

ภาคผนวก

ภาคผนวก 1

ลำดับเบสของแต่ละแอสไปไรท์

haplotype (Sequence)	
1	ATTCTAATTTAAACTATTCTCTGTTCTTTTCATGGGGAAGCAGATTGGGTACCACCCAAGTATTGACTCACCCTATCAACAACCGCTATGTAATTCGTA CATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCTCCCCAT GCTTACAAGCAAGTACAGCAATCAACCCCTCAACTATCACACATCAACTGCAACCCCAAGCCACCCCTCACCCTAGGATACCAACAAACCCACC CACCCTTAACAGCACATAGTACATAAAGCCATTTACCGTACATAGCACATTATAGTCAAATCCCTTCTCGTCCCATGGATGACCCCCCTCAGATAG GGGTCCTTGACCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCTCCTCGCTCCGGGCCATAACACTTGGGGGTAGCTAAAGT GAACTGTATCCGACATCTGGTTCCTACTTCAGGGCCATAAAGCCTAAATAGCCACACGTTCCCTTAAATAAGACATCACGATG
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3	ATTCTAATTTAAACTATTCTCTGTTCTTTCA?GGGGAAGCA?ATTTGGGTACCACCCAAGTATTGACTCACCCTATCAACAACCGCTATGTAATTCGTAC ATTACTGCTA?CCACCATGAATATTGTACAGTACCATAAATACTTGACCACCTGTAGTACATGAAAACCCAACCCACATCAAAACCCCTCCCATGTC TTACAAGCAAGTACAGCAATCAACCCCTCAACTATCACACATCAACTGCAATTCCAAAGCCACCCCTCACCCTAGGATACCAACAAACCTACCCAC CCTTAACAGCACATAGTACATAAAGCCATTTATCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCATGGATGACCCCCCTCAGATAGGG TCCCTTGACCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCTCCTCGCTCCGGGCCATAACACTTGGGGGTAGCTAAAGTGAA CTGTATCCGACATCTGGTTCCTACTTCAGGGCCATAAAGCCTAAATAGCCACACGTTCCCTTAAATAAGACATCACGATG
4	ATTCTAATTTAAACTATTCTCTGTTCTTTTCATGGGGAAGCAGATTGGGTACCACCCAAGTATTGACTCACCCTATCAACAACCGCTATGTAATTCGTA CATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCTCCCAT GCTTACAAGCAAGTACAGCAATCAACCTTCAACTATCACACATCAACTGCAACTCCAAGCCACCCCTCACCCTAGGATACCAACAAACCTACCC ACCCCTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCATGGATGACCCCCCTCAGATAGG GGTCCCTTGACCACCATCCTCCGTGAAATCAATATCCCGCACAAGAGTGCTACTCTCCTCGCTCCGGGCCATAACACTTGGGGGTAGCTAAAGTG AACTGTATCCGACATCTGGTTCCTACTTCAGGGCCATAAAGCCTAAATAGCCACACGTTCCCTTAAATAAGACATCACGATG
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[illegible]

[illegible]

[illegible]

ภาคผนวก 2

จำนวนของแฮปโบลไท์ที่พบในแต่ละประชากร

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[illegible]

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Haplotype40	0	1	0	0	0	0	0	0	0	0	1
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Haplotype112	0	0	0	0	0	1	0	0	0	0	1

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Haplotype141	0	0	0	0	0	0	2	2	0	0	4
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Haplotype145	0	0	0	0	0	0	1	0	0	0	1
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Haplotype149	0	0	0	0	0	0	0	1	3	0	4
Haplotype150	0	0	0	0	0	0	0	5	0	0	5

ภาคผนวก 3

Manuscript

Title: Geography has more influence than language on maternal genetic structure of various Northeastern Thai ethnicities

Contributor's names: Wibhu Kutanan^{1*}, Silvia Ghirotto², Giorgio Bertorelle², Suparat Srithawong¹, Kanokpohn Srithongdaeng¹, Nattapon Pontham¹, and Daoroong Kangwanpong³

¹Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

²Department of Life Science and Biotechnology, University of Ferrara, Ferrara 44100, Italy.

³Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

***Correspondence:** Dr. Wibhu Kutanan, Department of Biology, Faculty of Science, Khon Kaen University, 123 Moo 16, Mitthapap Road, Mueang District, Khon Kaen 40002, Thailand.
Email: wibhu@kku.ac.th

Running Title: Maternal lineage in the Northeast of Thailand

Keywords: mtDNA-HVRI/ genetic structure/ Approximate Bayesian Computation/ Austro-Asiatic/ Tai-Kadai/ Sakon Nakhon Basin/ Korat Basin/ Northeastern Thailand

Abstract

Several literatures have shown the influence of geographic and linguistic factors in shaping genetic variation patterns, but their relative impact, if any, in the very heterogeneous Northeastern region of Thailand has not yet been studied. This area, called Isan, is geographically structured in two wide basins, the Sakon Nakorn Basin and the Korat Basin, serving today as home to diverse ethnicities encompassing two different linguistic families, i.e., the Austro-Asiatic; Suay (Kui), Mon, Chaobon (Nyahkur), So and Khmer, and the Tai-Kadai; Saek, Nyaw, Phu Tai, Kaleung and Lao Isan. In this study, we evaluated the relative role of geographic distance and barriers as well as linguistic differences as possible causes affecting the maternal genetic distances among Northeastern Thai ethnicities. A 596-bp segment of the hypervariable region I (HVRI) mtDNA was utilized to elucidate the genetic structure and biological affinity from 433 individuals. Different statistical analyses agreed in suggesting that most ethnic groups in the Sakon Nakorn Basin are closely related. Mantel test revealed that genetic distances were highly associated to geographic ($r = 0.445$, $P < 0.01$) but not to linguistic ($r = 0.001$, $P > 0.01$) distances. Three evolutionary models were compared by Approximate Bayesian Computation. The posterior probability of the scenario which assumed an initial population divergence possibly related to reduced gene flow among basins was equal or higher than 0.87. All analyses exhibited concordant results supporting that geography was the most relevant factor in determining the maternal genetic structure of Northeastern Thai populations.

Introduction

Northeastern Thailand or Isan is geographically located on the Khorat Plateau. Nearly exactly as wide as one third of Thailand, with almost the same population size, Isan shares borders with Laos to the north and the east and with Cambodia to the southeast. The Phu Phan Mountain Range straddles its northeastern interior, separating it into two wide basins, the Khorat Basin in the southwest and the Sakon Nakhon Basin in the northeast (Figure 1). Numerous archaeological excavations, e.g. in Ban Chiang sites, indicate that Isan was primarily inhabited by prehistoric people. The Chaobon and the Suay, who speak languages classified in the Austro-Asiatic family, sub-family Mon-Khmer, are regarded as the original inhabitants in this region before any prosperous civilizations.¹ During the historical period multiple evidence show that, prior to Angkor influence, the Isan region was dominated by two competing kingdoms: Dvaravati, a Mon-Buddhist culture from central Thailand, and Chenla, a Khmer-Hindu culture from Cambodia. During the early 9th century A.D., the Angkorian Khmer state was established. Isan was then integrated into the state as reflected by several remarkable archaeological records, particularly abundant in the Khorat Basin.¹⁻² In the 14th century A.D., the Khmer civilization declined and was unable to resist to the Tai-Kadai speaking people who established the Kingdom of Lan Xang centered in Luang Prabang, in the present-day Laos. Between the late 18th and the early 19th century, during the war within the Lao kingdom, the dissidents began taking refuge into the area of Isan; this represents the first documented evidence of migration from Lao to Isan region. Again in 1827 A.D., an enormous number of Lao people were forcibly migrated to Isan³, thus increasing the dominance of Lao culture in the Isan region but, at the same time, weakening the populations of modern-day Laos.⁴ At that time, besides the Lao people, other ethnic groups from Laos and Vietnam migrated to the area of Northeastern Thailand, including several Tai-Kadai speaking groups, e.g., Phu Tai, Saek, Nyaw and Kaleung as well as the So,

one of the Mon-Khmer speaking populations. Most of them lived in villages along the Mekong River and its tributaries in the Sakon Nakhon Basin. In 1893 A.D., the Isan region became part of the Kingdom of Siam (Thailand) as a result of the Franco-Siamese War.^{1,3}

