รายงานวิจัยฉบับสมบูรณ์

โครงการ บทบาทของยีนในนิวเคลียสต่อการแสดงออกของโรคไมโตคอนเดรีย: Leber Hereditary Optic Neuropathy (LHON)

โดย

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กันยายน 2545-สิงหาคม 2548

บทคัดย่อ

โรค Leber hereditary optic neuropathy (LHON) เป็นโรคไมโตคอนเดรียที่ถ่ายทอดทางพันธุกรรม ผู้ป่วยโรคนี้จะมีอาการตาบอดและจะมีอาการเมื่ออยู่ในช่วงอายุ 15-25 ปีและเป็นในผู้ชายมากกว่าผู้หญิง โรคนี้เกิดจากการกลายพันธุ์ของยีนไมโตคอนเดรีย ตำแหน่งที่เกิดการกลายพันธุ์แล้วทำให้เกิดโรคมีอยู่หลายตำแหน่ง จากการที่พบโรคนี้มากในผู้ชาย และสมาชิกในครอบครัวของผู้ป่วยที่ถึงแม้จะมีการกลายพันธุ์นี้หลายคนไม่เกิดมีอาการของโรค ทำให้เชื่อว่าน่าจะมีปัจจัยอื่นเกี่ยวข้องกับการแสดงออกของโรคนอกเหนือจากการกลายพันธุ์ของยีนไมโตคอนเดรียแต่เพียงอย่างเดียว งานศึกษานี้มีจุดประสงค์จะค้นหายีนในนิวเคลียสที่จะมีผลต่อการแสดงออกของโรคไมโตคอนเดรียโรคนี้ จึงได้ทำการศึกษาในครอบครัวผู้ป่วยที่มีขนาดใหญ่จำนวน 8 ครอบครัว โดยทำ genome scan ด้วย microsatellite marker จำนวน 400 ตัว ผลการศึกษาโดยใช้ nonparametric linkage analysis พบว่ามีส่วนที่อยู่บนโครโมโซม 1, 3, 12, 13, 18 และ โครโมโซมเอกซ์ น่าจะมียีนที่อาจมีความสำคัญต่อการแสดงออกของโรคนี้ ส่วนบนโครโมโซมทั้ง 6 ส่วนนี้ได้ถูกนำไปวิเคราะห์ในแต่ละครอบครัวและพบว่าในแต่ละส่วนมีผลต่อการแสดงออกของโรคในแต่ละครอบครัวไม่เท่ากันซึ่งเป็นไปตามลักษณะดังที่คาด

Abstract

Leber hereditary optic neuropathy (LHON) is a mitochondrial genetic disease that commonly causes blindness in young adult males. Three primary mitochondrial DNA mutations are found in more than 95% of LHON cases world wide. The marked incomplete penetrance and gender bias of this disease indicates that additional genetic and/or environmental factors are required for the phenotypic expression of the pathogenic mtDNA mutations in LHON. In this study, we conducted a genomewide linkage scan in order to search for nuclear genes influenzing the expression of LHON in our 8 large LHON pedigrees of Thai or Chinese-Thai origin and one of Indian origin. Using the 16 different allele scoring models of multipoint nonparametric linkage analysis, 13 regions in 12 chromosomes which showed Z1r score > 2 ($p<0.05$) were found. The maximum Z1r was 2.87 ($p=0.002$) at the marker D12S352 in chromosome 12 using exp mnallele equal model. However, when compared all the 16 models, only 4 regions on chromosomes 3, 12, 13 and 18 showed Z1r > 2 fairly consistently across several models. The peak of Z1r > 2 at marker D1S207 in chromosome 1 was excessively contributed in one of our LHON family whereas the peak at marker DXS1227 in chromosome X was also worth looking at. We then ended up with 6 interesting chromosomal region on chromosome 1, 3, 12, 13, 18 and X which could be promising candidates for the nuclear modifier gene (s) in Thai LHON. These 6 regions were analysed in each individual of each family and it was found that different families contributed to the overall allele sharing score at different extent. Our result, to our knowledge, is the first genomewide scan analysis for the nuclear modifier genes of LHON.

EXECUTIVE SUMMARY

ชื่อโครงการ

(ภาษาไทย) บทบาทของยีนในนิวเคลียสต่อการแสดงออกของโรคไมโตคอนเดรีย: Leber Hereditary Optic Neuropathy (LHON)

(ภาษาอังกฤษ) Nuclear modifier for a mitochondrial DNA disorder: Leber Hereditary Optic Neuropathy (LHON)

ความสำคัญและที่มาของปัญหา

While there has been a major progress in the identification of a large number of disease-related mutations in the mitochondrial DNA (mtDNA), the pathobiology of these mutations in general is still poorly understood. The frequent lack of correlation between the mutations and their biochemical expression, their relatively tissue specific clinical manifestation despite the mitochondrial oxidative energy metabolism being essential for the function of almost all cell types, and the incomplete and often low penetrance of the mutations, all point to the complexity of the pathogenesis of the resulting disorders. Indeed, it might be more appropriate to consider these disorders as polygenic, with the clinical expression of the causal mtDNA mutations being modulated by other genetic factors, both in the nuclear and in the mitochondrial DNA, as well as by environmental factors.

Leber hereditary optic neuropathy (LHON) is a maternally inherited disorder of the optic nerves causing subacute onset of bilateral centro-cecal scotoma and optic atrophy that lead to acute or subacute bilateral visual loss. LHON is one of the common causes of blindness in young men. The onset of the disease is usually in the second to third decade of life. LHON affects almost exclusively the optic nerve, although there have been reports of associated electrocardiographic changes and various neurological signs.

The disease is associated with three primary mutations in the mtDNA G3460A, G11778A and T14484C affecting subunits of the respiratory enzyme complex I (ND1 Ala52Thr, ND4 Arg340His and ND6 Met64Val, respectively). Two additional mtDNA mutations, G14459A and G15257A, resulting in ND6 Ala72Val and *Cytb* Asp171Asn amino acid replacements are also provisionally considered as primary LHON mutations. The first three primary or class I mutations account for about 90% of the LHON cases reported; about 50% of LHON cases in the European and about 95% in the Japanese populations carry the causal G11778A mutation. Whereas in Thailand, all of the LHON patients detected so far carry G11778A mutation in their mtDNA. Other, so called secondary or class II mutations in mtDNA have been described in LHON, but they occur in the normal population and in association with the primary mutations.

There are some features of LHON that cannot simply be explained by mitochondrial inheritance. First, while mtDNA heteroplasmy may be a factor determining the penetrance of the disease, in most cases both patients and their uneffected relatives from LHON families have very high amounts of mutant mtDNA (>95%). Second, there is an excess of males affected by LHON, with a male:female ratio of 4.2:1 and yet unaffected females transmit the disease. Third, the later onset of the disease in females (6-30 years later in comparison with males) is observed in several pedigrees. This incomplete penetrance and predilection for males to lose vision clearly indicate that additional genetic and/or environmental factors must modulate the phenotypic expression of LHON, although the mtDNA mutation is necessary but insufficient on its own for disease manifestation. However, the secondary precipitating factors modulating the phenotypic expression of LHON remain poorly defined at present.

For LHON patients in Thailand, ~50% of males and ~10% of females who harbour a homoplasmic G11778A mtDNA mutation actually develop the optic neuropathy, which is similar to that previously reported. Many of Thai LHON pedigrees, in the same pedigree, males of the same generation harbour the same homoplasmic mtDNA mutation and yet some express the disease and some do not. This phenomenon is also found in female patients. Since they are the same sex, eliminating gender factor, they carry the same homoplasmic load of mtDNA mutation, excluding heteroplasmic factor, they are from the same pedigree, having the same mtDNA background as their mother. This, then, strongly suggest that the nuclear background plays an important role in the expression of LHON in these families. We, therefore, propose to explore the nuclear background of our 30 LHON pedigrees in order to identify the nuclear factors influencing the expression of LHON in our population.

The examination of nuclear background influencing the expression of LHON will give us better characterisation, better understanding of the relationship between mtDNA mutations, mitochondrial biogenesis and optic nerve dysfunction. This would clarify the unclear pathophysiology of LHON to some extent leading to both improved genetic counseling and the development of future therapeutic strategies.

วัตถุประสงค์

1. To get the preliminary information on what chromosome or what region that contain the modifier of the mtDNA mutation in Thai LHON

2. To generate the knowledge for the understanding of nuclear-mitochondria interaction using the well-characterized mitochondrial disease (LHON) as a model.

3. To get the SNPs database of the genes analysed among Thai population, may be patented if the disease association is found.

วิธีดำเนินการวิจัย

1. Sample collection

Blood samples (5 ml EDTA-blood) of patients with optic neuropathy clinically similar to LHON were sent to our laboratory by the ophthalmologists following informed consent. All of the patients were screened for the most common primary LHON mutations G11778A. Pedigree information of the patients who were positive for the mutation was investigated and, when possible, blood samples from their family members were also collected for the study with informed consent.

2. Mitochondrial DNA analysis

Total leukocyte DNA was extracted from at least 5 ml of whole blood sample containing EDTA or ACD-A using the standard phenol/chloroform method. The 11778 mutation was tested in all available family members. One sample in the maternal lineage of each family was tested for secondary LHON mutations. Degrees of heteroplasmy of the G11778A mutation were quantitated using radioactive restriction analysis and densitometric scanning.

3. Genome widescan analysis

Genome-wide scan was performed with 400 microsatellite markers. Microsatellite genotyping was carried out at the Australian Genome Research Facility (AGRF) under supervision of Dr Kelly Ewan-White. The data analysis was carried out in the Department of Bioinformatics, Walter Eliza Hall Institute of Medical Research under supervision of Dr Melanie Bahlo and Dr Jim Stankovich.

ผลการวิจัย

Pedigrees

Thirty G11778A LHON pedigrees were identified in the first two year. All the pedigrees are of Thai or Chinese ethnic origins except for one pedigree with Indian ethnic origin. Half of these pedigrees contained only one affected individual according to the current information. Of the pedigrees with two or more affected members, 6 of them were large pedigrees comprising 4-7 generations in the pedigree structures with blood samples being collected from at least 3 generations. From the pedigree structures, 935 individuals were identified. Of these, 247 were available for direct evaluation of clinical phenotypes, environmental exposure, and mtDNA mutations. Among these, 166 samples (81 males and 85 females) were positive for the

G11778A mutation consisting of 65 affected, 2 possibly affected (the affected status was difficult to be assigned owing to cataract both eyes), and 99 unaffected individuals

Heteroplasmy of the 17778 Mutation

Eleven (37%) of our 30 LHON pedigrees contained at least one individual with the heteroplasmic 11778 mutation (heteroplasmic pedigree). The number of such pedigrees might be underestimated owing to the fact that in 11 of our 19 homoplasmic pedigrees, only blood samples from probands were obtained. Therefore, other family members whose blood samples were not available could be heteroplasmic for the mutation if they were tested. The association between heteroplasmy and the disease status was analysed.

Genome widescan

Genetic Map

The genetic map generated from Human MSD program for the 400 microsatellite markers used in this study is shown in Table 6. The average marker spacing was 9.2 cM with 26 intervals larger than 15 cM (5 intervals larger than 20 cM).

Nonparametric linkage analysis

From overall-family results of the multipoint nonparametric linkage analysis using the 16 different allele scoring models as described above, with modified marker allele frequency, and without removing any errors reported by Merlin, no significant linkage was found. However, there were 13 regions in 12 chromosomes which showed Zlr score > 2 ($p<0.05$). These regions were at markers D1S207 (107 cM), D2S126 (233 cM), D3S1565 (178 cM), D4S406 (115 cM), D7S507 (32 cM), D9S287 (99 cM), D12S352 (7.6 cM), D13S1265 (114 cM), D14S70 (36 cM), D15S1007 (29 cM), D18S68 (89 cM), DXS1227 (150 cM), and DXS8091 (167 cM). The maximum Zlr was 2.87 ($p=0.002$) at the marker D12S352 in chromosome 12 using exp mn allele equal model. However, when all the 16 models were compared, only 4 regions on chromosome 3, 12, 13 and 18 showed Zlr > 2 fairly consistently across several models. The 4 regions where Zlr > 2 occurred fairly consistently across several models were the first set of candidate regions that was worth looking at. In addition, the peak of Zlr > 2 at marker D1S207 in chromosome 1 was excessively contributed one family F30 and it might be worth investigating around the marker in this family. Chromosome X has long been suspected in LHON and the peak at marker DXS1227 was also worth looking at. The peaks for these two markers were not seen in exp pairs equal model and are shown separately in Figure 5. The peak at marker DXS8091 was of less interest since it might occur by genotyping errors as

reported by Merlin. I ended up with 6 interesting chromosomal region on chromosome 1, 3, 12, 13, 18 and X, which could be promising candidates for the nuclear modifier gene(s) in Thai LHON. The results for each individual family were analysed in these 6 regions and it was found that different families contributed to different extent to the overall allele sharing score.

งานที่จะทำต่อในอนาคต

Since we can identify 6 candidate regions that could possibly be the regions (in the nuclear genome) carrying LHON modifying gene(s), in our LHON pedigrees. We are interested to examine these regions closely in order to find the modifying gene(s). First, we would like to do the fine mapping of these regions using a set of markers covering these areas. The gene(s) in the promising area(s) of the genome will be identified using the combination of the following methods, either *ab initio* program to look for the special signatures of genes in genomic sequences and to distinguish genes from intergenic sequences or database similarity search techniques or comparative genomic alignment. Once the genes (estimated to be 15-20 per Mb) have been identified, they will be examined based on their function. Each potential candidate gene will be screened in the families.

