CHAPTER 3

MATERIALS AND METHODS

1. Studied populations

This research project was approved by the Human Experimentation Committee, Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, Thailand. The studied populations were three hilltribes, i.e. the Karen, the Hmong and the Iu-Mien in northern Thailand. Information on linguistic and cultural aspects, village history and migration, especially the post-marital migration of these populations were collected from interviewed sessions and literature searches (Technical Service Club Tribal Research Institute, 1995).

Karen

The Karen, the largest hilltribe population in Thailand, immigrated from Tibet via Myanmar into northern and western Thailand. They live along Thai border close to Myanmar and have still migrated back and fort across the border. Karenic is their language and categorized as a sub-family of the Sino-Tibetan linguistic family. They are matrilocal, the husbands move into the wife's families after married, built their new residence and stay as a nuclear family. The Karens look like Mongolians. They compose of four groups, the Skaw Karen (Pga-gan Yaw), the Pwo Karen, the Kayah Karen (Bwe) and the Thoungthu Karen (Pa-o) classified by their spiritual beliefs.

The Skaw Karen in this study lives in Mae Sawan Noi village, Mae Hau sub-district, Mae Sariang district, Mae Hong Son province. The village was established 130 years ago as a temporary camp during the agriculture season, then settled and expanded through the continuous immigration.

The Pwo Karen lives in Tung Loy village, Omkoi sub-district, Omkoi district, Chiang Mai province. Most of the members moved from a neighboring area of old Tung Loy village, whereas the people migrated from Myanmar about a hundred years ago, to avoid its inconvenient transportation.

The Kayah Karen lives in Nai Soy refugee camp, Pang Moo sub-district, Muang district, Mae Hong Son province, supported by the United Nations (UN). All are refugees immigrated from Kayah state, Myanmar.

The Thoungthu Karen lives in Mai Lun village, Pang Mapha sub-district, Pang Mapha district, Mae Hong Son province. The village is located near Myanmar boarder and its populations are mixed between the Tai and Thoungthu Karen. The Thoungthu had immigrated from Muang Pin, Myanmar, about 10 years ago and became farmers and common laborers.

Hmong

The Hmong is the second largest group of hilltribe in Thailand. They originated in China and migrated through Vietnam and Laos into Thailand around 50-60 years ago. Their language is categorized in the Hmong-Mien linguistic family. They are patrilocal, the wifes move into the husband's families after married, and live in extended families.

The Hmong look like Chinese. They are categorized by their beliefs into two groups, the Black Hmong (Hmong Njua) and the White Hmong (Hmong Daw).

The Black Hmong are from 3 villages in northern Thailand:

1. Laoli-Mae Tho village, Bou Sali sub-district, Hod district, Chiang Mai province. People of this group had migrated from Doi Chiang Dao, Chiang Dao district, Chiang Mai province, about 40 years ago.
2. Khun Heuy Mae Pao village, Mae Pao sub-district, Phaya Mengrai district, Chiang Rai province. Most of the people are farmers and came from Thoeng district, Chiang Rai province. The village is about 30 years old.
3. Pang Kha Neur village, Pha Chang Noi sub-district, Pong district, Phayao province. This group included people from different agricutural regions, i.e. Chiang Khong district, Chiang Rai province; Chiang Kham district, Phayao province and Thung Chang district, Nan Province, since about 20 years ago.

The White Hmong are from 2 villages:

1. Khun Klang village, Ban Loung sub-District, Chom Thong district, Chiang Mai province. The people of the 60-year-old village practiced slash and burn cultivation, which resulted in a non-permanent settlement. Only about 25 years ago, the village is established at the mentioned location.
2. Kra Lae village, Mae Pao sub-district, Phaya Mengrai district, Chiang Rai province. This group came from Phayao and Nan on agricultural purpose. The village is about 30 years old.

Iu Mien

The Iu Mien originated in central China and expanded around Guangxi Zhuang autonomous region, Hunan, Yunnan, Guangdong, Guizhou and Jiangxi provinces. They immigrated via Northern Vietnam through Laos into Thailand. The Iu Mien in Thailand has no subgroup and is categorized in the Hmong-Mien family. The Iu Mien appearance is Chinese-liked. They are patrilocal and live in both nuclear and extended families. The Iu Mien has a tradition of adopting children, for domestic services, from either the same tribe or the others. In this study, the Iu Mien blood samples were collected from 3 provinces.