Isan's long history as well as variety of ethnicities (approximately eighteen groups populated in two distinct geographic locations) make this region an excellent area to elucidate genetic variation and its tentative influencing factors such as geography, language, and culture. A general and simplifying assumption when studying linguistic variation among populations is that a common language frequently signifies a common origin and a related language indicates a common origin further back in time.⁵ Such linguistic relationships should be reflected by genetic variation and might be correlated with geographic distances according to a model of Isolation by Distance (IBD hereafter). Under IBD, current patterns of genetic variation would basically result from the interaction between genetic drift (i.e. random fluctuation of allele frequencies in time) and dispersal of individuals between populations, neglecting all gene flow processes other than those in which movements of individuals from their birthplaces are local and random.⁶⁻⁸ The correspondence between geographic, genetic and linguistic distances would hence be explained by this simple model, except in those cases in which complicating dynamics would affect the expected relationship between geographical distances and genetic and linguistic diversity. These complicating dynamics can be represented by processes of linguistic assimilation during migrations or by the presence of migration resistance factors i.e. geographical barriers to gene flow. In some cases, even language differences themselves can somehow act as a barrier to free gene flow, enhancing the genetic differentiation.⁹⁻¹² In Thailand, where both geographic and ethno-linguistic diversities exist, our previous researches showed the influence of both linguistics and geography on genetic diversity of peoples residing exclusively in the North of

Thailand.¹³⁻¹⁵ However, it is still not clear how, and to what extent, these two factors are related with the genetic variation of Northeastern Thai populations

Maternal inherited mitochondrial DNA (mtDNA) has been proven to be a powerful genetic marker to infer population history in regional and continental frameworks¹⁶⁻¹⁸, however, until now, only four studies on genetic variation of five Northeastern Thai populations (i.e., Phutai, Chaobon, Thai Khon Kaen, Thai Khorat, Thai Isan) have been published.¹⁹⁻²¹

In the present study, we analyzed new mtDNA data of ten Isan ethnicities speaking languages belonging to two major families, namely the Tai-Kadai (Saek, Nyaw, Phu Tai, Kaleung, and Lao Isan) and the Austro-Asiatic (So, Suay, Mon, Chaobon and Khmer), and inhabiting two geographically separated wide basins, namely the Sakon Nakhon Basin (Saek, Nyaw, Phu Tai, Kaleung and So) and the Khorat Basin (Lao Isan, Suay, Mon, Chaobon and Khmer) to evaluate the relative role of geographic distance and barriers and linguistic differences as possible causes affecting the maternal genetic distances among Northeastern Thai ethnicities.

Materials and methods

Samples and DNA extraction

We studied 433 maternally unrelated individuals (for at least three generations) from ten ethnic groups, namely Khmer (KHM), Mon (MON), Suay (SUY), Chaobon (BON), So (SOA), Lao Isan (LAO), Phu Tai (PUT), Nyaw (YOH), Saek (SAK) and Kaleung (KAL), of the Northeast of Thailand. The studied populations were linguistically classified into 2 groups, Austro-Asiatic (AA) and Tai-Kadai (TK), and geographically separated into two groups, Sakon Nakhon (SN) Basin and Khorat (KR) Basin (Table 1 and Figure 1). General information about the studied populations are listed in Table 1. Prior to sample collection,

information on linguistic, cultural aspects, village and individual history was obtained by interview and the informed consent was signed. Buccal swabs were collected from each subject by using a brush embedded in Gentra Puregene Buccal Cell Kit (Qiagen, Hilden, Germany). Genomic DNA was extracted from the collected buccal cells using Gentra Puregene Buccal Cell Kits according to the manufacturer's protocols. The use of human subjects for this study was approved by Ethics Committee for Human Research of Khon Kaen University, Thailand.

Inserted Figure 1 here

Inserted Table 1 here

mtDNA amplification and sequencing

The mtDNA control region (np15704-430) of the ten ethnic groups was amplified using published primer pairs (LLmt-A, 15704-CATAGCCAATCACTTTATTG-15723; LHmt-E, 430-CTGTTAAAAGTGCATACCGCC-410).²² PCR reactions were performed by using *n*Pfu-Forte DNA polymerase (Enzynomics, Daejeon, Korea). Each PCR reaction mix had a final volume of 50 µl consisting of 5 µl of 10X *n*Pfu-Forte buffer, 5 µl of 200 µM dNTP mixture, 2.5 µl of each 5 µM PCR primer, 0.5 µl of 2.5U/µl *Pfu* polymerase, 0.5 µl of 50 ng genomic DNA and 34 µl of distilled water. PCR reactions were performed under the following conditions: 2 min at 95°C for an activation step, followed by 35 cycles of 30 second denaturation at 95°C, 1 min primer annealing at 56°C and 1 min extension at 72°C, and 5 min at 72°C for a final extension step. After visualization on a 1% agarose gel with a 100 bp DNA ladder (Norgen Biotek Corp, Thorold Ontario, Canada), amplicons (approximately 1,200 bp) were sent for purification and sequencing of hypervariable region I (HVRI) (np 15897-100) with a published set of primers¹⁹ (SeqLmt-A, 15897-GTATAAACTAATACACCAGTCTTGT-15921; SeqHmt-E, 100-CAGCGTCTCGCAATGCTATCGCGTG-76) at Macrogen Inc., Seoul, Korea. The

sequencing results were edited, assembled and aligned with the revised Cambridge Reference Sequence²³ using SeqScape software v2.7 (Applied Biosystem, Foster City, CA). The HVRI sequences of all samples were submitted to GenBank (accession numbers KJ205639-KJ206068).

Statistical analyses

Genetic variation within population and demographic parameters

We identified the polymorphic sites of the mtDNA sequences of 596 nucleotides (np 16001-16569) using DnaSP v.5 software.²⁴ Parameters of genetic diversity within populations, i.e. mean pairwise differences (MPD) or intra-MPD, number of segregating sites (S), nucleotide diversity (π), number of observed haplotypes, and the haplotype diversity (h)²⁵ were calculated by Arlequin v.3.5.²⁶ The demographic expansion parameters, i.e., a raggedness index value (r)²⁷ as well as neutrality estimators such as Fu's F_s ²⁸ and Tajima's D ²⁹, were computed by employing the same software. The number of shared haplotypes was determined for each of the 45 possible population pairs by a simple gene-count method.