ผลงานวิจัยที่ตีพิมพ์และอยู่ในระหว่างดำเนินการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

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(1) บทนำ (Introduction) ระบุเนื้อหาของเรื่องที่เคยมีผู้ทำการวิจัยมาก่อน ความสำคัญและที่มาของปัญหา วัตถุประสงค์และขอบเขตการวิจัย วิธีดำเนินการวิจัยโดยสรุป ทฤษฎีและ/หรือแนวความคิดที่นำมาใช้ในการวิจัย ประโยชน์ที่คาดว่าจะได้รับ ฯลฯ

(2) เนื้อเรื่อง (Main body) ระบุรายละเอียดเกี่ยวกับวิธีดำเนินการวิจัย (Material & Method) , ผลการวิจัย (Result) ฯลฯ

(3) ข้อวิจารณ์ (Discussion) ที่ได้นำผลการทดลอง (ผลการวิจัย)ที่ได้ในข้อ (2) มากล่าวทั้งหมด (ทั้งที่เป็นและไม่เป็นไปตามสมมติฐานที่ตั้งไว้)

(4) สรุปและข้อเสนอแนะ (Conclusion and recommendation) โดยสรุปเรื่องราวในการวิจัย พร้อมทั้งเสนอแนะเกี่ยวกับการวิจัยในขั้นต่อไป ตลอดจนประโยชน์ในทางประยุกต์ของผลงานวิจัยที่ได้

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บทนำ

While there has been a major progress in the identification of a large number of disease-related mutations in the mitochondrial DNA (mtDNA), the pathobiology of these mutations in general is still poorly understood. The frequent lack of correlation between the mutations and their biochemical expression, their relatively tissue specific clinical manifestation despite the mitochondrial oxidative energy metabolism being essential for the function of almost all cell types, and the incomplete and often low penetrance of the mutations, all point to the complexity of the pathogenesis of the resulting disorders. Indeed, it might be more appropriate to consider these disorders as polygenic, with the clinical expression of the causal mtDNA mutations being modulated by other genetic factors, both in the nuclear and in the mitochondrial DNA, as well as by environmental factors.

Leber hereditary optic neuropathy (LHON) is a maternally inherited disorder of the optic nerves causing subacute onset of bilateral centro-cecal scotoma and optic atrophy that lead to acute or subacute bilateral visual loss [Howell, 1998]. LHON is one of the common causes of blindness in young men. The onset of the disease is usually in the second to third decade of life. The decreased visual acuity is often preceded by peripapillary microangiopathy. LHON affects almost exclusively the optic nerve, although there have been reports of associated electrocardiographic changes and various neurological signs.

The disease is associated with three primary mutations in the mtDNA G3460A [Howell et al., 1991; Huoponen et al., 1991; Huoponen et al., 1993], G11778A [Wallace et al., 1988; Singh et al., 1989] and T14484C [Johns et al., 1993; Mackey and Howell, 1992] affecting subunits of the respiratory enzyme complex I (ND1 Ala52Thr, ND4 Arg340His and ND6 Met64Val, respectively). Two additional mtDNA mutations, G14459A [Jun et al., 1994; Shoffner et al., 1995] and G15257A [Brown et al., 1992], resulting in ND6 Ala72Val and *Cyt*b Asp171Asn amino acid replacements are also provisionally considered as primary LHON mutations. The respiratory chain enzyme activity was reported to be decrease in LHON patients carrying these mutations [Chalmers and Schapira, 1999]. The first three primary or class I mutations account for

about 90% of the LHON cases reported [Brown and Wallace, 1994; Mackey et al., 1996]; about 50% of LHON cases in the European [Harding et al., 1995; Newman et al., 1991] and about 95% in the Japanese populations carry the causal G11778A mutation [Mashima et al., 1998]. Whereas in Thailand, all of the LHON patients detected so far carry G11778A mutation in their mtDNA [Lertrit et al., 1998]. Other, so called secondary or class II mutations in mtDNA have been described in LHON, but they occur in the normal population and in association with the primary mutations.

There are some features of LHON that cannot simply be explained by mitochondrial inheritance. First, while mtDNA heteroplasmy may be a factor determining the penetrance of the disease [Holt et al., 1989; Smith et al., 1993; Black et al., 1996; Tanaka et al., 1998], in most cases both patients and their uneffected relatives from LHON families have very high amounts of mutant mtDNA (>95%) [Cormier et al., 1991; Zhu et al., 1992; Chinnery et al., 2001]. Second, there is an excess of males affected by LHON, with a male:female ratio of 4.2:1 [Newman et al., 1991; Harding and Riordan-eva, 1995; Macmillan et al., 1998; Johns et al., 1992; Johns et al., 1993] and yet unaffected females transmit the disease. Third, the later onset of the disease in females (6-30 years later in comparison with males) is observed in several pedigrees [Harding et al., 1995; Newman et al., 1991; Macmillan et al., 1998; Johns et al., 1992; Johns et al., 1993; Kerrison and Newman, 1997].

This incomplete penetrance and predilection for males to lose vision clearly indicate that additional genetic and/or environmental factors must modulate the phenotypic expression of LHON, although the mtDNA mutation is necessary but insufficient on its own for disease manifestation. However, the secondary precipitating factors modulating the phenotypic expression of LHON remain poorly defined at present [Taanman, 2001]. The schematic representation of the pathways leading to optic nerve degeneration in LHON is shown in Figure 1 [Man et al., 2002].

For LHON patients in Thailand, ~50% of males and ~10% of females who harbour a homoplasmic G11778A mtDNA mutation actually develop the optic neuropathy, which is similar to that previously reported [Seedorff, 1985; Nikoskelainen, 1994; Brown and Wallace, 1994]. Many of Thai LHON pedigrees, in the same pedigree, males of the same generation harbour the same homoplasmic mtDNA mutation and yet

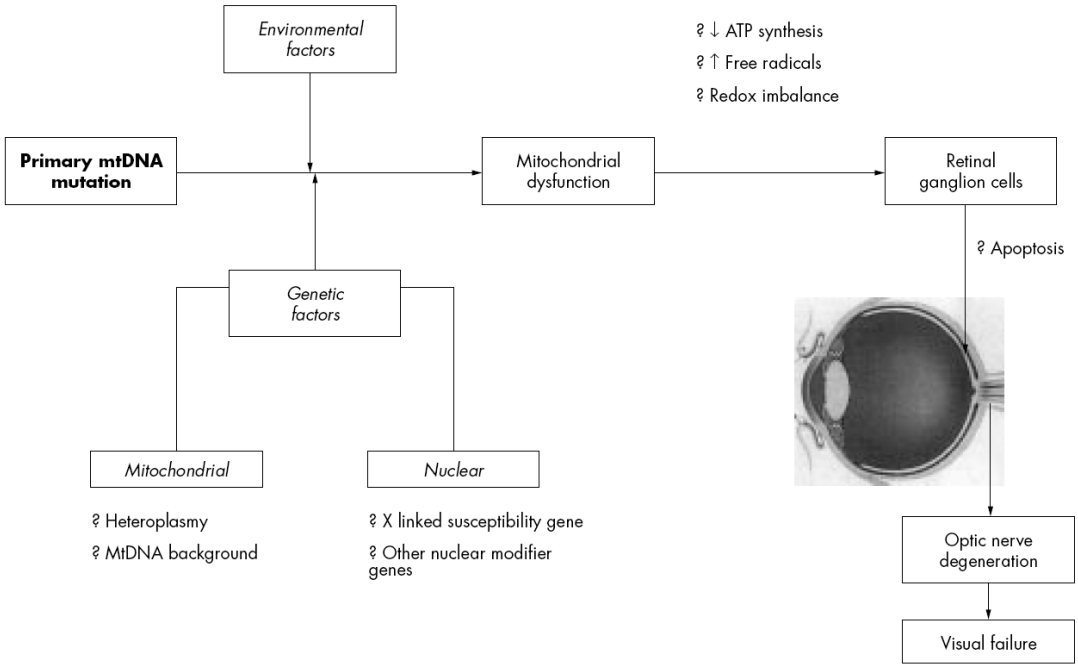

Figure 1. Schematic representation of the pathways leading to optic nerve degeneration in LHON [Man et al., 2002].

some express the disease and some do not. This phenomenon is also found in female patients. Since they are the same sex, eliminating gender factor, they carry the same homoplasmic load of mtDNA mutation, excluding heteroplasmic factor, they are from the same pedigree, having the same mtDNA background as their mother. This, then, strongly suggest that the nuclear background plays an important role in the expression of LHON in these families. We, therefore, propose to explore the nuclear background of our 30 LHON pedigrees in order to identify the nuclear factors influencing the expression of LHON in our population.

The examination of nuclear background influencing the expression of LHON will give us better characterisation, better understanding of the relationship between mtDNA mutations, mitochondrial biogenesis and optic nerve dysfunction. This would clarify the unclear pathophysiology of LHON to some extent leading to both improved genetic counseling and the development of future therapeutic strategies.

วัตถุประสงค์ของโครงการ

1. To get the preliminary information on what chromosome or what region that contain the modifier of the mtDNA mutation in Thai LHON

2. To generate the knowledge for the understanding of nuclear-mitochondria interaction using the well-characterized mitochondrial disease (LHON) as a model.

3. To get the SNPs database of the genes analysed among Thai population, may be patented if the disease association is found.

วิธีดำเนินการวิจัย

1. Sample collection

Blood samples (5 ml EDTA-blood) of patients with optic neuropathy clinically similar to LHON were sent to our laboratory by the ophthalmologists following informed consent. All of the patients were screened for the most common primary LHON mutations G11778A. Pedigree information of the patients who were positive for the mutation was investigated and, when possible, blood samples from their family members were also collected for the study with informed consent.

In each pedigree, the clinical data were obtained by direct examination by the ophthalmologists wherever possible, or indirectly, by interviews with one or more of the family members. Affected status in unseen relatives was based on a history of acute visual loss without other known causes.

Environmental factors of smoking, alcohol consumption, and head injuries in the family members were recorded. For affected persons, these factors were included in our analyses only if they occurred before the age of onset of vision loss. For smoking and alcohol consumption, the information collected included age at which they started consumption, average consumption (cigarettes/drinks per day), and duration of consumption. The amount of consumption was graded as no consumption, mild, and heavy consumption. Smoking of at least 10 cigarettes per days and/or continuous smoking for more than 10 years were classified as heavy smoking, while other smoking habits were regarded as mild smoking. Continuous alcohol consumption of more than 1 bottle (750 ml) per day and/or continuous drinking for more than 10 years were

classified as heavy drinking, whereas other drinking habits were regarded as mild. Other potential precipitating environmental exposure such as toxins was also noted.

2. Mitochondrial DNA analysis

Total leukocyte DNA was extracted from at least 5 ml of whole blood sample containing EDTA or ACD-A using the standard phenol/chloroform method. The 11778 mutation was tested in all available family members. One sample in the maternal lineage of each family was tested for secondary LHON mutations. Primary and secondary LHON mutations were detected by either Restriction Fragment Length Polymorphism (RFLP) or direct sequencing of the mtDNA as details in Lertrit *et al*, 1998 and Sudoyo *et al*, 2002. Degrees of heteroplasmy of the G11778A mutation were quantitated using radioactive restriction analysis and densitometric scanning. MtDNA was amplified using primer L11728* and H11942 for detection of the 11778 mutation. The PCR was carried out as in the RFLP analyses above [Lertrit et al., 1998] except for the last cycle. After 29th cycle, 1 μl of 12.5 μCi/ml of ^{35}S-dATP and 1 μl of *Taq* polymerase were added. The labelled PCR product was purified and digested with restriction enzyme *Bcl*I and run in 8% polyacrylamide gel. The gel was dried and exposed to X-ray film at –80° C for appropriate time. The autoradiogram was developed and gel pattern was visualized and analysed by densitometry to estimate the relative proportion of mutant to total mitochondrial DNA population. In order to be certain that all pedigrees are genetically unrelated, the hypervariable region I (HV I) in the mtDNA D-loop (nt 57 to nt 372) from the proband of each family were sequenced.

3. Genome widescan analysis

Genome-wide scan was performed with 400 microsatellite markers. Microsatellite genotyping was carried out at the Australian Genome Research Facility (AGRF) under supervision of Dr Kelly Ewan-White. The data analysis was carried out in the Department of Bioinformatics, Walter Eliza Hall Institute of Medical Research under supervision of Dr Melanie Bahlo and Dr Jim Stankovich.

3.1 Subject

Guidelines for selecting sample for the genome scan

From our previous power study performed by Mr Nopasak Phasukkijwattana, a Ph.D. student, at Department of Bioinformatics, Walter Eliza Hall Institute of Medical

Research under supervision of Dr Melanie Bahlo and Dr Jim Stankovich, we then tried to construct a model that fit our LHON dataset and estimate penetrance parameters and allele frequencies of a nuclear modifier allele from the model under autosomal dominant mode of inheritance. The result from simulation and parametric linkage analysis from the estimated parameters was not promising. Therefore, we were more optimistic with a nonparametric affected-only analysis and the guidelines for selecting sample were conducted in favour of nonparametric analysis.

Therefore, from our 37 pedigrees, we only included only pedigrees with at least two affected blood samples. We included all affected samples in those pedigrees, their parents and also individuals that connected those people in the pedigree, in order to gain as much information as possible about inherited haplotypes in the affected people. For unaffected people, we prefer to select the ones who provided more information of being truly unaffected, i.e., old age, male sex, and high G11778A mutation load are preferred.

Using the above guidelines, we ended up with 91 samples from 9 pedigrees included in the genome scan. Details of these pedigrees are shown in Table 1. All of the families included in the genome scan were of Thai or Chinese-Thai genetic background except for one family F15 which was Indian. In some certain programs, the pedigree size of F28 exceeded the programs' limitation and the pedigree was splitted into F28a (descended from individual II8) and F28b (descended from individual II11). These programs included Merlin and Allegro.

Table 1. Details of 9 pedigrees included in the genome scan.