1. **Chiang Rai:** Mae Yao sub-district, Muang district, and Tad Kwun sub-district, Phaya Mengrai district. This group settled in Mae Yao about 50 years ago and in Tad Kwun about 30 years ago.

2. **Phayao:** Ban Mai Pang Kha village, Pha Chang Noi sub-district, Pong district. This Iu Mien village was established around 40 years ago. This group previously lived on the Phu Lang Kha mountain in Chalerm Prakeat district, Nan province, and due to a political conflict between the Thai government and the communists, along with a search for more convenient transportation, the group then moved to the present location.

3. **Nan:** Ban Nam Khong, Sa-Nean sub-district, Muang district. This Iu Mien community are the mixture of Iu Mien from Pong district, Phayao province, and Thung Chang and Pua districts, Nan province. The village is around 40 years old.

According to a personal interview with Mr. Khravan Srisombut, a descendant of the leader who led the first Iu Mien group to immigrate into Thailand, and therefore, an expert in his own ethnic history, the Iu Mien history was clarified. The first Iu Mien population arrived Thailand about 170 – 180 years ago. They were from Yunnan in Southern China Laos and settled first in Lao. Their leader, who in the meantime became Phaya Khiree Srisombut, the renowned warlord and the Iu Mien ruler, led part of the community out of Lao to Phu Wae (in present day Chiang Klang district, Nan province). Later the group moved further to Phu Lang Kha mountain (in present day Chalerm Prakeat district, Phayao province). Nowadays, Mr. Khravan Srisombut, the ruler' descendants and his group are at Ban Mai Pang Kha village, Pha Chang Noi sub-district, Pong district, Phayao province.

Mr. Khravan Srisombat informed the research team that all the Iu Mien living now in Thailand were from the original group. The Iu Mien in Nan is Phaya Khiree Srisombut's group from Lao that remains there, while his group moved to Phayao province. There were some members of Phaya Khiree Srisombut's group, who immigrated later into Thailand along the same route from Laos, joined his group in Pong district. The Chiang Rai community came from Lao into Thailand via two migration routes, the majority via Chiang Khong district, Chiang Rai province and the rest via Nan province.

The map of sampling areas and the hilltribe populations is shown in Fig 1.