To compare the genetic variation among populations, we calculated the mean pairwise differences among populations (inter-MPD) and a measure of genetic distance between pairs of populations based on pairwise difference (F_{st} , significance tested by permutation). To characterize population affinity, we plotted in two dimensions the so calculated genetic distance matrix by means of a multidimensional scaling (MDS) using the available Statistica v.10 demo (StatSoft Ltd.). Spatial analysis of molecular variance (SAMOVA) in SAMOVA v.1.0 program was used to infer the most supported genetic structure of the sample, defining groups of populations that are geographically and genetically very similar.³⁰

Genetic variance at three hierarchical subdivisions (within individuals of population, among populations within a group, and among groups of populations), was assessed by the analysis of molecular variance (AMOVA) procedure³¹ as implemented in Arlequin v. 3.5. In

this analysis, studied populations were grouped by both geography and language (See Table 1)

Mantel test

The correlations and partial correlations between distance matrices of genetics-geography, genetics-language, and geography-language were performed by the Mantel test.³² Table 2 shows the matrices of geographic and linguistic distance we used for the Mantel test, whereas genetic distance (F_{st}) matrix is shown in Table 3. Geographic distances in Km between the approximate locations of each population were computed as great-circle distances calculated from their latitudinal and longitudinal coordinates. Linguistic distances between pairs of populations were defined as simple dissimilarity indices on the basis of the hierarchical classification of languages reported in Ethnologue.³³ Populations speaking languages belonging to different subfamilies, i.e., AA and TK, were assigned dLAN of 4 while different branches within subfamilies were assigned dLAN of 3. Different sub-branches within branch were assigned dLAN of 2 and then dLAN of similar sub-branches was 1.

Inserted Table 2 here

Approximate Bayesian Computation

To deeply investigate the evolutionary relationship among populations, an Approximate Bayesian Computation (ABC) procedure was applied.³⁴ An ABC approach, which combines the analysis of large genetic data sets and realistic models, can be briefly summarized as follows: millions of genetic datasets with the same features as the observed one, i.e., number of individuals, type of genetic markers, length of sequences, are generated according to the coalescent theory for each demographic model, taking into account the associated prior distributions. The pattern of genetic variation in the observed and simulated data, summarized by a certain number of statistics, is then compared by Euclidean distance.

The coalescent-based simulations were performed by combinations of parameters for a specific demographic model. Those coalescent-based simulations which generated summary statistics closest to the observed ones, as shown by smallest Euclidean distances, were then considered for calculating the posterior probabilities of each model using two different approaches, acceptance-rejection procedure (AR) and weighted multinomial logistic regression (LR).³⁵⁻³⁶ Under the AR, the posterior probability of a model is obtained by considering only a certain number of “best” simulations, and then simply counting the proportion of these retained simulations that have been generated by each model under investigation. This method can be considered reliable only when applied to a few simulations showing an excellent fit with the observed data, i.e. few hundreds.³⁶ Under LR procedure, a logistic regression is fitted where the model is the categorical dependent variable in the ABC simulations and the summary statistics are the predictive variables. The regression is local around the vector of observed summary statistics, and the probability of each model is finally evaluated at the point corresponding to the observed vector of summary statistics. The β coefficients of the regression model are estimated by maximum likelihood and the standard errors of the estimates might be taken as a measure of the accuracy of the method. To evaluate the stability of the models’ posterior probabilities, we considered different thresholds by considering different number of retained simulations for both the model selection procedures (100, 200, 300, 500 best simulations for AR and 25000, 50000, 75000, 100000 best simulations for LR). To generate the simulated datasets we used the software package ABCtoolbox³⁷, running 500,000 simulations for each model. To calculate the models’ posterior probabilities we used R scripts from <http://code.google.com/p/popabc/source/browse/#svn%2Ftrunk%2Fscripts>, modified by SG. To summarize the genetic information contained in the data we calculated the following

statistics within and between populations: the number of haplotypes (h), the number of private polymorphic sites (S), Tajima's D , intra- and inter-MPD, and pairwise F_{st} .

Testing the best-fit demographic models and type I error

Based on geographic locations and linguistic affiliations of the studied populations, SOA and LAO were variable populations whose languages differ from their geographically grouped neighbors. Therefore, three demographic models were proposed to describe different aspects of the evolutionary relationships among studied populations, in which geography or language was fitted to describe mtDNA data (Figure 2). In Model 1, the separation of the lineages follows the linguistic affiliation, with a first split (Ts1) involving the AA and the TK groups, and a subsequent separation by geographic location at Ts2 (AA and SOA), and Ts3 (TK and LAO). In Model 2, the “driving force” of the genetic variation is represented by geography. A first separation (Ts1) is started between populations from KR Basin and from SN Basin. Within each geographical group, the LAO and SOA, who speak different languages from their neighbors, were subsequently separated at Ts2 and Ts3, respectively. Model 3 extends Model 1, in which after Ts2 and Ts3, geographically closer populations (LAO-AA; SOA-TK) start to exchange migrants at a certain rate [$m1$ ($m1_b$) and $m2$ ($m2_b$)]. The effective population sizes were assumed to be constant in time; the prior distributions were all uniform (log-uniform for the effective population sizes), and, where possible, based on historical records (see supplementary Table 1).

We estimated the probability that the true null hypothesis would be rejected by evaluating the type I error. The proportion of cases in which 1,000 pseudo-observed datasets, generated under each model, is not correctly identified by the ABC analysis (both AR and LR procedures, 100 and 50000 retained simulations in turn). The power of the model choice

procedure has been evaluated using a wide range of decision probability thresholds to identify the support for a specific model, i.e. 0.5, 0.6, 0.7, 0.8, 0.9.

Inserted Figure 2 here

Results

Genetic diversity and demographic expansion

A total of 173 distinct mtDNA haplotypes were observed in 433 individuals. Among the observed haplotypes, 142 types were unique within populations, whereas the other 31 types were shared between two or more populations. Out of the 142 unique haplotypes, 34 were shared by two or more individuals within one group (multiple unique), whereas the remaining 108 haplotypes belonged to each individual (single unique). The highest number of shared haplotypes (6 haplotypes) was found between SOA-PUT, but none were shared among five pairs of populations: MON-BON, MON-LAO, MON-PUT, BON-KHM, and BON-SOA.

Genetic diversity within population and population expansion results are reported in Table 1. Haplotype diversity (h) varied from 0.9899 (LAO) to 0.7920 (SAK) which was in the same range as previous published populations in Thailand^{13-15,19-21}, albeit rather a low h value was found in SAK, indicating possible drift effect. The lowest nucleotide diversity (π) was observed in MON (0.0098), while PUT had the highest value (0.0153). The intra-MPD ranged from 5.5254 (MON) to 8.6956 (PUT), reflecting genetic homogeneity or recent diverged mtDNA within the MON and genetic heterogeneity in the PUT.