Pedigree	Maternal lineage			Not carrying G11778A	Heteroplamic family	Heteroplasmic persons		
	Affected	Unaffected	Unknown			Affected	Unaffected	Unknown
F1	3M	2F	0	2	Yes	0	2F	0
F9	2M	2M, 1F	1F	1	Yes		2M	1F
F11	5M, 1F	1M	0	0	No			
F15	3M	1M, 5F	0	1	No			
F18	3M, 5F	1M, 2F	0	0	No			
F19	2M, 2F	1M	1F	2	Yes	2M, 2F		1F
F28	5M, 4F	3M, 6F	1F	2	Yes (with a homoplasmic branch)	1M, 1F	1M, 3F	1F
F30	4M, 4F	2M, 4F	0	2	No			
F36	2M	1F	0	1	No			
Total	45	32	3	11		6	8	3

M = Male

F= Female

3.2 Genome widescan

1.2.1. PCR amplification

For each sample, 400 microsatellite markers spreading across the whole genome (with average coverage of every 10 cM) were amplified using fluorescently labeled primers (Linkage Mapping Set Version 2.5-MD10, Applied Biosystems). PCR was performed in 384-well microtitre plates (Abgene Thermofast, Integrated Sciences). Each PCR reaction (total volume of 6 ul) consisted of 3 ul of 5 ng/ul DNA template, 0.6 ul of 10x PCR buffer with 15 mM $MgCl_2$ (QIAGEN), 0.38 ul of 2mM dNTPs (dATP, dCTP, dGTP, and dTTP), 0.04 ul of 5 U/ul of HotStar Taq ™ (QIAGEN) DNA polymerase, and 0.2 ul of a fluorescently-labelled primer pair for each microsatellite marker (5 uM of each forward and reverse primers). Each primer pair is labeled with either 6FAM™, VIC®, or NED™ fluorescent dye. The amplification was performed in a PTC-225 DNA Engine Tetrad thermal cycler (MJ research, Inc.). The thermal profile consisted of pre-amplification denaturation at 95°C for 15 min, followed by 40 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and extension (72 °C for 1 min). In the last cycle, the extension time was extended for another 10 min at 72 °C to allow the complete extension of the amplified products. The ramp rate of increasing or decreasing temperature was set to 1 °C per sec.

1.2.2. Pooling the PCR products

After the PCR amplification, the PCR products of the same DNA sample were pooled together according to which panels they were in and then transferred into a new 96-well V-gottom plate (NUNC). A panel is a specific group of microsatellite PCR products that can be pooled together and run on the same lane during electrophoresis. Since they have non-overlapping size ranges or different fluorescent labeling, each of the PCR products in a panel can be distinguished from one another. The Linkage Mapping Set v2.5-MD10 primer pairs for amplifying 400 microsatellite markers were arranged into 28 panels according to the manufacturer.

1.2.3. Electrophoresis of the PCR products

Electrophoresis was performed on a 48-capillary ABI Prism® 3730 DNA Analyzer. First, the pooled PCR products were diluted with 200 ul of sterile distilled water. One ul of the diluted PCR products was then mixed with 10 ul of loading solution

(1000 ul of formamide and 6 ul of GeneScan™ –500 LIZ™ Size Standard (Applied Biosystems)) on a 96-well reaction plate (Micro Amp, Perkin Elmer). The plate was then heated at 95 °C for 5 min to denature the DNA, chilled on ice for 5 min, and put on the electrophoresis machine. The fluorescently labeled PCR products were then separated according to their sizes and detected by a fluorescence detecting system in the machine.

1.2.4. Microsatellite allele assignment

After the electrophoresis, the PCR products were visualized and their sizes were measured using ABI Prism® GeneMapper™ Version 3.0 (Applied Biosystems) genotyping software. Allelic sizes of microsatellite markers were assigned to by the software and they were manually checked to remove any miss specified alleles.

1.3. Determining genetic map

Several genome-wide genetic maps have been published and are in common usage. These includes Genethon map [Dib et al., 1996], Marshfield map [Broman et al., 1998] and DeCODE.map [Kong et al., 2002]. Genetic positions of the 400 microsatellite markers in this thesis were based on DeCODE map, which were constructed on the largest number of meiotic events to date and thus should be the most accurate. For markers which were not available in DeCODE map, their genetic positions were obtained by interpolating from the physical map position using the two closest flanking markers' genetic and physical map position as a scaffold. In some rare circumstances where both DeCODE map position and physical map position for a particular marker are unknown, but the genetic map position on either Genethon or Marshfield map is known, the genetic position in DeCODE map is then interpolated from genetic positions from these maps instead. The interpolation was performed using web-based program HumanMSD (Human MicroSatellite Database) [Bahlo et al., 2004] with some minor modification. The original program performed linear interpolation throughout the whole genome, while in this study the linear interpolation was employed unless the genetic distance between two flanking markers were more than 10 cM, where statistical LOESS procedure was used instead (Xing L, personal communication). All

the database of physical and genetic distances of the markers were retrieved from UCSC human genome database (http://genome.ucsc.edu/) assembly of May 2004.

1.4. Cleaning of data from the genome-wide scan

Before performing linkage analysis, it is important to minimize errors in genotype data or even in pedigree structures. Such errors could result in either reduced power or flase-positive evidence for linkage.

Genotyping errors or misspecified relationships in pedigrees could result in inconsistencies in Mendelian inheritance pattern. Such inconsistencies were checked using PedCheck v1.1 [O'Connell and Weeks, 1998]. Statistical test for misspecified relationships was also performed on the genome-screen data using PREST (Pedigree Relationship Statistical Test) program [McPeek and Sun, 2000], which determine whether the pattern of allele sharing for each relative pairs is consistent with the relationship specified in the pedigree. Genotypes which caused Mendelian errors but could not be corrected by revision of genotyping data were set to unknown, which allowed linkage analysis software to infer the unknown genotypes using information from genotypes of related individuals in the pedigree. Error detection was further performed using Merlin (Multipoint Engine for Rapid Likelihood Inference) [Abecasis et al., 2002], which detects genotypes that cause unlikely recombination events.

1.5. Nonparametric linkage analysis

Multipoint nonparametric linkage analysis was performed using Allegro v1.2 program [Gudbjartsson et al., 2000]. The program computes scores for allele sharing between affected relatives in the pedigree. There are several allele sharing scoring function (S), different weighting schemes, and linear (lin) and exponential (exp) methods of testing linkage, implemented in Allegro. The power to detect linkage using any particular allele sharing models can vary greatly depending on disease models and particular pedigree types [McPeek, 1999]. Although, the disease model of LHON is well-recognized as mitochondrial inheritance, none is known regarding the disease model by the effects of nuclear modifier gene(s) (if any). Therefore, several allele sharing models were tried using combination of 4 scoring functions (Spairs, Sall, Srobdom, and Smnallele), 2 methods of testing linkage (lin and exp), and 2 weighting schemes (weight each family equally, and weighting each family proportionally to the

standard deviation of the score function used, under the null hypothesis of no linkage, to the power 0.5 as suggested by Allegro). This gave rise to 16 different allele sharing models: exp pairs equal, exp all equal, exp robdom equal, exp mnallele equal, exp pairs 0.5, exp all 0.5, exp robdom 0.5, exp mnallele 0.5, lin pairs equal, lin all equal, lin robdom equal, lin mnallele equal, lin pairs 0.5, lin all 0.5, lin robdom 0.5, and lin mnallele 0.5. The results from all the models were compared. Owing to the lack of allele frequency information of the microsatellite markers in Thai population, the allele frequencies were estimated using genotypes of founders in the pedigrees.

The regions which showed interesting linkage result were investigated further by looking at haplotypes in that region. The haplotypes were constructed using Allegro for both genotyped individuals and ungenotyped individuals if they can be imputed.

ผลการวิจัย

Pedigrees

Thirty G11778A LHON pedigrees were identified in the first two year. All the pedigrees are of Thai or Chinese ethnic origins except for one pedigree with Indian ethnic origin. Half of these pedigrees contained only one affected individual according to the current information. Of the pedigrees with two or more affected members, 6 of them were large pedigrees comprising 4-7 generations in the pedigree structures with blood samples being collected from at least 3 generations. From these 30 families, 27 HV I mitochondrial haplotypes were detected. There were 3 HV I haplotypes which were shared by 2 families each. However, when the mitochondrial genome of these six families was subject to high resolution screening of polymorphic restiction sites and screen for 9-bp deletion, they all carried distinct mtDNA haplotypes (the results will be published elsewhere). These 30 families were then not closely genetically related.

From the pedigree structures, 935 individuals were identified. Of these, 247 were available for direct evaluation of clinical phenotypes, environmental exposure, and mtDNA mutations. Among these, 166 samples (81 males and 85 females) were positive for the G11778A mutation consisting of 65 affected, 2 possibly affected (the affected

status was difficult to be assigned owing to cataract both eyes), and 99 unaffected individuals

One family (F19) was found to have two genetic diseases simultaneously: LHON, a mitochondrial disease and facioscapulohumeral dystrophy (FSHD), an autosomal dominant disorder.

Age of Onset and Male:Female Ratio

In the 65 affected persons directly evaluated, 58 were documented with their age of onset. The mean age of onset was 22.6 ± 11.7 years (range: 6-53, median: 20 years) in all the patients. The mean age of onset in male patients was 20.7 ± 10.0 years (n = 44, range: 6-44, median: 19 years) and the value in female patients was 28.6 ± 14.6 (n = 14, range: 10-53, median: 30 years). It appeared that the mean age of onset in female was higher than in male in our patients. The difference was almost statistically significant (p=0.073; Mann-Whitney U test).

The 65 affected patients consisted of 47 males and 18 females, and the male:female ratio was 2.6:1. In other words, 72% of patients were male.

Disease Penetration

Excluding the 2 possibly affected persons, the directly evaluated 164 samples harbouring the 11778 mutation consisted of 40% (65/164) affected and 60% (99/164) unaffected individuals. When male and female groups were analysed separately, 58% (47/81) of males and 22% (18/83) of females carrying the mutation expressed the disease. It should be noted that 34% of the currently unaffected persons who were directly evaluated aged less than 24 years (the average age of onset of LHON in Thailand) and some of them might become affected later. In addition, these proportions of affected persons, calculated using only the directly evaluated persons, could be overestimated because affected persons were more likely to be ascertained than the unaffected.

To avoid the above ascertainment bias, the percentage of affected person was calculated in all individuals in maternal lineages of the pedigree structures. Therefore, 295 maternal family members whose disease status was known (either directly or

indirectly) were analysed, assuming (based on the principle of mitochondrial genetics) that the unseen maternal members should carry the mutation. However, it had to compromise on certainty regarding the disease status in the unseen persons. It was found that 24% (70/295) of all individuals, 37% (50/135) of males, and 13% (20/160) of females develop optic neuropathy. The ages of 90% of the unaffected persons analysed could be estimated and, again, it should be noted that 30% of those persons were less that 24 years old, and some of them might become affected later in their life.

The proportion of persons carrying the mutation and being affected widely varied in each individual pedigree from 7% to 77% with the mean of 26 ± 15%. This was calculated assuming that the unseen maternal members should carry the mutation and relying on the indirectly obtained disease status in the unseen persons. Considering only 10 large pedigrees with more than 10 maternal relatives spanning at least 3 generations, the disease penetrance varied from 9% to 45% with the mean of 19 ± 11%. In addition, our preliminary observation showed that the proportion also varied between different branches of the same large pedigree.

Next, we assessed whether or not affected mothers were likely to have more affected children than the unaffected mothers. Only sibships and their mothers, all with known disease status (either directly or indirectly), in which all the unaffected persons were at least 24 years of age (to assure that most of them would not become affected in the future), were included in this analysis. With this criterion, 19 sibships (and their mothers) were identified, comprising 13 sibships with unaffected mothers and 6 sibships with affected mothers (Table 2). The affected mothers seemed to be more likely to have affected children than the unaffected mothers (OR=2.51, p=0.12; Chi-square test) independent of gender of their children; 56% (9/16) of males born to affected mothers became affected, compared with 34% (10/29) of those born to unaffected mothers; whereas 33% (3/9) of females born to affected mothers developed optic neuropathy, compared with only 17% (4/23) of those born to unaffected mothers.

Table 2. Affection status of individuals categorized by affection status of mothers or G11778A heteroplasmy.

	Male		Female	
	Affected	Unaffected	Affected	Unaffected
Offspring from selected 19 sibships				
Offspring of 6 affected mothers (n=25)	9	7	3	6
Offspring of 13 unaffected mothers (n=52)	10	19	4	19
G11778A mutation load				
Heteroplasmic (n=44)	6	10	3	25
Homoplasmic (n=120)	41	24	15	40

Heteroplasmy of the 17778 Mutation

Eleven (37%) of our 30 LHON pedigrees contained at least one individual with the heteroplasmic 11778 mutation (heteroplasmic pedigree). The number of such pedigrees might be underestimated owing to the fact that in 11 of our 19 homoplasmic pedigrees, only blood samples from probands were obtained. Therefore, other family members whose blood samples were not available could be heteroplasmic for the mutation if they were tested.

Of the 166 individuals positive for the 11778 mutation, 28% (46/166) were heteroplasmic and 72% (120/166) were homoplasmic. Considering only the patients (affected persons), only 14% (9/65) were heteroplasmic, while in the unaffected group, 35% (35/99) were heteroplasmic. The result showed that the prevalence of heteroplasmy was higher in the unaffected group compared with the affected group.

The association between heteroplasmy and the disease status was analysed (Table IV). A total of 164 individuals positive for the 11778 mutation with known disease status (2 were not included because they were possibly affected) were included in the analysis. Our results supported the belief that heteroplasmy influences the expression of LHON. It was found that 20% (9/44) of heteroplasmic persons manifested the disease, compared with 47% (56/120) of the homoplasmic group (OR=3.40 p=0.004; Chi-square test). When sex was considered in the analysis, similar results were obtained. In the male group, 38% (6/16) of heteroplasmic persons developed the disease, compared with 64% (42/66) of homoplasmic ones. In the female group, only 11% (3/28) of heteroplasmic persons, compared with 27% (15/55) of homoplasmic ones, became affected.

Age of onset was compared between heteroplasmic and homoplasmic patients. In 8 heteroplasmic patients with known age of onset, the mean age of onset was 21.1 ± 10.3 years (range: 10-42, median = 19 years), whereas in 50 homoplasmic patients, the mean age of onset was 22.9 ± 11.9 years (range: 6-53, median = 20 years). Therefore, although heteroplasmy seemed to influence the disease manifestation in our patients, higher mutation load did not appear to accelerate the onset of the disease (p=0.77;

Mann-Whitney U test). However, this result should be taken with caution because the number of heteroplasmic patients in this analysis was small.