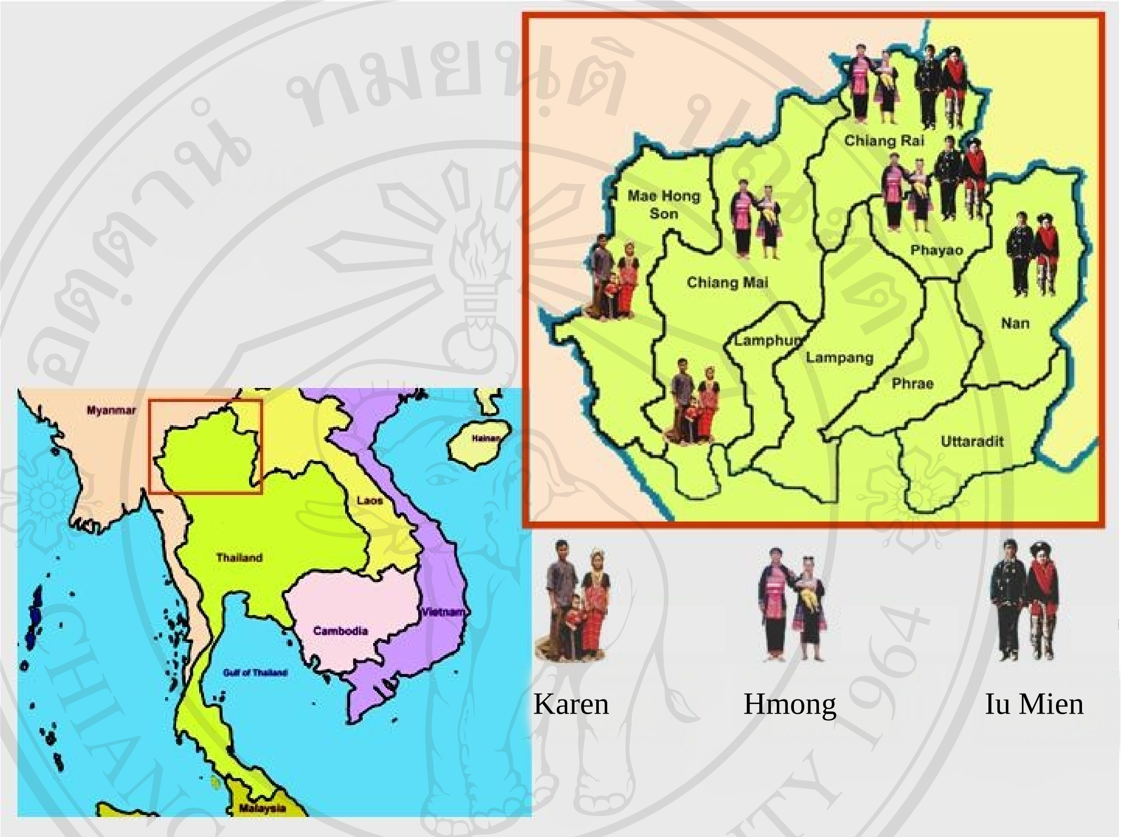

Figure 1 Map of sampling areas and the hill-tribes populations

2. Blood sampling and DNA extraction

2.1 Blood sample collection

Peripheral blood from each healthy unrelated volunteers tracing back for at least 2 generations, not more than 25 males and 25 females from each geographic site (village), was drawn using 5 ml vacutainer coated with anticoagulant-EDTA. The sample tubes were kept in an icebox during the field trip. The DNA should be extracted within 24 hours after sampling.

2.2 DNA extraction procedure

Total genomic DNA was extracted according to a standard inorganic salting out protocol (Seielstad *et al.*, 1999) as followed;

1. Transfer the whole blood into sterile 15 ml conical centrifuge tube, add 10 ml red cell lysis buffer (RCLB) and invert the tube until the solution is completely mixed.

RCLB: 1 mM NH_4HCO_3
115 mM NH_4Cl

2. Centrifuge the tube for 10 min at 1,200 rpm.
3. Discard the supernatant, add another 10 ml RCLB, resuspend the cell pellet and centrifuge as in step 2.
4. Discard the supernatant, add 1.8 ml white cell lysis buffer (WCLB) and resuspend the cell pellet. The white cell lysate should be milky-like now.

WCLB: 100 mM Tris-Cl (pH 7.6)
40 mM EDTA (pH 8.0)

50 mM NaCl

0.2 % SDS

5. Store the white cell lysate in microtube. The DNA should be stable in this form for several weeks at room temperature, although the refrigeration is recommended.

6. To perform the final extraction, add 5 µl of 20 mg/ml proteinase K stock solution to half of the white cell lysate (900 µl) and freeze the rest for future use.

7. Incubate the mixed solution for 2 hr at 50^{o}C with occasional shaking.

8. Add 300 µl of saturated NaCl (= 6M NaCl), vigorously shake the tube for 10 min and centrifuge for 5 min at 12,000 rpm.

9. Separate the supernatant into two 1.5 ml tubes, add an equal volume of pre-cooled isopropanol to each tube. Mix well by inverse the tube back and forth – the DNA precipitate should be visible at this point.

10. Centrifuge for 5 min at 12,000 rpm and discard the supernatant.

11. Wash DNA pellet with 1 ml of 70% ethanol, let the DNA dry and resuspend it in 100 µl of TE buffer.

3. Genetic variation detection

Genetic variation of X- and Y-chromosomal microsatellites, and the mtDNA were studied. Number of individual analyzed in each data set was shown in Table 1.

