ ) we can easily calculate the frequency of the other allele ( $q$ ) by  $1 - p = q$ .

In a diploid organism with alleles  $A$  and  $a$  at a given locus, there are three possible genotypes:  $AA$ ,  $Aa$ , and  $aa$ . If we use  $p$  to represent the frequency of  $A$  and  $q$  to represent the frequency of  $a$ , we can write the genotype frequencies as  $(p)(p)$  or  $p^2$  for  $AA$ ,  $(q)(q)$  or  $q^2$  for  $aa$ , and  $2(p)(q)$  for  $Aa$ . The equation for genotype frequencies is

$$p^2 + 2pq + q^2 = 1.$$

One approach to the study of genetic diversity is to look at allele and genotype frequencies of allozymes. Allozymes are enzymes that show different rates of movement in gel electrophoresis due to the presence of different alleles at a single locus; they are often denoted as F (fast-moving) and S (slow-moving) alleles. Allozyme variation is an indicator of genetic variation, and can be studied to quantify genetic variation among populations.

Lidicker and McCollum (1997) examined genetic variation in two populations of sea otters (*Enhydra lutris*) in the eastern Pacific. Sea otters were distributed throughout this region before fur hunting nearly led to their local extinction. Along the central California coast only one population of 50 or fewer individuals is thought to have survived; this population was protected in 1911 and has grown to its current size of approximately 1500 otters. Because of the extreme reduction in population size (a bottleneck), the population may have lost considerable genetic variation. A population from Alaska also experienced a bottleneck around that time but it was not as severe.

The table below (data from Lidicker & McCollum 1997) contains counts of the number of individuals with a given genotype for six variable (polymorphic) two-allele loci.

		California	Alaska
Locus	Genotype	<i>n</i>	<i>n</i>
	SS	37	3
EST	SF	20	3
	FF	7	2
	SS	48	7
ICD	SF	4	2
	FF	3	0
	SS	20	3
LA	SF	11	2
	FF	2	3
	SS	16	1
PAP	SF	7	3
	FF	10	2
	SS	16	1
ME	SF	11	2
	FF	5	1
	SS	17	3
NP	SF	4	1
	FF	5	0

We can use these data to calculate the allelic frequencies for a given locus, such as the EST locus in the California population ( $n = 64$ ). Each individual with the genotype *SS* has two copies of the *S* allele; therefore the 37 individuals with this genotype have a count of 74 *S* alleles. Heterozygote individuals (*SF*) have one of each allele, so there are 20 *S* alleles and 20 *F* alleles among them. Like the *SS* homozygotes, individuals with the *FF* genotype have two copies of the *F* allele, so these seven individuals contribute 14 *F* alleles to our count. In other words, among the 64 individuals in this sample there are 94 *S* alleles and 34 *F* alleles. To calculate the allelic frequencies we simply divide the



number of S or F alleles by the total number of alleles:  $94/128 = 0.734 = p =$  frequency of the S allele, and  $34/128 = 0.266 = q =$  frequency of the F allele.

If this population were in Hardy-Weinberg equilibrium, we would expect the genotype frequencies for SS, SF, and FF to be  $p^2$ ,  $2pq$ , and  $q^2$ :

$$p^2 = (0.734)^2 = 0.539$$

$$2pq = 2(0.734)(0.266) = 0.390$$

$$q^2 = (0.266)^2 = 0.071$$

For the 64 individuals in this sample, then, we would expect that approximately 34 individuals ( $p^2 * n = 0.539 * 64 = 34.496$ ) would have the SS genotype, 25 individuals ( $2pq * n = 0.390 * 64 = 24.960$ ) would have the SF genotype, and 5 individuals ( $q^2 * n = 0.071 * 64 = 4.544$ ) would have the FF genotype. How do these expected values compare to the observed numbers for genotype frequencies at the EST locus?

genotype	observed	expected
SS	37	34
SF	20	25
FF	7	5

Generally we would use a statistical test to compare our expected and observed counts. In this case we can see that the numbers are fairly similar, and in fact the authors have used a chi-square test and concluded that the observed and expected counts are not significantly different from one another.

#### Interpretation:

We can check our math to ensure that we have calculated the correct genotype frequencies:  $p^2 + 2pq + q^2$  should equal 1, and  $(0.734)^2 + 2(0.734)(0.266) + (0.266)^2$  does indeed equal 1. Similarly,  $p + q$  must equal 1 and  $0.734 + 0.266 = 1$ . Our results suggest that for the California sea otter population, the allele and genotype frequencies at the EST locus are in Hardy-Weinberg equilibrium. In other words, we can expect these allele frequencies to remain constant over time (barring any specific evolutionary forces acting upon this locus), thus ensuring genetic variation in the population at the EST locus. This equilibrium in the genetic structure of the population at the EST locus does not necessarily imply, however, that the population is not evolving; it merely

indicates that this particular locus is not changing. Even if the frequency of alleles at just a single locus is changing over the generations, the population is evolving.

**Conclusions:**

Natural populations with whole genotypes in Hardy-Weinberg equilibrium are rarely found; one or more of the assumptions are violated in most situations. If nothing else, most populations are under the influence of natural selection. Certainly no population can be infinite, but many populations are not even large enough to be functionally infinite. Oftentimes populations are not completely isolated from one another, and migration of individuals into or out of one population can change its genetic makeup. Mutations can potentially alter the gene pool significantly, although the majority are thought to have little or no effect (neutral mutations). Finally, individuals often mate selectively rather than randomly; for example, humans show assortative mating by height (tall people tend to marry tall people and short people tend to marry short people).

**Additional Questions:**

- 1) For which loci are the genotypes apparently not in Hardy-Weinberg equilibrium (note that  $n$  is different for each locus investigated)? Is this true for both populations?
- 2) What might affect the validity of your conclusions about the Alaska population?

*Extra credit:* Confirm some or all of your conclusions for #1 by performing a chi-square test. The null hypothesis you are testing is that the observed and expected values are not significantly different from one another (because your expected values are calculated based on an assumption of Hardy-Weinberg equilibrium, this is the same as saying that the population is in H-W equilibrium for the genotype being tested). The critical value for the chi-square in this case is 3.841; if your calculated value of the chi-square is equal to or greater than that, the probability of the null hypothesis being correct (i.e., the probability of the population being in H-W equilibrium at that genotype) is 0.05, and the null hypothesis is rejected.

## BIOGRAPHY

Mr. Chupong Ittiwut was born in Chiangrai, the northeast province of Thailand, in January 11<sup>th</sup>, 1978. In 1999, He received his bachelor degree in Genetics from Faculty of Science, Chulalongkorn University in Bangkok Thailand. Consequently, with his interests in human and molecular genetics, he had made one of his vigorous decision to study in curriculum of Medical Science in Faculty of Medicine for his master degree. Nowadays, he has been to Mahasarakham University to further his authority with his new status, the lecturer of genetics program in the Faculty of Science.



ASSOCIATION BETWEEN *MTHFR* POLYMORPHISMS AND FRONTOETHMOIDAL  
ENCEPHALOMENINGOCELE AND CLEFT LIP WITH OR WITHOUT CLEFT PALATE

Mr. Chupong Ittiwut

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