It should be noted that in 2 of our heteroplasmic families, 8 samples of maternal lineages were found to be negative for the 11778 mutation. This provided evidence that the heteroplasmic 11778 mutation could segregate to pure wild type. This supports the important of molecular mtDNA testing in family members seeking genetic counseling. This was also suggested by Man et al., 2003 [Man et al., 2003].

Other Primary and Secondary LHON Mutations in the 11778 LHON Pedigrees

A total of other 27 LHON associated primary and secondary mutations previously reported were screened in thirty 11778 LHON families as in Table 3. In the last family (F30), only 5 mutations were screened. Two families were found to possess mutations other than the G11778A; one family (F11) carried a C3497T mutation and the other (F19) carried the G3316A mutation as revealed by direct sequencing. Both mutations are classified as secondary mutation and are located in the *ND1* gene of the mitochondrial genome. No combination of more than 1 secondary mutation in the same family was observed.

Age of onset was compared between two groups of patients: one carrying the 11778 mutation plus the secondary mutation, and the other carrying only the 11778 mutation. The family F30 was not included in the analysis because only 5 mutations were screened. The mean age of onset in the former group (n = 10) was 16.4 ± 8.9 years (range: 8-33, median = 14.5 years), while in the latter group (n = 43) the mean age of onset was 23.5 ± 11.8 years (range: 6-53, median = 20 years). The result indicated that the secondary mutations, G3316A and C3497T, seemed to have synergistic deleterious effect with the 11778 mutation, accelerating the onset of the disease ($p=0.056$; Mann-Whitney U test).

Table 3. Spectrum of LHON-associated mutations in Thai familes with G11778A.

Family ID	Primary Mutations					Secondary Mutations																							
	G 3 4 6 0 A	G 1 1 7 7 8 A	G 1 4 4 5 9 A	T 1 4 4 8 4 C	G 1 5 2 5 7 A	G 3 3 1 6 A	T 3 3 9 4 C	G 3 4 9 6 T	C 3 4 9 7 T	G 3 6 3 5 A	A 4 1 3 6 G	T 4 1 6 0 C	C 4 1 7 1 A	T 4 2 1 6 C	A 4 9 1 7 G	G 5 2 4 4 A	G 7 4 4 4 A	G 9 7 3 8 T	G 9 8 0 4 A	G 1 3 7 0 8 A	G 1 3 7 3 0 A	C 1 4 4 8 8 A	C 1 4 4 8 8 A	A 1 4 4 9 9 C	T 1 4 4 9 9 C	C 1 4 4 6 6 T	A 1 4 5 9 1 T	G 1 5 8 1 2 A
F1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F4	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-
F5	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F6	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F7	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F8	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F9	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F10	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F11	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F12	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F13	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F14	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F15	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F16	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F17	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F18	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F19	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F20	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F21	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F22	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F23	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F24	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F25	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F26	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F27	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F28	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F29	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F30	NA	+	NA	NA	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-

-, negative for the mutation; +, positive for the mutation; NA, not available.

Environmental Factors

To evaluate the role of environmental factors in 11778 LHON expression, the presence of environmental factors was compared between the affected and unaffected groups. Since heteroplasmy appeared to influence the disease penetrance, only molecularly confirmed homoplasmic persons were considered in both groups. Furthermore, only environmental exposure before the onset of visual symptom was considered in the affected group.

Owing to the fact that LHON can occur in various ages, it was appropriate to take the age factor into consideration. Individuals were classified into different age groups and history of environmental exposure was compared between affected and unaffected people in the same age group. For affected people, their ages of onset (instead of their current ages) were used for classifying them into different age groups because we only considered environmental exposure before the age of onset. Therefore, the affected and the unaffected groups were age matched in terms of time and opportunity to have the environmental exposure.

We observed that the frequency of smoking was higher in the affected than the unaffected in young age groups but it was not different for old age groups (Table 4). In particular, for individuals with $\leq$30 years old, 19% (6/31) of affected persons were smokers compared with only 7% (2/27) in the unaffected group. For individuals with more than 30 years of age, the frequencies of smokers were 38% (6/16) for the affected group and 32% (7/22) for the unaffected group. Similar results were obtained for alcohol consumption (Table 4) with the frequency of drinkers being higher in the affected than the unaffected in young age groups but not different for old age groups. The frequencies of drinkers were 27% (8/30) in the affected group of $\leq$30 years old compared with 18% (5/28) in the unaffected group. The frequencies of drinkers for persons with more than 30 years old were 81% (13/16) for the affected group and 82% (18/22) for the unaffected group. However, the differences in the younger age group were not statistically significant, due to the small sample size in our analyses (for

smoking in young age (≤30) group, OR=3, p=0.26, Fisher exact test; for alcohol consumption in young age (≤30) group, OR= 1.67, p=0.62, Chi-square test).

Table 4. Smokers and alcohol drinkers in affected and unaffected groups with homoplasmic G11778A mutation.

Age	Affected individuals (n=47)			Unaffected individuals (n=49)			Affected individuals (n=46)			Unaffected individuals (n=50)		
(years)	Total smokers (%)	Mild	Heavy	Total smokers (%)	Mild	Heavy	Total drinkers (%)	Mild	Heavy	Total drinkers (%)	Mild	Heavy
<11	0/7 (0%)	0	0	0/6 (0%)	0	0	0/7 (0%)	0	0	0/6 (0%)	0	0
11-20	3/17 (18%)	3	0	0/14 (0%)	0	0	4/17 (24%)	4	0	2/15 (13%)	2	0
21-30	3/7 (43%)	2	1	2/7 (29%)	2	0	4/6 (67%)	4	0	3/7 (43%)	3	0
31-40	3/8 (38%)	0	3	3/9 (33%)	1	2	7/8 (88%)	6	1	8/9 (86%)	7	1
41-50	2/4 (50%)	1	1	0/5 (0%)	0	0	3/4 (75%)	2	1	3/5 (60%)	2	1
>51	1/4 (25%)	0	1	4/8 (50%)	1	3	3/4 (75%)	1	2	7/8 (88%)	5	2

Affected persons were categorized into different age groups by their ages of onset, while unaffected persons were categorized by their ages at the last examination

The role of head injuries in the disease expression was also analyzed (Table 5). The frequencies of head injuries were higher in the affected group than the unaffected group regardless of age. In young age (≤30) group, the frequencies were 27% (7/26) for the affected and 19% (5/26) for the unaffected, while in old age (>30) group, the difference was slightly greater with the frequencies of 25% (4/16) for the affected and only 9% (2/22) for the unaffected. However, with our small sample size, the differences were again not statistically significant (for young age group, OR=1.5, p=0.74, Chi-square test; for old age group, OR=3.3, p=0.21, Fisher exact test).

Genome widescan

Genetic Map

The genetic map generated from Human MSD program for the 400 microsatellite markers used in this study is shown in Table 6. The average marker spacing was 9.2 cM with 26 intervals larger than 15 cM (5 intervals larger than 20 cM).

Marker allelle frequency estimation

Marker allele frequencies were originally estimated (freq from the founders in the pedigrees. Alleles that were not presented in the founders were assigned the frequency 0.000001. However, there are only 12 founders (9 males, 3 females; 24 chromosomes for autosomal chromosomes and 15 chromosomes for X chromosome) who were genotyped and the alleles not presented in this 12 founders would become very rare (frequency 0.000001) which may not be true in Thai population, and linkage analysis programs would be sensitive to this very low allele frequency if anyone shared those alleles. Therefore, some modification to the originally estimated marker allele frequencies was done by adding an arbitrary number to each of the counts of the allele occurrence for each marker, and the allele frequencies were recalculated. This caused the frequency of the alleles not presented in the 12 founders to increase while reduced the frequency of common alleles. An arbitrary number of 0.5 was chosen to be the main for this study to increase the frequency of the alleles not presented in the founder while having a minimal effect on the other more common alleles which were more likely to be already well represented in the 24 chromosomes of the founders. Originally estimated allele frequencies and modified allele frequencies with an arbitrary

Table 5. Individuals with head injuries in affected and unaffected groups with homoplasmic G11778A mutation.

Age groups (years)	Persons with head injuries in affected group (%)	Persons with head injuries in unaffected group(%)
<11	1/7 (14%)	0/6 (0%)
11-20	5/15 (33%)	3/14 (21%)
21-30	1/4 (25%)	2/6 (33%)
31-40	2/8 (25%)	0/9 (0%)
41-50	1/4 (25%)	1/5 (20%)
>51	1/4 (25%)	1/8 (13%)

Affected persons were categorized into different age groups by their ages of onset, while unaffected persons were categorized by their ages last seen.

Table 6. Maps of the 400 microsatellite markers and their heterozygosities in this study.

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
Chromosome 1						
1	3608018	D1S468	4	6.2	4.22	78.90%
2	6896255	D1S214	12.05	16.4	14.04	75.60%
3	9519684	D1S450	16.61	22.9	20.61	77.50%
4	11421226	D1S2667	19.88	26.9	24.68	90.00%
5	16164755	D1S2697	29.25	39.9	37.05	52.20%
6	19702298	D1S199	37.48	47.7	45.33	76.70%
7	24896706	D1S234	44.49	56.6	55.1	75.60%
8	37318806	D1S255	58.66	66.6	65.47	43.70%
9	46645459	D1S2797	68.9	77.6	75.66	67.80%
10	57585420	D1S2890	80.9	87.7	85.68	64.40%
11	62314305	D1S230	88.12	97.4	95.31	56.70%
12	79199315	D1S2841	103.82	108.8	106.45	85.60%
13	82255369	D1S207	107.16	117.6	113.69	86.70%
14	93047527	D1S2868	116.64	129.9	126.16	56.70%
15	101397325	D1S206	122.64	137.6	134.2	80.50%
16	110896304	D1S2726	132.11	149	144.38	76.40%
17	117268812	D1S252	139.16	155.1	150.27	67.90%
18	148114568	D1S498	144.94	160.7	155.89	76.70%
19	157580383	D1S484	157.51	173.9	169.68	61.10%
20	162135023	D1S2878	165.78	181.7	177.86	77.30%
21	164335785	D1S196	169.4	186.4	181.49	64.80%
22	171234749	D1S218	176.3	196.5	191.52	72.20%
23	184877834	D1S238	188.55	206.7	202.73	81.80%
24	195352027	D1S413	194.98	216.5	212.44	68.90%
25	202446965	D1S249	207.07	225.1	220.65	68.50%
26	208471580	D1S425	215.07	235.3	231.11	23.30%
27	unknown	D1S213	228.26	246.2	242.34	86.70%
28	230765089	D1S2800	240.77	256.1	252.12	60.70%
29	237202723	D1S2785	257.46	269.7	266.27	85.10%
30	239198599	D1S2842	261.86	277.3	273.46	70.50%
31	243196118	D1S2836	271.84	290.1	285.75	74.40%
						Average = 70.5%
Chromosome 2						
1	4965344	D2S319	7.6	6	7.6	69.50%
2	7421975	D2S2211	17.73	14	15.61	64.40%
3	8810893	D2S162	22.73	21.3	20.03	77.50%
4	11396048	D2S168	28.92	28.6	27.06	83.30%
5	19340005	D2S305	41.52	40.7	38.87	75.60%
6	28514932	D2S165	50.92	50.7	47.43	74.40%
7	34352798	D2S367	57.89	58.3	54.96	77.80%
8	42908197	D2S2259	67.06	67.4	64.29	57.80%
9	46323018	D2S391	72.33	73.8	70.31	73.30%
10	61581506	D2S337	83.98	84.1	80.69	74.40%
11	67125255	D2S2368	89.24	89.2	85.48	84.40%
12	75253784	D2S286	99.99	98.4	94.05	77.80%

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
13	85399777	D2S2333	108.81	107.7	103.16	77.50%
14	88248902	D2S2216	111.48	115.3	111.21	61.80%
15	112714665	D2S160	124.94	127.4	122.96	72.20%
16	123966039	D2S347	135.31	135.7	131.51	63.30%
17	133042428	D2S112	145.71	145.8	141.62	75.30%
18	147620245	D2S151	157.5	156.4	152.04	83.30%
19	156108565	D2S142	165.19	166.3	161.26	61.10%
20	166522919	D2S2330	171.1	175.5	169.41	84.30%
21	172392002	D2S335	177.98	182.5	175.91	94.40%
22	182860038	D2S364	187.67	192.9	186.21	76.70%
23	195444208	D2S117	194.63	201.4	194.45	85.60%
24	208096141	D2S325	204.88	210.9	204.53	70.00%
25	216874070	D2S2382	213.82	220.7	213.49	64.40%
26	221842418	D2S126	223.35	228.8	221.13	71.10%
27	230509098	D2S396	235.36	240.2	232.9	93.30%
28	233533279	D2S206	240.03	248.3	240.79	80.50%
29	237017393	D2S338	248.37	258.7	250.54	74.40%
30	240888027	D2S125	258.19	269.5	260.63	70.00%
						Average = 75%
Chromosome 3						
1	2013402	D3S1297	4.94	2.5	8.31	62.20%
2	6894241	D3S1304	19.61	16.5	22.33	76.70%
3	11492246	D3S1263	29.62	30.4	36.1	89.80%
4	16824398	D3S2338	37.49	36.3	42.1	70.00%
5	27932374	D3S1266	50.53	46.9	52.6	64.40%
6	34630713	D3S1277	60.07	56.1	61.52	64.70%
7	54454496	D3S1289	73.73	69.1	71.41	87.80%
8	60484946	D3S1300	82.22	79	80.32	83.30%
9	64914149	D3S1285	89.28	91	91.18	75.60%
10	70381991	D3S1566	94.76	97.2	97.75	88.90%
11	79894319	D3S3681	107.43	108.8	109.22	88.20%
12	102217419	D3S1271	112.28	117.7	117.76	66.30%
13	116606843	D3S1278	122.74	131.8	129.73	84.40%
14	124525892	D3S1267	130.3	141.1	139.12	78.70%
15	133113006	D3S1292	138.82	148.7	146.6	93.30%
16	144854126	D3S1569	150.58	162	158.38	85.40%
17	152507945	D3S1279	160.19	173	169.6	77.80%
18	169692713	D3S1614	169.98	183.1	177.75	70.00%
19	174965369	D3S1565	177.94	193	186.04	76.70%
20	187706180	D3S1262	194.33	207.2	201.14	78.90%
21	190025494	D3S1580	202	213.7	207.73	86.50%
22	193159977	D3S1601	208.14	220.4	214.45	87.80%
23	198506285	D3S1311	220.19	230.7	224.88	76.40%
						Average = 78.9%
Chromosome 4						
1	3417660	D4S412	4.42	3.7	4.74	75.60%
2	6678952	D4S2935	12.85	12.2	13.96	58.90%
3	13427096	D4S403	26.71	24.9	25.9	46.70%