Table 1 Number of individual analyzed in each population and subgroup

Population	Number of individual		
	X chromosome (male and female)	Y chromosome (male)	mtDNA (male and female)
Karen	154	83	63
Kayah (KK)	44	24	11
Pwo (KP)	49	22	10
Skaw (KS)	44	23	31
Thougthu (KT)	17	14	11
Hmong	136	90	50
Black Hmong (Hm-B)	90	56	31
White Hmong (Hm-W)	46	34	19
Iu Mien	75	52	44
Chiang Rai (I-Ch)	25	17	26
Nan (I-Na)	28	22	8
Phayao (I-Ph)	22	13	10
Total	**365**	**225**	**157**

3.1 X chromosome variation

A total of 365 unrelated males and females volunteers from 9 subgroups of 3 hilltribe populations were sampled and analyzed. They were 44 Kayah Karen (KK), 49 Pwo Karen (KP), 44 Skaw Karen (KS), 17 Thoungthu (KT), 90 Black Hmong (Hm-B), 46 White Hmong (Hm-W) and the Iu Mien from 3 different locations - 25, 28 and 22 individuals from Chiang Rai (I-Ch), Nan (I-Na) and Phayao (I-Ph), respectively.

Fourteen X-linked dinucleotide (CA) repeats microsatellites loci: DXS1001, DXS1060, DXS1068, DXS1073, DXS1106, DXS1214, DXS1227, DXS8043, DXS8051, DXS8091, DXS987, DXS990, DXS991 and DXS993 were genotyped. The primers used were from a commercial set, ABI PRISM Linkage Mapping Sets v2.5 panel 28 (Applied Biosystems).

PCR reaction mix contained 9 μl of True Allele^{TM} PCR premix, 1 μl of primer mix (5μM each), 1.2 μl of 50-100 ng/μl genomic DNA, and the sterile deionized water to give a final volume of 15 μl. The PCR cycling conditions followed the company instruction, i.e. 12 min at 95^{o}C for an initial denaturation step, followed by 10 cycles of 15 sec at 94^{o}C, 15 sec at 55^{o}C, 30 sec at 72^{o}C, then 20 cycles of 15 sec at 89^{o}C, 15 sec at 55^{o}C, 30 sec at 72^{o}C, and a final extension step for 10 min at 72^{o}C.

Allelic size of the amplicons were determined on a 4.5% denatured polyacrylamide gel using an ABI 377 automated DNA sequencer. Genotype^{TM} ladder 50-500 and 60-500 ROX labeled (Gibco BRL) were used as internal size standard. Fragment sizes were analyzed by the GENESCAN^{TM} 3.1 and GENOTYPER^{TM} 2.5 softwares (Applied Biosystem).

3.2 Y chromosome variation

A total of 225 unrelated males from 9 subgroups of 3 hilltribe populations were sampled and analyzed. They were 24 KK, 22 KP, 23 KS, 14 KT, 56 Hm-B, 34 Hm-W, 17 I-Ch, 22 I-Na and 13 I-Ph.

Y-chromosomal microsatellites from 15 loci were amplified: i.e. DYS19, DYS388, DYS390, DYS391, DYS392, DYS393 (MS1 kit) and DYS389I, DYS389II, DYS426 (MS2 kit) (Thomas *et al*., 1999); DYS436, DYS437, DYS439 (Ayub *et al*., 2000); Y-GATA-A7.1, Y-GATA-A7.2 and Y-GATA-A10 (White *et al*., 1999). The DYS392, DYS426, DYS436 are trinucleotide repeats and the rest are tetranucleotide repeats.

PCR reaction using fluorescent labeled primers was performed in a 10 μl final reaction volume. It contained 1 μl of 10x PCR buffer II, 1.6 μl of 1.25 dNTP mix, 0.9 μl of 25 mM $MgCl_2$ 0.03 U of Ampli*Taq* Gold polymerase (Applied Biosystems) and 50-200 ng genomic DNA and double-sterile deionized water to give a final volume of 10 μl. Primer sequences and final concentrations used in each multiplex PCR set are shown in Table 2.