The highly significant negative values of the Fu's F_s ($P < 0.05$) were predictions of demographic expansion in KHM, MON, SOA, LAO, and PUT. The lower raggedness index (less than 0.03) as well as the unimodal mismatch distribution graph for these populations (data not shown) also provide congruent evidence for population growth and expansion.³⁸

Genetic relationships

Among 45 pairwise F_{st} comparisons, 36 (80%) were statistically significant ($P < 0.01$) (Table 3). The MON, BON, and SAK showed significant F_{st} values for all comparisons, indicated high genetic differentiation. The KHM had genetically differentiated from almost all other populations, except the LAO. It is interesting that SUY and BON has genetic similarity. Most F_{st} comparisons between populations in SN Basin were not statistically significant, particularly among the SOA, PUT, and YOH, reflecting genetic homogeneity. The corrected MPD among populations showed a similar pattern to F_{st} result (see supplementary Table 2), which indicate that the SAK were most differentiated while the next most respectively differentiated populations were the BON, MON and SUY.

Inserted Table 3 here

To visualize the genetic relationship among populations, we plotted a pairwise F_{st} matrix through MDS analysis and performed SAMOVA analysis. In the MDS as shown in Figure 3, most populations residing in the SN basin (YOH, PUT, SOA and KAL) were clustered in the center of the plot with the exception of the SAK which appear to be the most genetically differentiated population, even if still genetically more closely related to neighbors in the SN Basin than to populations from the KR basin. Surprisingly, although the LAO resided in the KR Basin, they clustered together with other SN dwelling populations. For the ethnicities located in the KR basin, the KHM were quite genetically proximate to the SUY and BON, while the MON was considerably distanced from other studied populations indicating their genetic distinction. In SAMOVA analysis, when number of group was increasing from 2-groups until 6-groups category, the SAK, MON, SUY, BON, and KHM respectively were partitioned from the other populations (Table 4). The maximal percent of variation with significant value was observed at 6-groups category (7.287%, $P < 0.01$): SAK, MON, SUY, BON, KHM, YOH-PUT-SOA-KAL-LAO. Interestingly, population grouping by SAMOVA was concordant to MDS plot.

Inserted Figure 3 here

Inserted Table 4 here

Correlation among genetics, geography and language

The AMOVA was used to infer the proportion of total genetic variation accounted by groups. Groupings were defined on the basis of geographic and linguistic classification (Table 5). When populations were grouped according to geography, the results revealed that it can be used to describe the genetic structure of studied populations, since the amount of observed variation among groups was 4.68% with statistical difference ($F_{ct}=0.0468$, $P<0.01$), whereas the proportion of variance among population within groups explain 7.429 % ($F_{sc}=0.07429$, $P<0.01$) and within populations explain 11.765% ($F_{st}=0.11765$, $P<0.01$). The average F_{st} of populations in the SN basin ($F_{st}=0.06902$, $P<0.01$) and in the KR basin ($F_{st}=0.07900$, $P<0.01$) were much lower than the overall F_{st} (0.09889, $P<0.01$). It seems evident that there is a certain level of genetic homogeneity among populations within each geographic region, with an higher homogeneity in populations from the SN basin than in populations from the KR basin.

Based on linguistic classification, the proportion of genetic variation among groups was considerably low (0.913 %) with no statistical significance ($F_{ct}=0.00913$, $P>0.01$), reflecting no relationship between genetic distance and linguistic affiliation. Most of the genetic variance (89.74%) was found within populations ($F_{sc}=0.09434$, $P<0.01$), while variance among populations within the linguistic groups was 9.35% ($F_{st}=0.10260$, $P<0.01$). We observed a slight higher value of average F_{st} of AA ($F_{st}=0.10681$, $P<0.01$) respect to Tai speaking group ($F_{st}=0.07820$, $P<0.01$), possibly indicating more genetic heterogeneity among Austro-Asiatic than among Tai-Kadai groups. A notable amount of genetic variance was found among geographic groups, which is higher than variance among linguistic groups.

Mantel testing showed that genetic distances strongly correlated to geographic distances by means of correlation test ($r = 0.4713$, $P < 0.01$) and partial correlation test ($r = 0.4449$, $P < 0.01$), whereas, we detected no correlation and partial correlation between genetic and linguistic distances ($r = 0.1735$, $P > 0.01$ and $r = 0.0008$, $P > 0.01$, respectively). Among geographic and linguistic matrices, no correlation ($r = 0.3667$, $P > 0.01$) and partial correlation ($r = 0.3281$, $P > 0.01$) was observed.

Inserted Table 5 here

Model Selection

Table 6 shows the posterior probabilities of the three considered evolutionary scenarios. Model 2, in which the geography has a major role in shaping the genetic variation, received the strongest support. The posterior probability of Model 2 was never lower than 87%, considering both AR and LR and remained stable over different number of retained simulations. To assess the reliability of the probabilities estimated, we also evaluated the models' posterior probabilities within two times the range of the standard error associated to the β coefficients of the regression model (in both directions). The support remained in favor of Model 2.

Inserted Table 6 here

To evaluate whether there is enough power in the data for these models to be discriminated, we calculated a type I error, i.e., the incorrect rejection of a true null hypothesis. This analysis has fundamentally verified the reliability of the estimated probabilities because the compared models were quite similar to each other, and only a single genetic locus was analyzed. The type I error analysis considered both AR and LR as criterion of model selection, and several probability thresholds to identify the support for a specific model (Table 7). The results of the logistic regression (50,000 best simulations) were in general better than those obtained with the acceptance-rejection, especially for higher

probability thresholds. The models appeared to be well recognized even when the decision probability threshold was 0.5, since the probability of recognize the right model was never lower than 0.6. Moreover, when the right model was not selected as the “true” one, the alternative models almost never reached a probability high enough to be supported by the ABC model selection procedure. This was particularly true when the decision probability threshold was very high (0.9), i.e., similar to the value we obtained from the real data for Model 2. All together these results can be considered highly significant, and the model that has been selected here (Model 2) can be confidently regarded as the best one.

Inserted Table 7 here

Discussion

MtDNA sequences data analyzed in the current study provide us a better understand about the level of genetic variation in a micro-geographic scale and about past population dynamics in several ethnicities of the Isan region or Northeastern Thailand. Whether geography or language most influenced genetic variation of populations within regional, continental, and worldwide scales have been long-standing questions for molecular anthropologists and human geneticists.³⁹⁻⁴³ To date, there has been no report aimed to answer the above question for populations residing in the Northeast of Thailand, addressed here for the first time. Results obtained from Mantel test, AMOVA, SAMOVA, and ABC procedures indicate that geography plays an important role to determine Northeastern Thai genetic structure, according to IBD model. Under IBD, current patterns of genetic variation would simply result from the interaction between genetic drift and dispersal of individuals between populations, thus resulting in a decrease of genetic similarities between populations when geographic distance increases⁴⁴⁻⁴⁵. Based on linguistic and archaeological data, each of the two different geographic regions in Isan was occupied by linguistically distinct groups of

people. The native AA populations were mainly resided in KR basin⁴⁶, while the TK people who migrated from Laos and Vietnam were centered in the SN Basin.³

Living in close geographical proximity, the SOA and their neighbors (PUT, SAK, KAL, and YOH), have languages of unrelated ancestry. Our genetic findings reveal the parallelism between genetic variation and geographic factors. The SOA's historical homeland is in the forested covered hills of Khammuan Province in Laos. Some of the tribe members migrated to Thai soil, in the area of Kusumal District, in 1844 A.D.¹ Based on ABC procedure (Figure2), the three demographic scenarios might be suggested to explain the degree of genetic resemblance between SOA and other SN populations, possibly linked to a recent common origin. The SOA and other neighbors within SN Basin might have shared genetic similarity from the time they resided in their historical homeland in Laos and Vietnam. At that time, they might have come into contact and after the migratory time with spatial and temporal different settlement in Thailand, their genetic homogeneity continued. The two greatest numbers of shared haplotypes between SOA-PUT and SOA-YOH, respectively, could be additionally explained by the same genetic source between the SOA and their neighbors.