Physical distance			Genetic distance (cM)			Heterozygosity from this study (calculated from
Order	(bp)	Marker	DeCODE	Genethon	Marshfield	Pedstats)
4	18525028	D4S419	34.97	32.6	33.42	78.90%
5	27288501	D4S391	47.33	43.2	43.59	77.80%
6	40193439	D4S405	60.23	56.7	56.95	74.40%
7	57522738	D4S1592	72.71	68.4	69.53	79.80%
8	70704052	D4S392	80.38	77.9	78.97	68.90%
9	81132491	D4S2964	88.45	87.1	88.35	73.00%
10	86665470	D4S1534	93.19	93.5	95.09	67.80%
11	92795838	D4S414	100.16	99.2	100.75	82.20%
12	104127191	D4S1572	107.52	106.3	107.95	80.00%
13	112075979	D4S406	114.74	115.8	117.06	83.10%
14	120505782	D4S402	122.1	123.5	124.45	74.00%
15	135147960	D4S1575	131.59	131.9	132.05	40.40%
16	142555250	D4S424	138.87	143.8	144.56	75.60%
17	158710759	D4S413	152.24	157.9	157.99	73.90%
18	170217844	D4S1597	163.65	169.1	169.42	44.40%
19	176062869	D4S1539	170.01	181.2	176.19	31.10%
20	179086249	D4S415	173.24	185	181.36	64.40%
21	185610898	D4S1535	189.38	198.5	195.06	77.80%
22	189482800	D4S426	202.69	211	206.98	55.60%
						Average = 67.4%
Chromosome 5						
1	1207413	D5S1981	1.19	0.6	1.72	77.50%
2	5047042	D5S406	12.36	10.7	11.85	78.90%
3	9434789	D5S2095	23.44	18.6	19.67	92.00%
4	16772994	D5S416	36.73	27.9	28.76	68.90%
5	26694222	D5S419	46.2	39.5	39.99	84.40%
6	34798552	D5S426	56.81	51.6	51.99	77.80%
7	40051523	D5S418	64.56	58.1	58.55	67.80%
8	56030499	D5S407	71.92	65	64.67	74.40%
9	66282905	D5S647	78.89	74.7	74.07	76.70%
10	76193474	D5S424	90.87	82.8	81.95	61.10%
11	82038651	D5S641	99	92.3	92.38	85.60%
12	85446379	D5S428	101.88	95.4	95.4	77.20%
13	95838449	D5S644	108.55	104.5	104.76	83.30%
14	103990521	D5S433	112.58	112.2	111.97	71.90%
15	111173317	D5S2027	118.12	118.9	119.5	71.10%
16	119076842	D5S471	124.98	129.6	129.83	67.80%
17	134747146	D5S2115	136.75	138.6	138.64	61.10%
18	145184110	D5S436	147.14	147.2	147.49	75.60%
19	152755167	D5S410	158.44	156	156.47	58.00%
20	162086436	D5S422	166.12	163.9	164.19	88.90%
21	168375447	D5S400	177.01	174.3	174.8	79.80%
22	179920770	D5S408	205.05	195.8	195.49	75.60%
						Average = 75.2%
Chromosome 6						
1	5957131	D6S1574	15.18	8.7	9.18	56.70%
2	8169920	D6S309	20.5	13.6	14.07	92.20%
3	10133700	D6S470	23.75	17.7	18.22	65.60%

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
4	15389917	D6S289	34.61	29.6	29.93	64.00%
5	20478015	D6S422	42.83	35.7	35.66	70.00%
6	24293780	D6S276	47.93	44.9	44.41	81.10%
7	39367524	D6S1610	58.47	53.9	53.81	68.20%
8	56026395	D6S257	79.52	80	79.92	90.70%
9	80407562	D6S460	90.58	90	89.83	78.90%
10	90985231	D6S462	97.76	99	99.01	43.30%
11	102542632	D6S434	106.2	109.2	109.19	90.00%
12	119595297	D6S287	119.55	122	121.97	74.40%
13	131772324	D6S262	130.73	129.8	130	84.30%
14	136356906	D6S292	136.39	138.2	136.97	85.60%
15	141298257	D6S308	144.82	145.5	144.46	64.40%
16	153906250	D6S441	160.64	155.3	154.1	77.30%
17	160247178	D6S1581	169.88	165	164.78	79.80%
18	166679600	D6S264	180.95	179.1	179.07	46.70%
19	169815439	D6S281	188.38	201.1	190.14	65.60%
20	170469932	D6S446	189.16	188.4	189	71.10%
						Average = 72.4%
Chromosome 7						
1	2995502	D7S531	7.51	4.8	5.28	85.40%
2	4271155	D7S517	8.69	7.8	7.44	79.30%
3	11424476	D7S513	22.62	unknown	17.74	89.50%
4	17370306	D7S507	31.51	29.1	28.74	86.70%
5	21578329	D7S493	36.92	35	34.69	66.30%
6	27970934	D7S516	44.27	42.1	41.69	70.20%
7	35058145	D7S484	54.65	55.6	53.5	75.60%
8	38962951	D7S510	60.48	60.5	59.93	87.60%
9	45889234	D7S519	68.66	70.5	69.03	75.30%
10	66501686	D7S502	79.12	79.6	78.65	78.40%
11	77517086	D7S669	89.39	90.9	90.42	78.40%
12	88088243	D7S630	99.93	98.7	98.44	76.40%
13	92450836	D7S657	103.47	105.2	104.86	82.70%
14	101297209	D7S515	111.29	112.9	112.32	79.80%
15	115488625	D7S486	122.44	125.3	124.08	84.30%
16	128796370	D7S530	130.7	136.4	134.55	62.90%
17	132096915	D7S640	138.75	139.7	137.83	86.50%
18	138000940	D7S684	146.68	149.6	147.22	73.00%
19	143039542	D7S661	151.86	157.5	155.1	75.60%
20	150136884	D7S636	162.09	165	162.33	94.40%
21	152249427	D7S798	169.87	171.3	168.98	71.90%
22	155614160	D7S2465	181.67	182.1	180.24	83.70%
						Average = 79.2%
Chromosome 8						
1	2117695	D8S264	3.36	0.7	0.73	87.60%
2	6504083	D8S277	15.23	8.4	8.34	77.50%
3	10918925	D8S550	20.09	20.4	21.33	73.30%
4	15693947	D8S549	26.31	30.7	31.73	45.60%
5	20411446	D8S258	34.88	40.3	41.55	80.00%

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
6	25497030	D8S1771	43.74	49.6	50.05	67.80%
7	34570315	D8S505	54.23	60	60.87	78.20%
8	57229610	D8S285	68.31	70.6	71	85.60%
9	61984334	D8S260	73.6	78.8	79.36	87.50%
10	93089545	D8S270	97.29	102.1	103.69	75.60%
11	106172348	D8S1784	112.44	116.8	118.15	78.80%
12	123811283	D8S514	124.62	128.9	130	70.10%
13	131580804	D8S284	139.79	142.7	143.82	83.10%
14	137804459	D8S272	150.14	152.5	154.02	79.50%
						Average = 76.4%
Chromosome 9						
1	3941638	D9S288	8.1	8.8	9.83	83.30%
2	8043377	D9S286	17.77	16.8	18.06	80.00%
3	16067944	D9S285	33.56	27.9	29.52	85.60%
4	17618218	D9S157	36.26	31.8	32.24	81.10%
5	24524208	D9S171	45.57	42	42.73	25.80%
6	27622317	D9S161	51.5	50.3	51.81	48.90%
7	33849592	D9S1817	56.48	57.9	59.34	82.20%
8	69768948	D9S273	66.75	64.5	65.79	70.00%
9	75177108	D9S175	70.64	68.8	70.33	66.70%
10	83013561	D9S167	81.01	82.4	83.41	81.10%
11	89643864	D9S283	92.91	93.2	94.85	75.60%
12	95545644	D9S287	98.7	103.3	103.42	87.60%
13	101179621	D9S1690	104.08	106.5	106.63	80.00%
14	109017080	D9S1677	112.85	117.8	117.37	83.10%
15	115038976	D9S1776	121.62	124.2	123.33	71.10%
16	122072737	D9S1682	128.77	132.9	132.09	60.00%
17	128607005	D9S290	136.4	141.1	140.86	66.70%
18	133285484	D9S164	146.92	148.1	147.91	83.70%
19	135674212	D9S1826	157.73	160.2	159.61	85.40%
20	136324992	D9S158	158.3	163	161.71	59.60%
						Average = 72.9%
Chromosome 10						
1	270790	D10S249	1.18	0	2.13	75.30%
2	4399165	D10S591	14.38	12.3	13.49	60.00%
3	6761879	D10S189	19.78	17.3	19	83.30%
4	10590415	D10S547	27.79	28.1	29.15	69.70%
5	15717837	D10S1653	37.75	38.8	40.36	72.20%
6	18761141	D10S548	43.4	43.4	45.7	60.70%
7	26566886	D10S197	50.04	50.5	52.1	68.90%
8	31720082	D10S208	59.94	60.2	60.64	70.80%
9	51812273	D10S196	70.07	72.5	70.23	63.30%
10	64077500	D10S1652	80.61	83.3	80.77	47.80%
11	72065333	D10S537	89.16	93.8	91.13	74.20%
12	85556280	D10S1686	105.03	109.2	105.04	79.80%
13	95178112	D10S185	113.34	123.3	116.34	80.00%
14	102426189	D10S192	120.75	131.2	124.27	91.00%
15	111220774	D10S597	126.7	137.6	128.73	46.70%

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
16	119434826	D10S1693	138.39	146.1	137.39	85.60%
17	125178441	D10S587	148.87	156.6	147.57	80.00%
18	129430167	D10S217	159.85	167.2	157.89	86.00%
19	132582543	D10S1651	171.95	178.3	168.77	56.70%
20	134299489	D10S212	177.19	180.7	170.94	42.20%
						Average = 69.6%
Chromosome 11						
1	1920210	D11S4046	1.26	3.9	2.79	90.00%
2	5944487	D11S1338	9.77	14.9	12.92	74.40%
3	17445017	D11S902	25.69	24.7	21.47	83.00%
4	26637090	D11S904	43.64	37	33.57	76.70%
5	35979739	D11S935	52.94	49.6	45.94	74.20%
6	40930848	D11S905	57.39	55.7	51.95	75.90%
7	59756134	D11S4191	64.96	63.4	60.09	83.30%
8	67649916	D11S987	72.17	unknown	67.48	83.50%
9	72000791	D11S1314	78.75	77.5	73.64	74.20%
10	77531965	D11S937	83.73	84.6	79.98	87.80%
11	81522196	D11S901	88.48	89.8	85.48	73.00%
12	89891627	D11S4175	93.04	96.3	91.47	79.50%
13	100561653	D11S898	103.59	103.1	98.98	36.70%
14	114792510	D11S908	116.46	112.5	108.59	46.70%
15	120333420	D11S925	124.09	123.5	118.47	87.50%
16	125797180	D11S4151	133.15	132.9	127.33	65.20%
17	131427605	D11S1320	146.94	147.2	141.91	61.10%
18	133323585	D11S968	152.45	152.8	147.77	57.80%
						Average = 72.7%
Chromosome 12						
1	531651	D12S352	0	0	0	68.50%
2	5434814	D12S99	15.2	13.9	12.6	82.60%
3	9385295	D12S336	24.04	unknown	19.68	77.80%
4	13724569	D12S364	31.16	31.7	30.6	81.10%
5	18864801	D12S310	36.23	36.1	36.06	56.80%
6	24991278	D12S1617	45.91	45.1	44.03	91.10%
7	32216079	D12S345	55.25	54.4	53.09	89.80%
8	45622953	D12S85	60.49	62.7	61.34	77.80%
9	50917730	D12S368	66.55	67.3	66.03	61.10%
10	59175649	D12S83	74.03	76.5	75.17	77.50%
11	76476264	D12S326	91.2	87.6	86.4	67.80%
12	90407774	D12S351	101.06	97.1	95.56	64.40%
13	98030790	D12S346	111.02	106.1	104.65	53.90%
14	102766931	D12S78	116.12	113.3	111.87	81.10%
15	114523147	D12S79	131.88	126.1	125.31	75.90%
16	117633041	D12S86	138.06	135.1	134.54	86.50%
17	125149965	D12S324	148.64	148.3	147.17	58.90%
18	127940970	D12S1659	159.6	157.2	155.94	55.60%
19	130487603	D12S1723	169.54	165.7	164.63	90.00%
						Average = 73.6%
Chromosome 13						