Table 2 Primer sequences and final concentrations used in each multiplex PCR set

set	Locus	primers and fluorescent dye labeled	primer sequence	final concentration (μM)	allele size ranges
1	DYS19	DYS19R DYS19L 6FAM	ATGGCATGTAGTGAGGACA CTACTGAGTTTCTGTTATAGT	0.236 0.236	178-206
	DYS388	DYS388R DYS388L 5TET	CAGATCGCAACCACTGCG GTGAGTTAGCCGTTTAGCGA	0.318 0.318	120-145
	DYS390	DYS390R 6FAM DYS390L	TGACAGTAAAATGAACACATTGC TATATTTTACACATTTTTGGGCC	0.2 0.2	188-224
	DYS391	DYS391Rna DYS391L 6FAM	ACATAGCCAAATATCTCCTGGG CTATTCATTCAATCATACACCCATAT	0.384 0.384	154-186
	DYS392	DYS392Rna 5HEX DYS392L	CAGTCAAAGTGGAAAGTAGTCTGG AAAAGCCAAGAAGGAAAACAAA	0.15 0.15	158-182
	DYS393	DYS393R 5HEX DYS393L	AACTCAAGTCCAAAAAATGAGG GTGGTCTTCTACTTGTGTCAATAC	0.07 0.07	107-135
2	DYS389I and DYS389II	DYS389R DYS389La 5TET	CCTGAGTAGCAGAAGAATGTCATA CCAACTCTCATCTGTATTATCTATGTG	0.8 0.8	146-166 and 104-140
	DYS426	DYS426Ra DYS46L 5HEX	CTCAAAGTATGAAAGCATGACCA GGTGACAAGACGAGACTTTGTG	0.06 0.06	90-96
3	DYS436	DYS436R DYS436L 6FAM	GCAATCCAACTTCAGCCAAT CCAGGAGAGCACACACAAAA	0.0125 0.0125	128-143
	DYS437	DYS437R DYS437L 5HEX	AGACCCTGTCATTCACAGATGA GACTATGGGCGTGAGTGCAT	0.05 0.05	186-202

	DYS439	DYS439R DYS439L 5TET	GCCTGGCTTGGAATTCTTTT TCCTGAATGGTACTTCCTAGGTTT	0.1 0.1	238-258
4	Y-GATA-A7.1	Y-GATA-A7.1 F 5HEX Y-GATA-A7.1 R	CAAATTTGCCAAACTCTTTC TCTATCCTCTGCCTATCATTTATTA	0.4 0.4	161-181
	Y-GATA-A7.2	Y-GATA-A7.2 F 5TET Y-GATA-A7.2 R	AGGCAGAGGATAGATGATATGGAT TTCAGGTAAATCTGTCCAGTAGTGA	0.4 0.4	174-190
	Y-GATA-A10	Y-GATA-A10 F 6FAM Y-GATA-A10 R	ATAAATGGAGATAGTGGGTGGATT CCTGCCATCTCTATTTATCTTGCATATA	0.4 0.4	160-172

The PCR cycling conditions of each multiplex set were;

The first set (MS1 kit): 10 min at 95°C pre-incubation for enzyme activation and an initial denaturation step, followed by 38 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, and 10 min at 72°C for a final extension step (Thomas *et al*., 1999).

The second set (MS2 kit): 10 min at 95°C for enzyme pre-incubation and an initial denaturation step, followed by 38 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and 10 min at 72°C for a final extension step (Thomas *et al*., 1999).

The third set: 10 min at 95°C for enzyme pre-incubation and an initial denaturation step, followed by 8 cycles of the TouchDown protocol: 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, the annealing temperature was decreased by 0.5°C in each cycle, then 30 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, and 10 min at 72°C for a final extension step (Ayub *et al*., 2000).

The fourth set: 10 min at 95°C for enzyme pre-incubation and an initial denaturation step, followed by 32 cycles of 30 sec at 94°C, 30 sec at 59°C and 30 sec at 72°C, and 10 min at 72°C for a final extension step.

Allelic size of the amplicons were determined on a 4.5% denatured polyacrylamide gel using an ABI 377 automated DNA sequencer. Genotype™ ladder 50-500 and 60-500 TAMRA labeled (Gibco BRL) were used as internal size standard. Fragment sizes were analyzed by the GENESCAN™ 3.1 and GENOTYPER™ 2.5 softwares (Applied Biosystem).

3.3 MtDNA sequence variation

A total of 157 mtDNA sequences at the HVS-I in males and females were analyzed. The subjects were from 11 KK, 10 KP, 31 KS, 11 KT, 31 Hm-B, 19 Hm-W, 26 I-Ch, 8 I-Na and 10 I-Ph.