In accordance with pairwise F_{st} , MPD and SAMOVA, the peculiar genetic divergence of the SAK made this population particularly interesting. Historically, the SAK originated in Vietnam and then with the influence of the Kinh (the vast majority of Vietnamese) they moved westward to Laos around 380 years ago. The majority of SAK are centered in Khammuan Province of Laos and they migrated across the Mekong river into Nakorn Panom Province of Thailand about 200 years ago.¹ The greatest differentiation as seen in the SAK is likely a consequence of genetic drift associated with female immigrants during the settlement period. The limited genetic diversity, as reflected by the lowest haplotype diversity and second lowest intra-MPD (Table 1), were regarded as reliable indicators of a genetic

bottleneck.⁴⁷ The debates on the origin of the SAK have arisen in linguistic classification. At first, the language of the SAK was classified as belonging to the AA family in the Mon-Khmer sub-family, but later most linguists classified the SAK language to the TK family in the Northern Tai branch, spoken mainly by the Tai in Guangxi Province of China.^{1,48} The SAK exhibited closest genetic relationship to the SOA. It might be indicated that the SAK are genetically more closely related to AA than to TK groups. Thus, based on several articles reporting the strong association between linguistic and genetic classifications⁴⁹⁻⁵², to our knowledge, the present-day SAK language classification is not in agreement with genetic affinity. However, it should be cautioned that the genetic ancestry of the SAK might be blurred by strong influences of the geographic factor.

Almost all AA groups, KHM, BON, SUY, and MON, as well as the only TK village of the LAO, were dispersedly situated in the KR basin. LAO or Lao Isan refers to peoples who are ethnically Lao but are Thai citizens.⁴ They comprise the majority of inhabitants and are widely distributed in all provinces of Northeastern Thailand. Most of Lao Isan people were forcibly migrated from their historical homeland in the present-day Laos during 1827-1870 A.D.³ Although the LAO village in this study was located within the area of KR Basin, close genetic affinity between the LAO and populations in the SN Basin was detected. Through previous massive migration, the LAO in the SN and KR Basins might have still preserved genetic similarity, thus, close genetic relationship might have resulted in low levels of differentiation between LAO populations in the SN and KR Basins. Future study with more broadly samples of LAO from the SN Basin will be helpful to evaluate this assumption.

Interestingly, non-significant pairwise F_{st} between LAO and KHM could be plausibly explained by extensive gene flow, concordant with an earlier genetic study²⁰, and socio-linguistic research.⁵³⁻⁵⁵ Although current study's results support that geography explains genetic variation and relationship among populations, we somehow detect significant genetic

differentiations among populations within the KR Basin. It might be suggested that geographic proximity determined the genetic homogeneity among AA populations in the past, but later on the factors of cultural and linguistic differences as well as evolutionary factors, like drift effect, inbreeding, and genetic exchange, overcame the influence of spatial isolation, as reflected in KHM, BON, SUY, and MON.

A certain degree of inbreeding is evident particularly in the Chaobon (BON), alternately called Nyahkur. Chaobon inhabited the area that is now Thailand preceding the coming of the Khmer and the Tai groups. They now lived in Thailand only in Chaiyabhum, Petchaboon, and Nakorn Rachasima provinces. The bulk of these people live in Chaiyabhum Province, scattered among different deep jungle and mountainous villages.^{1,56-57} The most original Chaobon tribe in Wang Ai Pho village in Chaiyabhum Province, who still preserved their language and culture, was sampled in this study. Loss of genetic diversity, as indicated by low values of h , S and intra-MPD, might reflect consanguineous marriage due to cultural isolation. This study has documented the sequential genetic effects from preserved cultural practice within this population before they may be possibly erased by the opportunity for admixture with Lao Isan people. Based on linguistic research, Chaobon are believed to be the remaining descendants of the ancient Mon from the historic Dvaravati period. Contrary to our expectation, the present results do not support the genetic bond between the extant BON and MON.

The Mon are one of the oldest settlers in Southeast Asia. Their origin is uncertain. It is known that they once lived in Southwest China, and moved down to upper Myanmar early in the Christian era. They were politically driven southward to settle in Pegu and Thaton, in Myanmar and eastward to the present-day Central and Southern Thailand, respectively. The great Mon Dvaravati Kingdom with an advanced civilization was founded between the 3rd and 10th century A.D. in the area of Central Thailand¹. The prosperous Mon Kingdom

expanded to present-day Southern, Northern and Northeastern Thailand. In 1775 A.D., the first group of studied Mon migrated from Myanmar to settle down in Nakorn Rachasima, further increasing in population size to approximately 2,500 around 1793 A.D. The studied MON who historically migrated from Myanmar was indeed different from Dvaravati Mon in Central Thailand, therefore a genetic link between BON and MON was not apparent. Another important finding emerged from the results of genetic diversity and demographic expansion parameters which exhibit the lowest π , intra-MPD, and number of multiple unique haplotypes. These, as well as positive signals of population growth in the MON (Table 2), provide congruent evidence for a recent bottleneck followed by an expansion in the population, which have not yet been recognized in socio-linguistic and historic literatures.

Worthy of attention is the genetic ancestry of the Suay (SUY). MDS result reveals the close genetic relatedness between SUY and KHM, while pairwise F_{st} indicates non-significant genetic difference between SUY and BON. These results seem to be congruent with previous historic research documenting connections between SUY and KHM in language, history, society, and ancestry. The Suay or Kui, called Kamen-boran (meaning ancient Khmer) by Khmer people, are the original inhabitants of part of Thailand, Laos, and Cambodia, predating the invasion of the Khmer and the Tai group. Nowadays Suay in Thailand have been adopted a Thai-Lao language referred to as Lao-Suay or a Khmer language referred to as Khmer-Suay.¹ The current studied Suay from Surin Province migrated at first from Southern Laos during 1656-1688 A.D. and then sporadically moved until around 1760 A.D. when the mass migration period occurred.⁵⁸ However, it has been proposed by some scholars¹⁴ that SUY share ancestry with BON, now strengthened by our investigation.

To summarize, this study highlighted some main aspects of maternal genetic structure of various populations in Northeastern Thailand. Genetic findings obtained through this

study made it possible to infer the influence of geographic factors in shaping patterns of genetic variations and affinity among linguistically diverse populations. Genetic divergence between populations was primarily influenced by geography. Then, within the same geographic location different driving forces, including language and culture as well as evolutionary driven factors, like genetic drift from founder effect, inbreeding, and admixture are considered to be the plausible additional factors. Our results remain open to future investigations with further mtDNA sequences from other populations and genetic data from different genetic markers to gain more insight into genetic history of Northeastern Thai people.

Conflict of Interest

The authors declare no conflict of interest.