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
1	19746379	D13S175	0.33	7.4	6.03	67.80%
2	28269790	D13S217	22.17	19.1	17.21	79.80%
3	32151911	D13S171	31.07	27.3	25.08	67.80%
4	37930230	D13S218	39.34	35.3	32.9	67.40%
5	40978919	D13S263	43.24	40.4	38.32	84.30%
6	47788734	D13S153	51.62	47.5	45.55	90.90%
7	73555425	D13S156	69.73	57.3	55.85	66.70%
8	80007094	D13S170	76.17	65.4	63.9	91.10%
9	89170920	D13S265	80.82	70.6	68.73	80.90%
10	97851594	D13S159	91.96	81.5	79.49	84.40%
11	102774397	D13S158	98.96	86.9	84.87	83.70%
12	106604889	D13S173	109.36	95.9	93.52	66.70%
13	108126457	D13S1265	114.46	101.7	98.82	79.80%
14	111843382	D13S285	123.78	112.8	110.55	84.40%
						Average = 78.2%
Chromosome 14						
1	19910227	D14S261	4.33	0	6.46	68.90%
2	21757254	D14S283	14.7	7.5	13.89	82.00%
3	25766612	D14S275	22.21	21.9	28.01	63.30%
4	33528944	D14S70	36.37	32.9	40.11	69.70%
5	43171518	D14S288	44	39.1	47.51	87.80%
6	54752768	D14S276	54.41	47	56.36	80.00%
7	63720759	D14S63	63.5	59	69.18	72.20%
8	69652604	D14S258	68.54	65.8	76.28	72.20%
9	77728132	D14S74	78.19	76.4	87.36	74.20%
10	87498456	D14S48	86.54	unknown	95.89	76.40%
11	91252619	D14S280	94.42	95.5	105	64.40%
12	96691224	D14S65	106.52	108.1	117.3	62.20%
13	100366288	D14S985	115.83	117.1	126.61	84.40%
14	103666456	D14S292	122.82	124.2	134.3	68.90%
						Average = 73.3%
Chromosome 15						
1	22681797	D15S128	5.82	6.1	6.11	72.20%
2	25523216	D15S1002	15.05	14.5	14.58	74.40%
3	29047841	D15S165	22.64	20.2	20.24	53.30%
4	31524978	D15S1007	28.86	25.9	25.86	76.70%
5	36794818	D15S1012	38.12	35.3	35.95	56.70%
6	38369343	D15S994	42.62	40	40.25	87.60%
7	47061967	D15S978	48.18	45.5	45.62	91.00%
8	56266838	D15S117	56.46	50.8	51.21	81.10%
9	64346916	D15S153	68.1	62.1	62.4	82.20%
10	68970829	D15S131	76.52	70.7	71.28	67.80%
11	82021671	D15S205	89.09	77.4	78.92	84.40%
12	89198603	D15S127	97.26	84.8	86.81	93.20%
13	92512173	D15S130	108.21	98	100.59	71.10%
14	97409502	D15S120	126.52	109.6	112.58	81.10%
						Average = 76.6%
Chromosome 16						

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
1	5983141	D16S423	14.05	8.4	10.36	82.20%
2	9625612	D16S404	24.89	16.7	18.07	76.70%
3	12116698	D16S3075	29.51	21.8	23.28	90.00%
4	17380963	D16S3103	37.97	31.1	32.07	51.10%
5	20793898	D16S3046	42.99	39.3	40.65	66.70%
6	25468101	D16S3068	49.5	46.6	48.53	72.20%
7	49263733	D16S3136	60.35	60	62.11	56.70%
8	52228161	D16S415	66.97	65.6	67.62	73.30%
9	62156288	D16S503	81.79	81.8	83.55	70.00%
10	75074528	D16S515	92.06	90.2	92.1	80.00%
11	77681516	D16S516	98.52	98.3	100.39	76.70%
12	81537950	D16S3091	109.59	109.1	111.12	84.40%
13	85073612	D16S520	122.84	123.3	125.82	82.20%
						Average = 74.1%
Chromosome 17						
1	379286	D17S849	0.63	0.6	0.63	71.10%
2	1857149	D17S831	7.22	6.6	6.6	80.00%
3	6189991	D17S938	17.06	14.8	14.69	75.60%
4	10456225	D17S1852	30.67	23.2	22.24	86.70%
5	13111680	D17S799	37	32.8	31.96	71.10%
6	14201323	D17S921	41.47	37.3	36.14	57.80%
7	16355941	D17S1857	46.39	44.1	43.01	55.60%
8	28313924	D17S798	55.6	53.9	53.41	54.40%
9	44539744	D17S1868	74.53	65.1	64.16	80.00%
10	50636883	D17S787	81.32	75.7	74.99	77.80%
11	58789905	D17S944	92.76	84.2	82.56	45.60%
12	65976926	D17S949	102.96	94.9	93.27	84.40%
13	71942894	D17S785	115.34	104.7	103.53	80.00%
14	75416715	D17S784	129.62	117.7	116.86	68.90%
15	77846127	D17S928	135.67	128.7	126.46	80.00%
						Average = 71.3%
Chromosome 18						
1	636459	D18S59	1.39	0.1	0	73.30%
2	3428519	D18S63	9.57	7.9	8.3	68.90%
3	5819472	D18S452	17.62	17.7	18.7	76.70%
4	9951257	D18S464	32.42	32.4	31.17	63.30%
5	11482729	D18S53	37.67	40.4	41.24	77.80%
6	23401301	D18S478	50.83	52.3	52.86	61.10%
7	33177089	D18S1102	58.16	61.7	62.84	78.70%
8	46948679	D18S474	72.83	71.3	71.32	88.90%
9	55577010	D18S64	82.18	83	84.8	71.80%
10	59688619	D18S68	88.78	94.4	96.48	82.20%
11	65587036	D18S61	97.71	102.8	105.03	71.10%
12	70399261	D18S1161	108.45	112	114.26	68.90%
13	73264439	D18S462	116.37	118	120.05	78.90%
14	75965205	D18S70	121.65	123.8	126	63.30%
						Average = 73.2%
Chromosome 19						

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
1	3265328	D19S209	11.38	10.8	10.97	87.60%
2	4900356	D19S216	16.72	19.1	20.01	66.70%
3	8055954	D19S884	25.67	26	26.37	76.40%
4	12573741	D19S221	32.39	35.5	36.22	55.70%
5	14494399	D19S226	36.35	41.7	42.28	83.30%
6	36606563	D19S414	53.81	53.2	54.01	55.60%
7	43123390	D19S220	63.58	61.4	62.03	86.70%
8	48500599	D19S420	68.94	66	66.3	83.30%
9	53023839	D19S902	76.15	76.2	72.72	81.10%
10	57988748	D19S571	93.01	87.7	84.08	19.50%
11	60237645	D19S418	104.01	97.5	92.56	77.80%
12	61711332	D19S210	108.59	104.9	100.01	72.20%
						Average = 70.9%
Chromosome 20						
1	603091	D20S117	2.9	2.9	2.83	71.90%
2	3894952	D20S889	11.93	11	11.2	82.20%
3	7607866	D20S115	24.66	20.9	21.15	47.80%
4	11471794	D20S186	34.58	33.2	32.3	82.20%
5	17263937	D20S112	44.35	39.3	39.25	73.30%
6	31289325	D20S195	56.36	50.2	50.81	84.40%
7	38315924	D20S107	61.78	54.9	55.74	94.40%
8	43082263	D20S119	69.4	61	61.77	85.60%
9	45985333	D20S178	73.83	65.5	66.16	65.50%
10	48994970	D20S196	78.47	74.5	75.01	86.70%
11	53747459	D20S100	88.96	83.4	84.78	75.30%
12	57241283	D20S171	98.63	94.4	95.7	71.10%
13	58311347	D20S173	100.39	96.5	98.09	72.20%
						Average = 76.4%
Chromosome 21						
1	18244526	D21S1256	12.9	8.6	9.72	60.90%
2	24544187	D21S1914	24.37	23	19.39	80.00%
3	31143785	D21S263	32.52	31.4	27.4	87.60%
4	36748728	D21S1252	42.96	38.7	35.45	64.40%
5	41606426	D21S266	56	49.9	45.87	78.90%
						Average = 74.4%
Chromosome 22						
1	16233834	D22S420	2.96	0	4.06	72.20%
2	20582333	D22S539	14.68	9	14.44	70.10%
3	24340393	D22S315	22.59	16.2	21.47	76.70%
4	31533925	D22S280	37.03	25.9	31.3	74.40%
5	35075204	D22S283	42.07	33.4	38.62	70.00%
6	38706685	D22S423	49.14	40.2	46.42	80.00%
7	43589652	D22S274	56.47	45.5	51.54	80.00%
						Average = 74.8%
Chromosome X						
1	5269502	DXS1060	12.71	10.1	15.12	75.00%
2	9308965	DXS8051	17.73	15.7	17.29	82.10%
3	14468958	DXS987	28.2	25.5	22.18	87.80%

Order	Physical distance (bp)	Marker	Genetic distance (cM) DeCODE	Genethon	Marshfield	Heterozygosity from this study (calculated from Pedstats)
4	22707078	DXS1226	38.7	36.8	27.59	77.50%
5	31020404	DXS1214	46.21	46.2	33.54	78.00%
6	38664205	DXS1068	60.25	56.2	37.33	73.20%
7	40903890	DXS993	64.18	66.1	42.21	65.90%
8	55402059	DXS991	79.2	86.9	52.5	75.60%
9	79187174	DXS986	86.84	95.9	57.37	84.60%
10	92806741	DXS990	94.92	104.9	60.62	53.70%
11	102538076	DXS1106	103.56	115.1	66.58	53.70%
12	114477867	DXS8055	114.85	126.8	70.91	31.70%
13	119618407	DXS1001	120.35	139.4	75.79	65.00%
14	128800831	DXS1047	131.5	150.3	82.07	90.20%
15	140527900	DXS1227	150.37	164.7	88.33	63.40%
16	143734058	DXS8043	160.62	176.7	94.22	56.10%
17	147308345	DXS8091	167.3	186.3	96.14	43.90%
18	153392551	DXS1073	188.22	196.5	102.35	73.20%
						Average = 68.3%

Table 7. Examples of marker allele frequencies.

						Allele frequency		
Marker	Allele	Allele code	Allele count			Original	+0.5 count	+3 counts
D1S468	192	1	4	of	24	0.166667	0.160700	0.145800
D1S468	194	2	1	of	24	0.041667	0.053600	0.083300
D1S468	200	3	1	of	24	0.041667	0.053600	0.083300
D1S468	202	4	7	of	24	0.291667	0.267900	0.208300
D1S468	204	5	1	of	24	0.041667	0.053600	0.083300
D1S468	206	6	2	of	24	0.083333	0.089300	0.104200
D1S468	208	7	4	of	24	0.166667	0.160700	0.145800
D1S468	210	8	4	of	24	0.166667	0.160700	0.145800
D1S214	123	1	3	of	24	0.125000	0.118600	0.105300
D1S214	125	2	0	of	24	0.000001	0.016900	0.052600
D1S214	137	3	1	of	24	0.041667	0.050800	0.070200
D1S214	138	4	1	of	24	0.041667	0.050800	0.070200
D1S214	139	5	13	of	24	0.541667	0.457600	0.280700
D1S214	140	6	0	of	24	0.000001	0.016900	0.052600
D1S214	141	7	1	of	24	0.041667	0.050800	0.070200
D1S214	142	8	2	of	24	0.083333	0.084700	0.087700
D1S214	143	9	2	of	24	0.083333	0.084700	0.087700
D1S214	144	10	1	of	24	0.041667	0.050800	0.070200
D1S214	145	11	0	of	24	0.000001	0.016900	0.052600

numberof 3 were also used and the results from different allele frequencies were compared. Examples of original and modified marker allele frequencies were shown in Table 7.

Heterozygosities of the marker set were calculated using Pedstat which was included in the Merlin distribution. The heterozygosities in this Thai data set was around 75% as shown in Table 6.

Cleaning of data from the genome scan

Mendelian inconsistencies were detected by PedCheck. First, genotypes in chromosome 1 of all samples were run in the program to initially screen any misspecification of relationship in the pedigrees. PedCheck found 46 mendelian inconsistencies and most of the inconsistencies were from pedigree F30. The genotypes of 21 markers out of 31 markers in chromosome 1 were found to be inconsistent in F30. The mendelian inheritance errors in F30 were then investigated and found that most of the errors were most likely to be originated from one person F30.III6. Further investigation was done to test the possible relationship of this individual to the other pedigree members, and to test if there were any mispecified relationships in all the pedigrees since this might not show in mendelian inheritance errors if not all members in the pedigree were typed. The genome scan data were then put in PREST program, which estimated the probability of each pair of pedigree members sharing 0, 1, or 2 alleles IBD from the genotype data and performed statistical tests to determine if the pattern of allele sharing was consistent with the relationship specified in the pedigree. It was found that the individual F30.III6 did not essentially share any allele IBD with other people in the pedigree. Therefore, it was most likely that this individual was unrelated to the pedigree and was dropped out from the analyses. There was no marked significant deviation of allele sharing pattern from the relationship specified in the 9 pedigrees of all 90 samples left.

After dropping out the individual F30.III6, PedCheck were run for all 23 chromosomes. A total of 55 mendelian inheritance errors were detected (Table 8). Each error was manually investigated. The genotypes that were most likely to cause the errors were recoded to unknown and PedCheck was run again. The process was repeated until PedCheck found no mendelian errors. A total of 82 genotypes were recoded to unknown to remove those 55 mendelian errors. Error detection was further performed by looking for genotypes that cause unlikely recombination events using Merlin. Merlin calculated an error statistic for each individual and reported only individuals with the error statistic less than 0.025 to be likely to have genotype errors. However, in 10 cM genome scan, Merlin might not have much power for this error detection since the distance between adjacent markers was not close enough and errors reported by Merlin might not be real errors. In our samples, Merlin reported 46 genotype errors with the error statistics. Then a histogram was plotted (Figure 2) to observe the distribution of the error statistics. From the histogram, a level of 0.002 was selected to be a cut-off value. Bearing in mind that 11 errors (Table 9) with the error statistics less than 0.002 could be the real genotype errors, the main analysis was pursued without removing (setting to unknown) these errors. Analysis with removing these errors was also conducted and compared with the results from the main analysis.

Yield of the microsatellite genotyping

Excluding the mispecified samples F30.III6, the expected total number of genotypes would be 36,000 genotypes (90 samples, each with 400 genotypes). In the actual genome scan, 262 genotypes failed during the genotyping process and 82 genotypes were removed to eliminate 55 mendelian inheritance errors. In total, 344 (0.96%) genotypes were missing, resulting in the yield of microsatellite genotyping in this study being 99.04%.