The primers reported by Schurr *et al*. (1999) with a slightly modified protocol were used to sequence the mtDNA HVS-I. The whole control region of about 1,121 bp was amplified using primer complimentary to the light (5' CTACGCCAATCACTTT ATTG 3') and the heavy (5' CTGTTAAAAGTGCATACCGCC 3') strands. Each PCR reaction was performed in 50 µl final volume. The PCR reaction mix contained 5 µl of 10x PCR buffer II, 16 µl of 2.5 dNTP mix, 8 µl of 25 mM $MgCl_2$, 1.3 µl of each 20 µM PCR primer 1.25 U of Ampli*Taq* Gold polymerase (Applied Biosystems), 50-100 ng genomic DNA and double-sterile deionized water to give a final volume of 50 µl. PCR cycling conditions were 12 min at 95^{o}C for an initial denaturation step, followed by 30 cycles of 1 min at 94^{o}C, 1 min at 56^{o}C and 1 min at 72^{o}C, and 7 min at 72^{o}C for a final extension step.

To digest the excess PCR primer, 2l µl of the PCR product was added to 3.5 µl of 10 U/µl Exonuclease I and 7.5 µl 1 U/µl Shrimp Alkaline Phosphatase. The reaction tube was incubated at 37^{o}C for 30 min and another 15 min at 95^{o}C. Then 8.0 µl of the digested PCR products were directly sequenced using the two mentioned primers and the BigDye^{TM} V3.0 terminator ready sequencing kit (Applied Biosystems). The sequencing cycling conditions were 25 cycles of 10 sec at 96^{o}C, 5 sec at 50^{o}C, and 4 min at 60^{o}C.

The signal of fluorescent-labeled nucleotide were detected on a 4.5% denatured polyacrylamide gel using an ABI 377 automated DNA sequencer. SEQUENCHER software (Genecode) was used for bases calling and sequence strands assembling. About 384 bps of mtDNA HVS-I (nt positions 16037 to 16420 according to the revised Cambridge Reference Sequence — available at http://www.mitomap.org, and the HVS-I of the Cambridge Reference Sequence was shown in the appendix F) from each individual was analyzed.

4. Data analyses

4.1 Allelic distribution

Allele frequencies from every microsatellite locus in X and Y chromosomes, and the haplotype frequencies of the Y chromosome were calculated. Common alleles of every microsatellite locus in X and Y chromosomes and common Y-chromosomal haplotypes were considered in each hilltribe population and subgroup.

4.2 The genetic diversity

1) Genetic diversity of the X chromosome

Since an effective population size of the X chromosome is unequal between male and female, the genetic diversity of the X chromosome was estimated in 3 set of data, all subjects, females and males.

Genetic diversity indices of the X chromosome were total alleles of all loci, average diversity of all loci, and total diversity.

The total number of alleles was counted directly from the observed alleles. This index was used to measure the amount of variation but not the differences across populations. It is useful only for multi-allelic markers that have the variation in number of alleles (Rosenberg *et al*., 2003).

The average diversity of all loci and the total diversity were calculated using the Microsat 2.1 program (available at http://hpgl.stanford.edu/projects /microsat/).

2) Genetic diversity of Y chromosome

Genetic diversity ($D= 1- \Sigma p_i^2$) of each locus in each population and subgroup were estimated (Nei, 1973), where p_i is the frequency of each allele at the locus. Haplotype diversity (H) from 15 Y-chromosomal microsatellite loci was estimated using the same equation, where p_i was the haplotype frequency. Other genetic diversity indices of the Y chromosome were total alleles of all loci (*N*), average diversity of all loci and number of haplotypes. The average diversity of all loci was calculated using the Microsat 2.1 program.

3) Genetic diversity of the mtDNA sequence

MtDNA diversity that calculated from the frequency of HVS-I sequence is known as mtDNA haplotype diversity. The diversity ($H = 1- \Sigma p_i^2$) in each population and subgroup was estimated, where p_i was the frequency of HVS-I haplotype.