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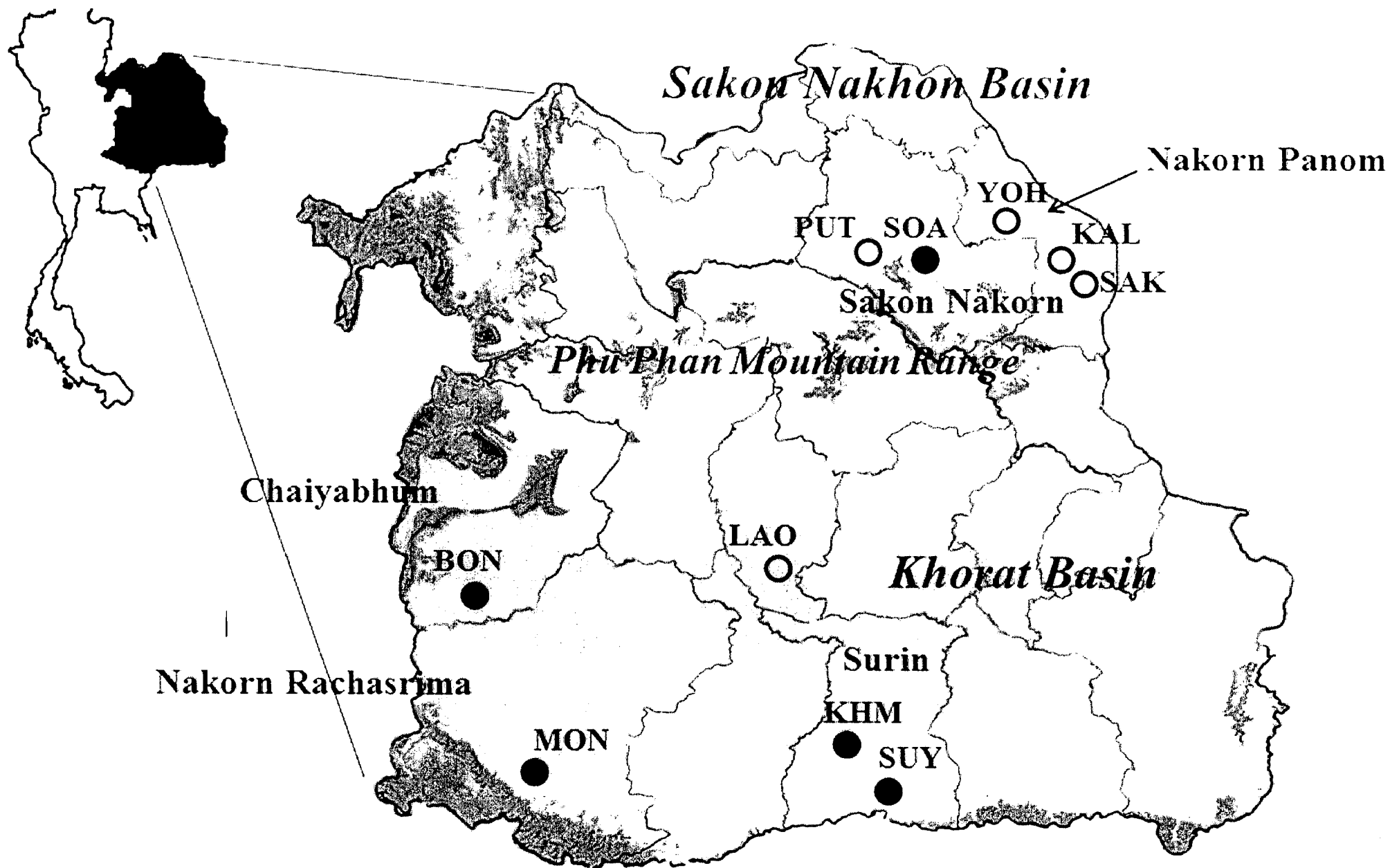
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Titles and legends to figures

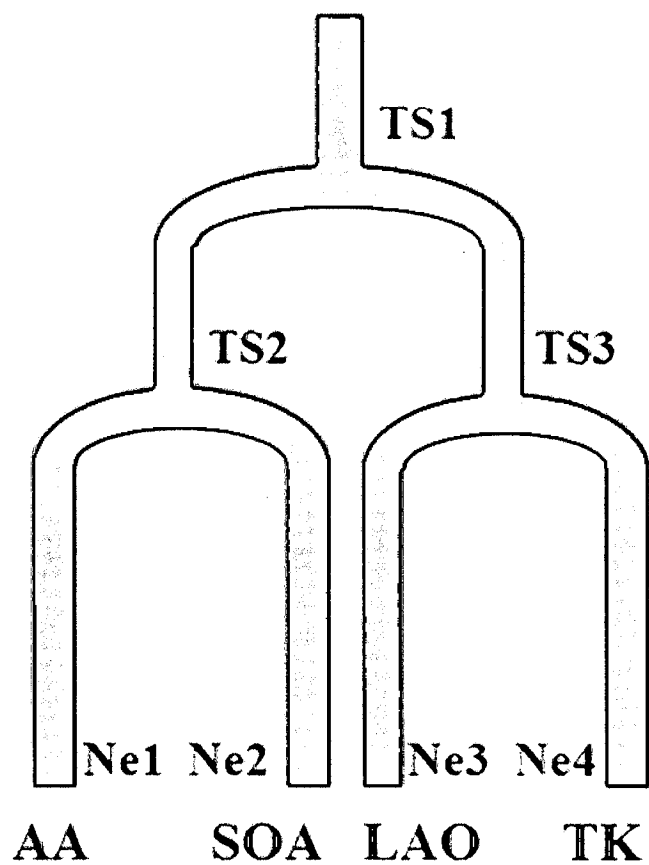
Fig. 1 Map of Khorat Plateau showing the locations of studied populations in different geographic areas of Northeastern Thailand. Population codes are given in Table 2. Filled circles: Austro-Asiatic linguistic family; Empty symbols: Tai-Kadai linguistic family.

Fig. 2. Schematic presentation of the three models described by language (Model 1), geography (Model 2), and recent migration (Model 3). N_e , T_s and m are the effective population sizes, separation times and the migration rates, respectively. Population codes are given in Table 2.

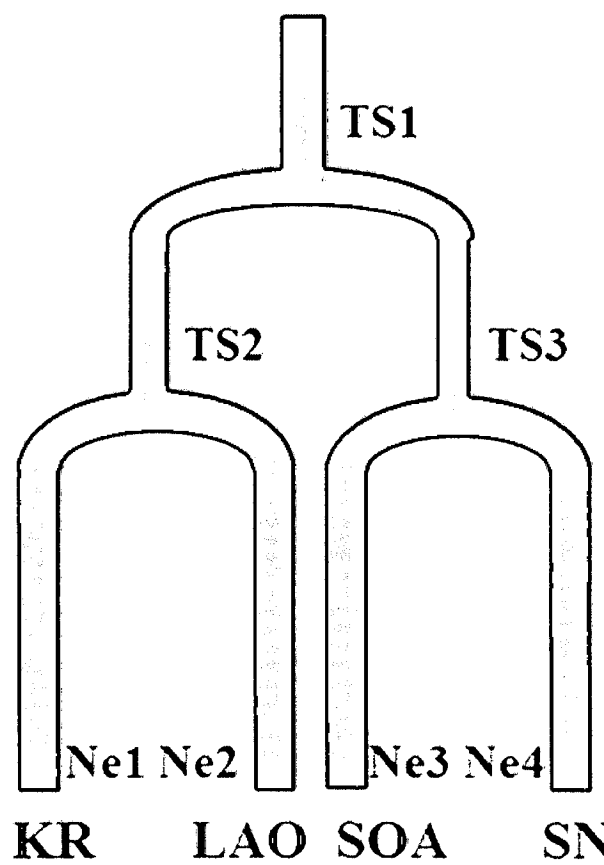
Fig. 3. Three dimensional scaling plot (3D-MDS) constructed based on pairwise F_{st} . Population codes are given in Table 1. Stress value for MDS = 0.0339.