Nonparametric linkage analysis

From overall-family results of the multipoint nonparametric linkage analysis using the 16 different allele scoring models as described above, with modified marker allele frequency, and without removing any errors reported by Merlin, no significant linkage was found. However, there were 13 regions in 12 chromosomes

which showed Zlr score > 2 ($p<0.05$). These regions were at markers D1S207 (107 cM), D2S126 (233 cM), D3S1565 (178 cM), D4S406 (115 cM), D7S507 (32 cM), D9S287 (99 cM), D12S352 (7.6 cM), D13S1265 (114 cM), D14S70 (36 cM), D15S1007 (29 cM), D18S68 (89 cM), DXS1227 (150 cM), and DXS8091 (167 cM). The maximum Zlr was

Table 8. Number of mendelian errors detected by PedCheck.

	Number of errors in the pedigree									
Marker	F1	F9	F11	F15	F18	F19	F28	F30	F36	Total
D1S214								1		1
D1S252			1					1		2
D1S2878								1		1
D1S238	1		1		1					3
D3S1263		1								1
D3S2338	1				1					2
D3S1271								1		1
D3S1569								1		1
D3S1580								1		1
D3S1311								1		1
D4S1592									1	1
D4S1575								1		1
D5S1981								1		1
D6S289								1		1
D6S257							1			1
D7S517								1		1
D7S516								1		1
D7S502								1		1
D7S657									1	1
D7S486							1	1		2
D7S530								1		1
D7S661							2			2
D7S798							1			1
D8S264								1		1
D8S277							1			1

Marker	Number of errors in the pedigree									Total
	F1	F9	F11	F15	F18	F19	F28	F30	F36	
D9S164			1							1
D9S158				1						1
D10S217		1								1
D11S902				1						1
D11S905								1		1
D11S987					1					1
D12S345					1					1
D12S79			1					1		2
D13S217								1		1
D13S218				1						1
D13S153							1			1
D13S158			1				1			2
D13S173								1		1
D15S127	1									1
D19S571	1			1						2
D20S178					1					1
D21S1256									1	1
D22S539			1					1		2
DXS8051								1		1
DXS986								1		1
Total	4	2	6	4	5	0	8	23	3	55

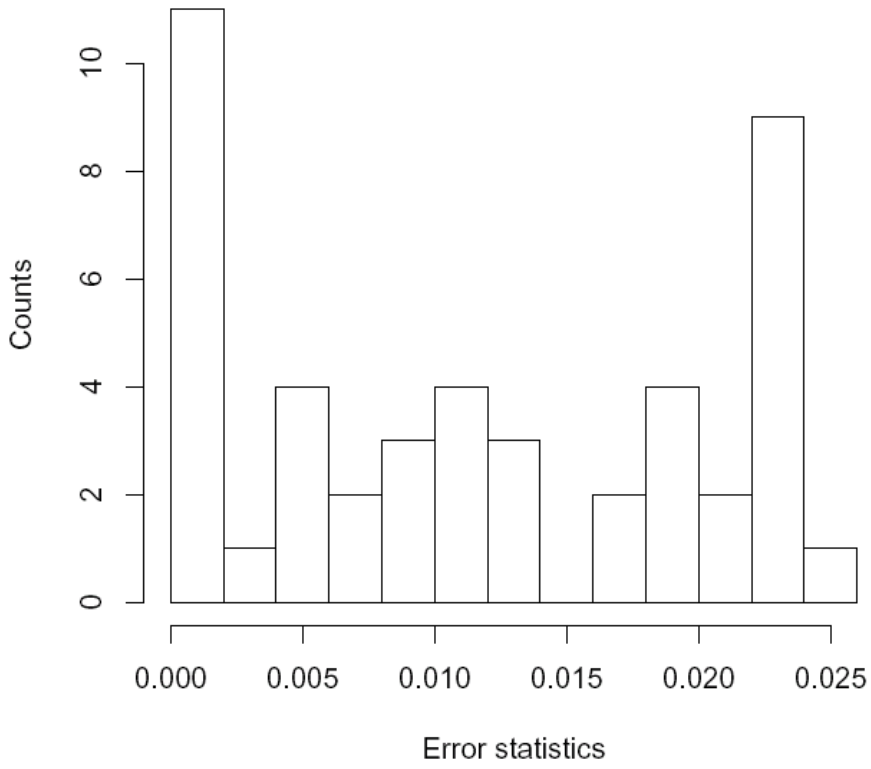

Figure 2. A histogram of error statistics from Merlin

Table 9. Eleven individuals most likely to be errors as reported by Merlin.

Individual	Chromosome	Marker	Genetic distance (cM)	Error statistic
F19.II6	3	D3S2338	37	0.00165
F19.IV9	3	D3S2338	37	0.000849
F30.III2	4	D4S392	80	0.0000249
F28.IV96	6	D6S262	131	0.00162
F18.II12	7	D7S661	152	0.00155
F18.II8	9	D9S287	99	0.00000041
F18.II13	9	D9S287	99	0.000313
F28.III25	20	D20S173	100	0.00111
F30.II5	X	DXS1060	13	0.000000000673
F30.II8	X	DXS8051	18	0.0000851
F30.III2	X	DXS8043	161	0.00149

2.87 (p=0.002) at the marker D12S352 in chromosome 12 using exp mnallele equal model. However,when all the 16 models were compared, only 4 regions on chromosome 3, 12, 13 and 18 showed Zlr > 2 fairly consistently across several models (Table 10). Curves of Zlr scores plotted against location (Figure 3) were presented for an exp pairs equal model which represented the overall of the information quite well. This was in accordance with McPeek's review [McPeek, 1999], in which Spairs was recommended as a compromised choice for an allele sharing statistic that performed well over all disease models in general.

Removing the mostly likely 11 errors reported by Merlin did not have much effect to the results. There were no additional Zlr scores of more than 2, however, there were 2 markers D9S287 and DXS8091, at which Zlr dropped to lower than 2. Zlr curves of the analysis without the 11 errors for exp pairs equal model were shown in Figure 4.

The 4 regions where Zlr > 2 occurred fairly consistently across several models were the first set of candidate regions that was worth looking at. In addition, the peak of Zlr > 2 at marker D1S207 in chromosome 1 was excessively contributed one family F30 and it might be worth investigating around the marker in this family. Chromosome X has long been suspected in LHON and the peak at marker DXS1227 was also worth looking at. The peaks for these two markers were not seen in exp pairs equal model and are shown separately in Figure 5. The peak at marker DXS8091 was of less interest since it might occur by genotyping errors as reported by Merlin. I ended up with 6 interesting chromosomal region on chromosome 1, 3, 12, 13, 18 and X, which could be promising candidates for the nuclear modifier gene(s) in Thai LHON. The results for each individual family Twere analysed in these 6 regions and it was found that different families contributed to different extent to the overall allele sharing score. This was summarized in Table 11.

Table 10. Chromosomal regions showing Zlr > 2 in 16 allele sharing models.

Zlr in the chromosomal region where Zlr > 2																						
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
D1S207	D2S126	D3S1565	D4S406			D7S507		D9S287			D12S352	D13S1263	D14S70	D15S1007			D18S68					DXS1227
107cM	233cM	178cM	115cM			32cM		99cM			7.6cM	114cM	36cM	29cM			89cM					150cM
		2.09						2.01*			2.2						2.06					
2.32																						
2.51																						
		2.61									2.3	2.34					2.18					2.07*+
		2.47									2.16	2.4					2.4					
												2.2										
		2.24									2.87	2.11										2.42*+
		2.11															2.17					
														2.26								
2.01														2.3								
		2.2										2.08										2.2
		2.42				2.04						2.37					2.45					
	2.03	2.3									2.06	2.48					2.45					
	2.18	2.01	2.07									2.51	2.02				2.28					
		2.28										2.2					2.04					2.13

most likely errors detected by Merlin were removed

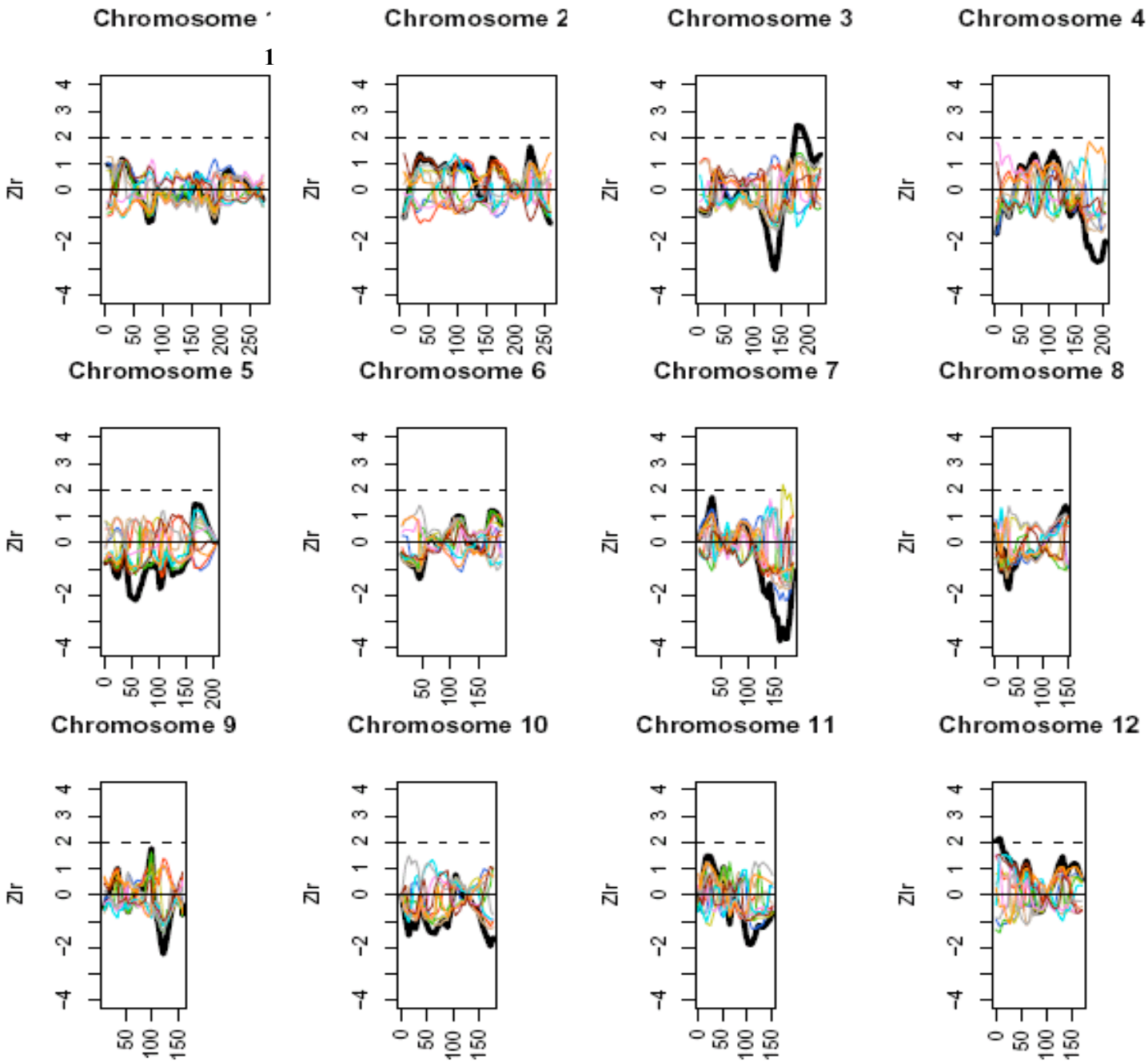

Figure 3. Zlr curves plotted against location (cM) for each chormosome from the analysis without removing the errors reported by Merlin, using exp pairs equal model. The thick

black lines are Zlr from the overall families. The thin coloured lines are family specific Zlr curves. The legend for each colour is presented next to chromosome X.