To evaluate the mtDNA polymorphism, identical sequences were primarily considered. The procedure was as follow: the sequence from each individual was imported into BioEdit software in order to convert sequence file to an appropriate format for the GeneStudio^{TM} software (GeneStudio, Inc). ClustalW 1.8 and Tree-puzzle programs in the GeneStudio^{TM} software package were used for multiple sequences alignment and identical comparison, respectively. The result from the GeneStudio^{TM} software was manually checked for confirmation.

4.3 The genetic distances among populations

1) Genetic distances for X- and Y- chromosomal microsatellite data

The delta mu square $(\delta\mu)^2$ distance was introduced to fit a two complicating properties of microsatellite loci. Firstly, a mutation of the microsatellite is based on the stepwise mutation model and secondly, allele size is constrained. The $(\delta\mu)^2$ is modified from the average square distance (ASD), which is a linear function of time under the strict stepwise mutation process, to remove its dependence on population size and to decrease its variance. The distance achieves the standardization with respect to the variation within populations without estimating additional parameters (Goldstein *et al.*, 1995).

The pairwise $(\delta\mu)^2$ distances between population as well as subgroups were calculated using the Microsat 2.1. 95% confidence intervals were estimated by analyzing 10,000 bootstrap replications.

Regardless of stepwise mutation model, the pairwise F_{st} was used as short-term genetic distances between populations. The population pairwise F_{st} distance based on a haplotype frequency of the Y chromosome was calculated using the ARLEQUIN 2.000 software (Schneider *et al.*, 2000: available at http://anthropologie.unige.ch/arlequin).

2) Genetic distance of the mtDNA sequence

The population pairwise F_{st} distance based on haplotype frequency was performed to measure the distance between populations and subgroups using ARLEQUIN 2.000 software.

4.4 The phylogenetic tree among populations

An unrooted neighbor-joining tree was reconstructed from the $(\delta\mu)^2$ and the F_{st} distances using the MEGA 2.1 software (Kumar *et al.*, 2001).

The tree topologies from the Y chromosome and from the mtDNA data sets were compared. Each tree was reconstructed from the matrix of F_{st} distances based on the haplotype frequencies of the Y-chromosomal microsatellites and the mtDNA HVS-I sequences.

4.5 The genetic structure of the population

Analysis of molecular variance (AMOVA) was performed to examine genetic structure of populations (Excoffier *et al.*, 1992). The calculation is a framework for the study of molecular variation within a single species. Information on DNA haplotype divergence is incorporated into an analysis of variance format, derived from a matrix of squared-distances among all pairs of haplotypes. The AMOVA produced the estimation of variance components, and *F*-statistic analogs reflected the correlation of haplotype diversity at different levels of hierarchical subdivision. The significance of the variance components is tested, using a permutational approach, to eliminate the normality assumption that is conventional for the analysis of variance but inappropriate for the molecular data. The method that requires the fewest assumptions is the permutational analysis of the null distribution for each variance component. Under the null hypothesis, samples are considered as a drawn from the global population with the variation due to the random sampling in the construction of populations.

To examine the genetic structure of populations at different levels of hierarchical subdivision whether there are genetic differences among different ethnic groups and language families, the AMOVA was performed for 3 populations and their 9 subgroups (Excoffier *et al*., 1992), using the ARLEQUIN 2.000 software (Schneider *et al*., 2000).

4.6 Migration rate and the effective number of migrant

Theory based on the island model of migration indicates that the fixation index (F_{st}) (approximated as the variation among subpopulations) is equal to $1/(1+4N_e\nu)$ for diploid system (X chromosome data), and $1/(1+N_e\nu)$ for haploid system (mitochondrial and Y chromosome data), where N is the effective population size and ν is the sum of migration (m) and mutation (μ; more exact is $\nu = m+ \mu - m\mu$). If the mutation rates for all systems were assumed to be very low and substantially lower than any estimations of the migration rate, then ν is equal to m. If N_e is the same for males and females, the male and female migration rates were estimated from each tribe. The fixation index among subgroups within the population was calculated via the AMOVA, using the ARLEQUIN 2.000 software.

The migration rates and the effective number of male and female migrants in matrilocal and patrilocal populations, estimated from the Y chromosome and mtDNA data sets, were considered.