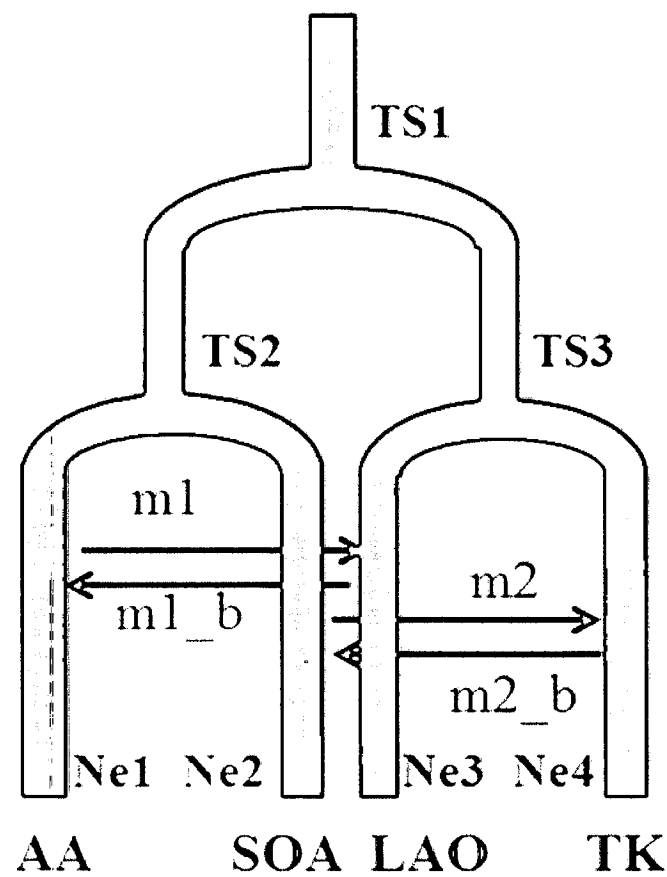
Model 1



Model 2



Model 3



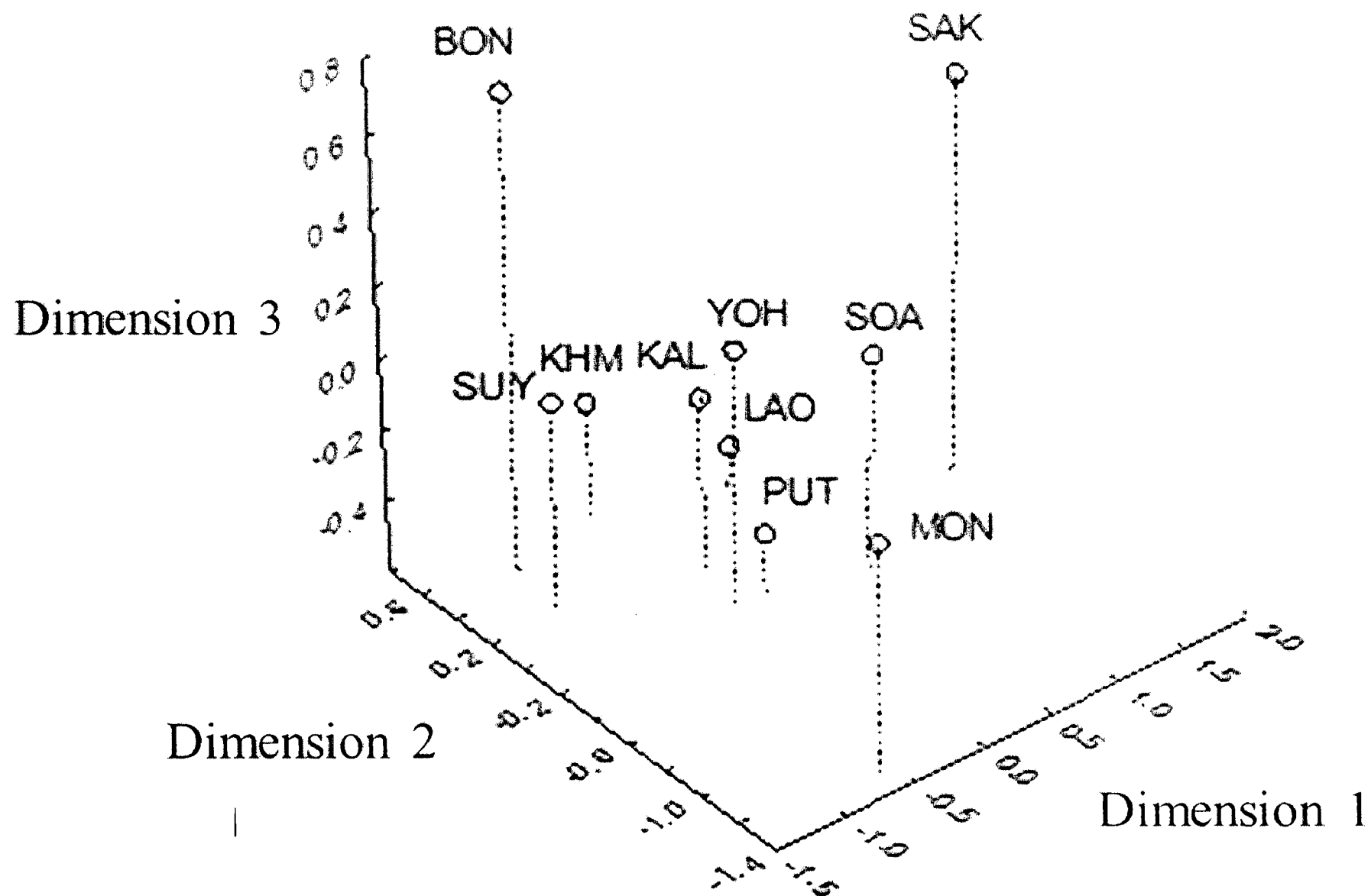


Table 1. General information of studied populations and summary statistics

Populations	Khmer	Mon	Suay	Chaobon	So	Lao Isan	Phutai	Nyaw	Saek	Kaleung
Code	KHM	MON	SUY	BON	SOA	LAO	PUT	YOH	SAK	KAL
Latitude	14.90	14.69	15.01	15.59	17.37	15.62	17.28	17.55	17.45	17.33
Longitude	103.49	102.06	103.94	101.46	104.30	103.50	103.65	104.09	104.74	104.59
Language	AA	AA	AA	AA	AA	TT	TT	TT	TT	TT
Geography	KR	KR	KR	KR	SN	KR	SN	SN	SN	SN
Location (District, Province)	Sangkla, and Chumpholburi, Surin	Pakthongchai, Nakorn Rachasrima	Sam Rong Tap, Surin	Thepsatit, Chaiyabhum	Kusuman, Sakon Nakorn	Kaset Wisai, Roiet	Waritchabhum, Sakon Nakorn	Nawa, Sakon Nakorn	Mueang, Nakorn Panom	Kuruku, Nakorn Panom
Sample size	68	44	44	42	47	35	38	41	28	46
^a Population size	1,266,828	1,000	407,724	6,283	71,532	11,135,493	457,411	406,738	3,535	68,431
Haplotype	37	23	22	12	27	30	23	20	11	21
Unique	24	19	12	10	16	21	14	9	6	11
Single unique	20	12	11	6	12	17	10	7	4	9
Multiple unique	4	7	1	4	4	4	4	2	2	2
Non-unique	13	4	10	2	11	9	9	11	5	10
<i>h</i>	0.9583	0.9545	0.9397	0.8583	0.9584	0.9899	0.9573	0.9402	0.7920	0.9063
π	0.0130	0.0098	0.0143	0.0121	0.0141	0.0149	0.0153	0.0131	0.0114	0.0115
Intra MPD	7.3995	5.5254	8.1057	6.8269	8.0324	8.4924	8.6956	7.4317	6.4929	6.5266
Polymorphic site	54	40	47	23	48	54	47	39	33	35
Tajima's D (p-value)	-1.0596 (0.1370)	-1.3277 (0.0710)	-0.9632 (0.1730)	1.1116 (0.8990)	-0.9327 (0.1850)	-1.3016 (0.0780)	-0.8134 (0.2270)	-0.6458 (0.2900)	-0.8067 (0.2200)	-0.5512 (0.3170)
Fu's <i>F_s</i> (p-value)	-17.1136 (0.0000)	-8.3834 (0.0080)	-3.8913 (0.1130)	1.4256 (0.7610)	-8.3333 (0.0080)	-19.0744 (0.0000)	-5.6044 (0.0400)	-3.3709 (0.1190)	0.3691 (0.5630)	-4.3474 (0.0760)
<i>r</i>	0.0204	0.0195	0.0332	0.0572	0.0154	0.0095	0.0108	0.0203	0.0694	0.0399

AA = Austro-Asiatic linguistic family; TT = Tai-Kadai linguistic family; KR= Khorat Basin; SN = Sakon Nakorn Basin

^aPopulation size estimated in Northeastern Thailand

h = haplotype diversity; π = nucleotide diversity; *r* = a raggedness index value

Table 2. Linguistic distance matrix (below diagonal) and geographic distance matrix (above diagonal) using the Mantel test. See the population abbreviation key in Table 1.

	KHM	MON	SUY	BON	SOA	LAO	PUT	YOH	SAK	KAL
KHM		155.63	50.07	237.46	286.91	79.85	264.69	300.36	312.93	293.54
MON	3		205.48	118.45	380.8	185.71	333.54	383.36	409.11	397.83
SUY	2	3		274.47	264.15	82.47	253.75	281.65	283.79	265.91
BON	3	3	3		361.54	219.26	300.24	354.87	406.89	385.84
SOA	2	3	1	3		211.57	69.04	29.61	48.38	31.53
LAO	4	4	4	4	4		184.99	222.63	242.41	221.96
PUT	4	4	4	4	4	1		54.84	117.38	99.7
YOH	4	4	4	4	4	1	1		70.31	58.55
SAK	4	4	4	4	4	2	2	2		21.41
KAL	4	4	4	4	4	1	1	1	1	

Table 5. AMOVA analysis

	No. of groups	No. of populations	% of variance			F_{st}	F_{sc}	F_{ct}
			Within populations	Among populations Within groups	Among groups			
Geography								
All samples	1	10	90.11	9.89		0.09889		
SN	1	5	93.10	6.90		0.06902		
KR	1	5	92.10	7.90		0.07900		
SN/KR	2	10	88.235	7.081	4.684	0.11765	0.07429	0.04684
Language								
All samples	1	10	90.11	9.89		0.09889		
TT	1	5	92.18	7.82		0.07820		
MK	1	5	89.32	10.68		0.10681		
TT/MK	2	10	89.74	9.35	0.91	0.10260	0.09434	0.00913

Bold letters indicate statistical significance at $P < 0.01$

F_{st} = Fixation index among populations and groups

F_{sc} = Fixation index among populations but within groups

F_{ct} = Fixation index among groups

AA = Austro-Asiatic linguistic family; TT = Tai-Kadai linguistic family; KR = Khorat Basin; SN = Sakon Nakorn Basin

Table 6. Posterior probabilities of three population models computing by acceptance-rejection procedure (AR) and weighted multinomial logistic regression (LR) approaches.

Threshold	Model 1	Model 2	Model 3
AR			
100	0.090	0.910	0.000
200	0.070	0.910	0.020
300	0.077	0.907	0.017
500	0.078	0.904	0.018
LR			
25000	0.009	0.873	0.118
50000	0.006	0.870	0.124
75000	0.005	0.883	0.112
100,000	0.004	0.906	0.090

Table 7. Type one error results for three best model emerging from an ABC analysis.

AR					LR				
probability threshold	probability of recognize the right model				probability threshold	probability of recognize the right model			
	Model 1			Not		Model 1			Not
	(true)	Model 2	Model 3	Assigned		(true)	Model 2	Model 3	Assigned
>0.5	0.49	0.1	0.01	0.4	>0.5	0.59	0.12	0.06	0.23
>0.6	0.4	0.02	0.01	0.57	>0.6	0.54	0.07	0.03	0.36
>0.7	0.33	0	0	0.67	>0.7	0.45	0.05	0.01	0.49
>0.8	0.2	0	0	0.8	>0.8	0.35	0.03	0	0.62
>0.9	0.08	0	0	0.92	>0.9	0.19	0	0	0.81
	Model 2		Not			Model 2		Not	
	Model 1	(true)	Model 3	Assigned		Model 1	(true)	Model 3	Assigned
>0.5	0.08	0.45	0.06	0.41	>0.5	0.07	0.61	0.18	0.14
>0.6	0.03	0.33	0.01	0.63	>0.6	0.04	0.5	0.07	0.39
>0.7	0.01	0.26	0	0.73	>0.7	0.01	0.41	0.03	0.55
>0.8	0	0.11	0	0.89	>0.8	0	0.32	0.02	0.66
>0.9	0	0.05	0	0.95	>0.9	0	0.17	0	0.83
	Model 3		Not			Model 3		Not	
	Model 1	Model 2	(true)	Assigned		Model 1	Model 2	(true)	Assigned
>0.5	0.02	0.08	0.59	0.31	>0.5	0.04	0.09	0.7	0.17
>0.6	0	0.05	0.37	0.58	>0.6	0.02	0.06	0.61	0.31
>0.7	0	0.02	0.16	0.82	>0.7	0	0.02	0.49	0.49
>0.8	0	0.01	0.04	0.95	>0.8	0	0.01	0.38	0.61
>0.9	0	0	0	1	>0.9	0	0	0.23	0.77