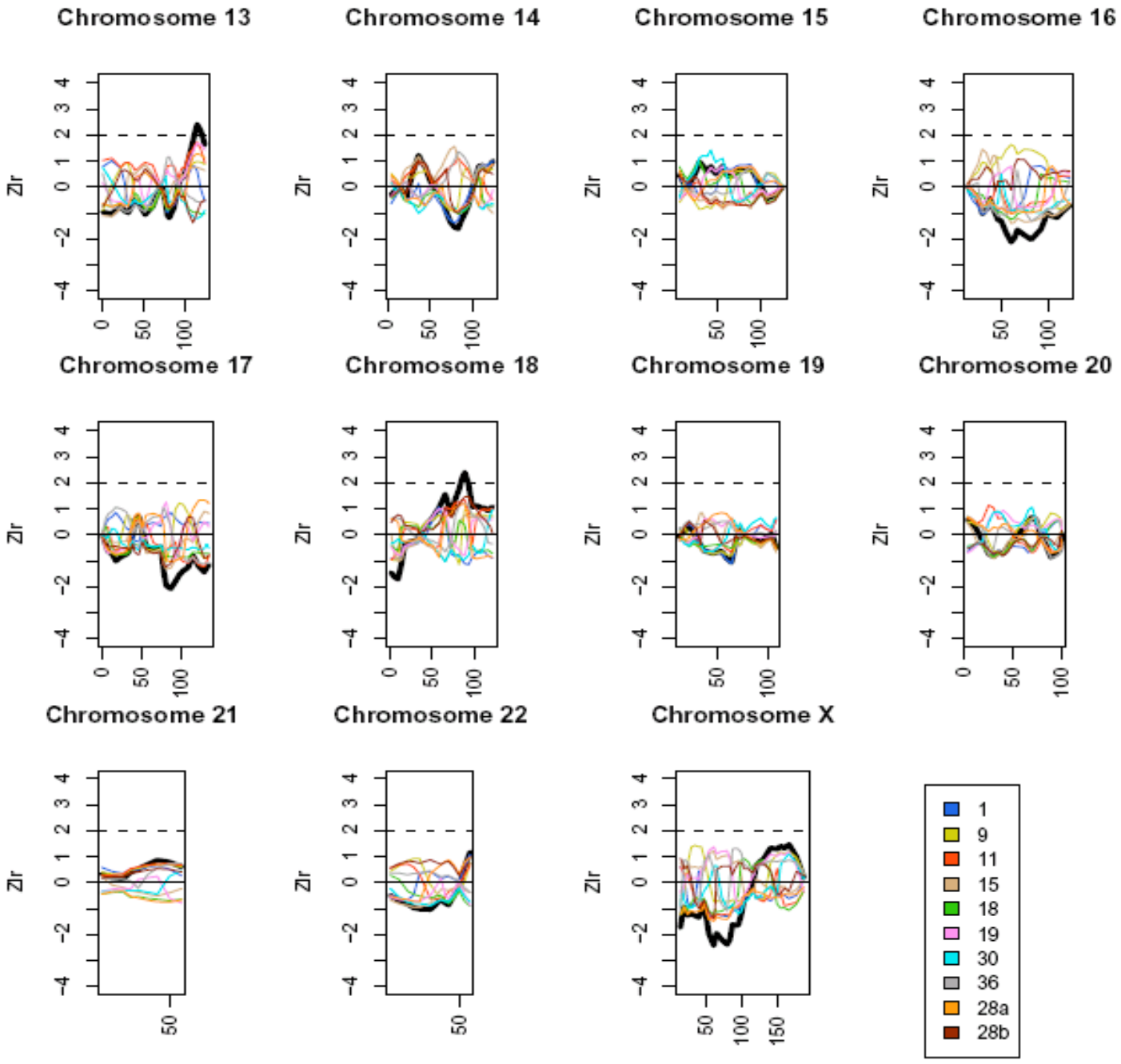

Figure 3. Zlr curves plotted against location (cM) for each chormosome from the analysis without removing the errors reported by Merlin…(cont)

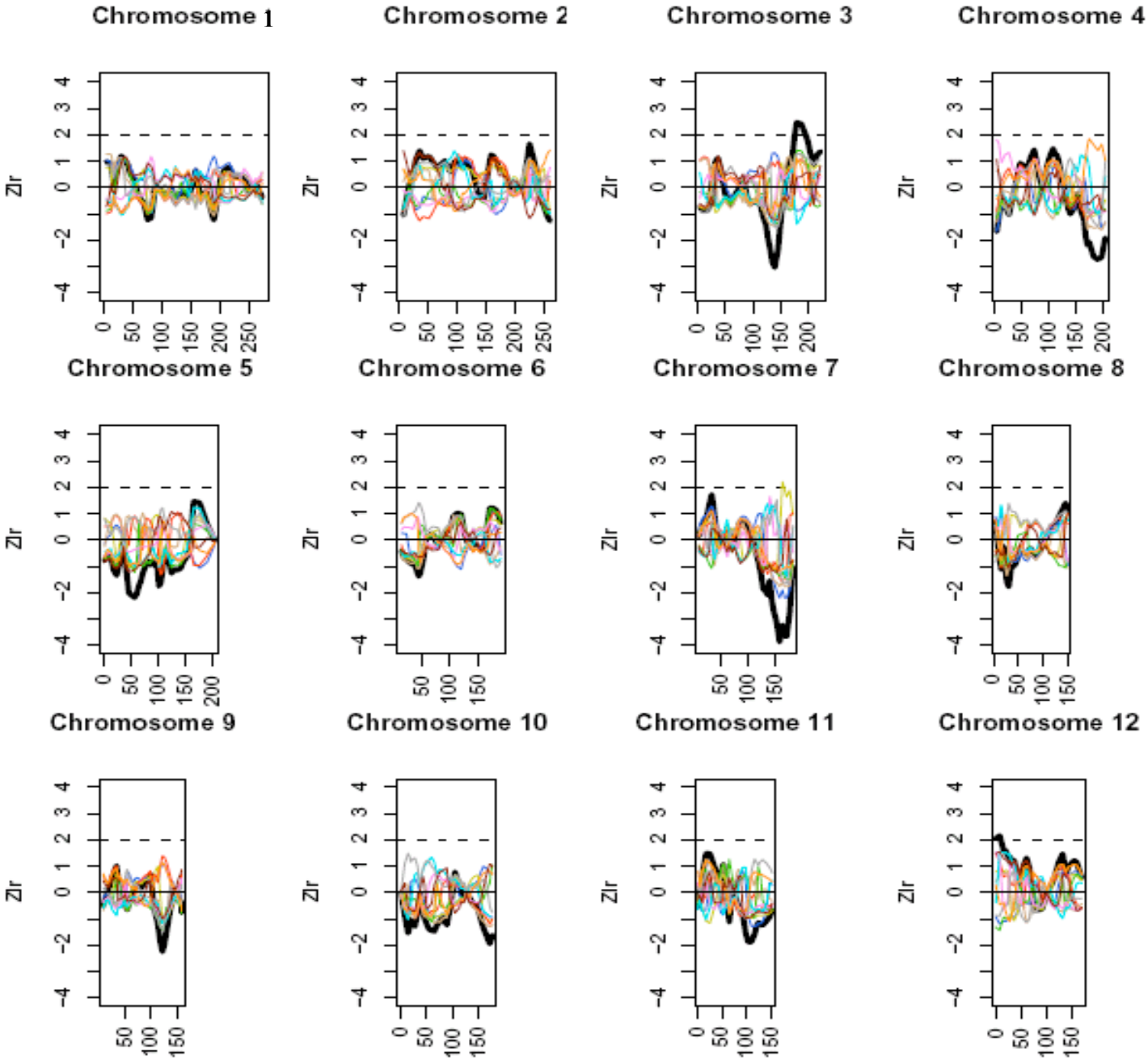

Figure 4. Zlr curves plotted against location (cM) for each chormosome from the analysis with the most likely 11 errors from Merlin removed, using exp pairs equal model. The thick black lines are Zlr from the overall families. The thin coloured lines are family specific Zlr curves. The legend for each colour is

presented next to chromosome X. Note some drops of peaks in chromosome 9 (99 cM) and X (167 cM) compared with the analysis without removing the 11 errors.

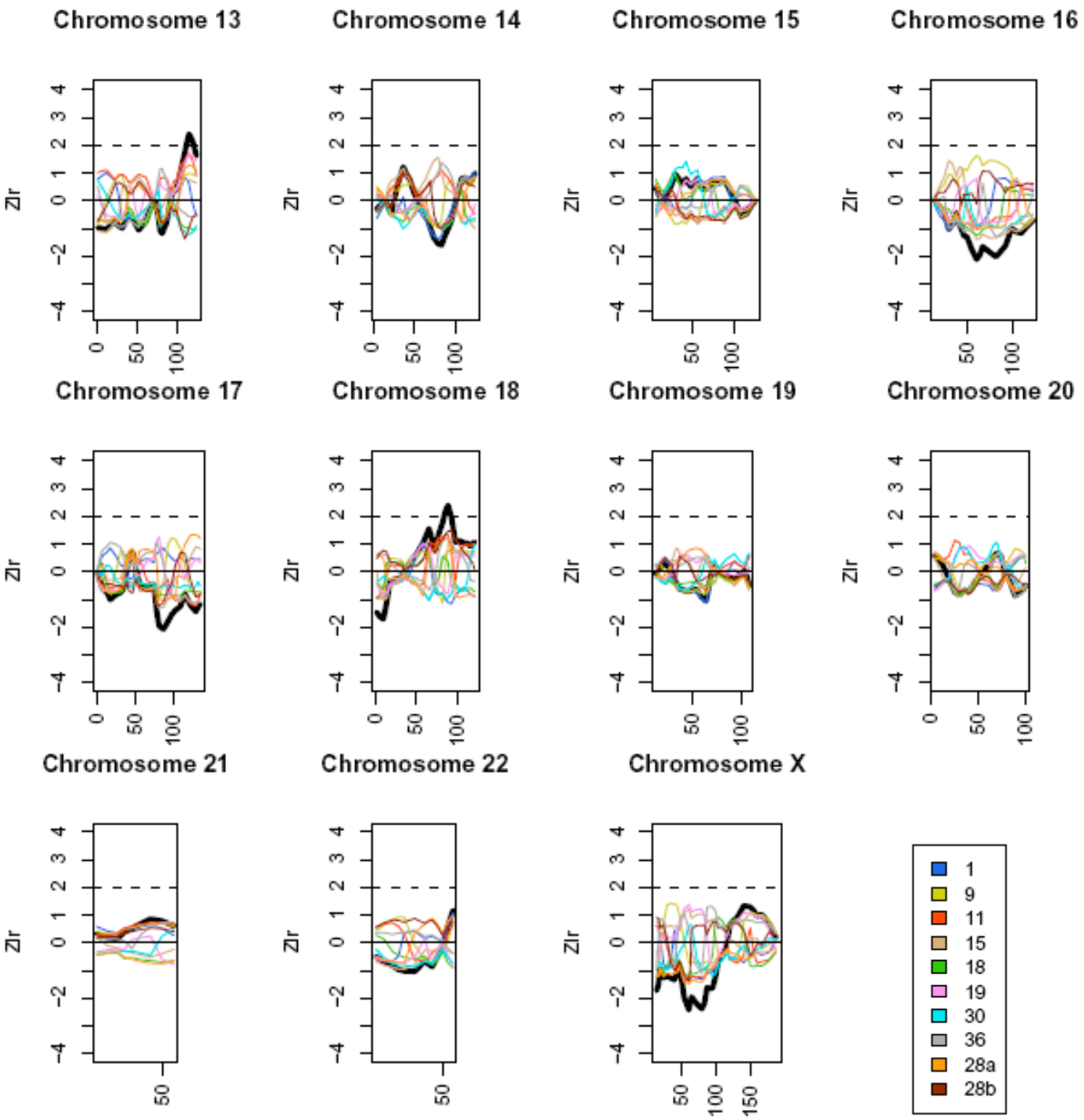

Figure 4. Zlr curves plotted against location (cM) for each chormosome from the analysis with the most likely 11 errors from Merlin removed…(cont)

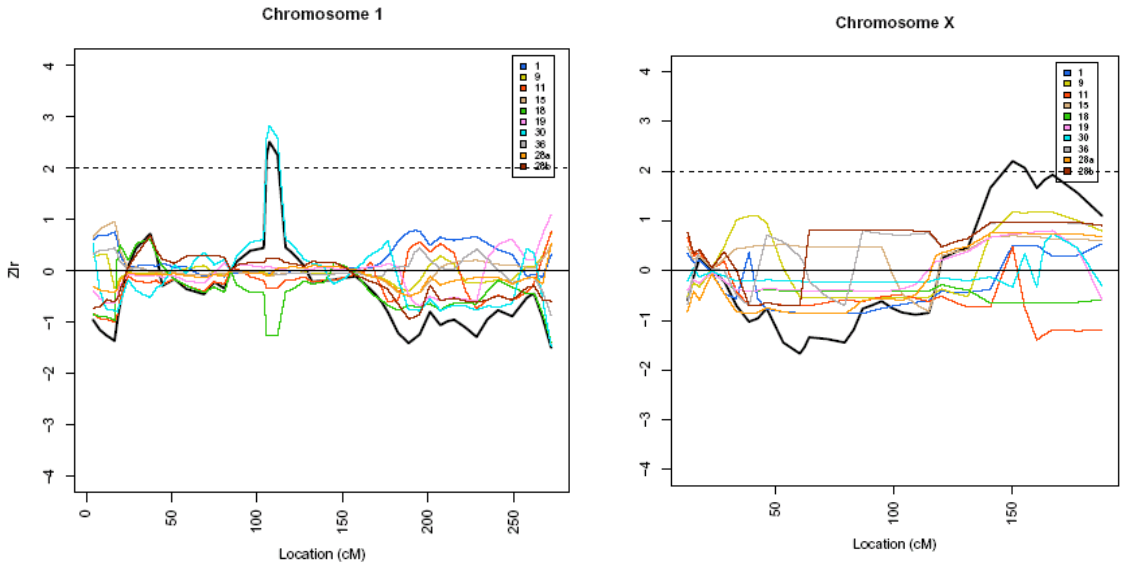

Figure 5. Two interesting regions at marker D1S207 (107 cM) and DXS1227 (150 cM) which showed up in relatively few models but were interesting as discussed in the text. The curve on chromosome 1 came from exp robdom 0.5 model while the curve on chromosome X came from lin mnallele 0.5 model.

Table 11. Six interesting candidate regions and families with major contribution to the linkage peak in each region.

Family	Interesting candidate region					
	1	3	12	13	18	X
	D1S207	D3S1565	D12S352	D13S1265	D18S68	DXS1227
	107cM	178cM	7.6cM	114cM	89cM	150cM
F1				√		
F9		√		√	√	√
F11		√	√	√	√	
F15		√		√	√	√
F18		√				
F19			√	√		√
F28a		√		√	√	√
F28b		√	√		√	√
F30	√		√			
F36		√			√	√

Sensitivity to maker allele frequencies

Marker allele frequencies would not have large impact on likage analysis if most of the people in the pedigree were typed. When some of the pedigree members are not available for typing, the linkage analysis programs try to estimate their genotypes and haplotypes using genotypes from their descendants and the marker allele frequency information. The increase in the rarity of an allele would result in the increase in the probablility of linkage since the rare allele would be more likely to be inherited form a common ancestor.

The sensitivity of our results to marker allele frequency was tested. Marker allele frequencies were changed by adding 3 counts (instead of adding 0.5) to all the allele occurrences in the 12 founders (24 alleles) and the allele frequencies recalculated. Given that a typical microsatellite marker contains 8 alleles, this addition of 3 to each allele was equal to adding extra 24 alleles to the existing founder alleles. In other words, it was similar to doubling the number of founder 12 to 24 with each added founder having equal chance of carrying any allele. The same multipoint nonparametric linkage analyses were performed using this set of new marker allele frequencies. There were no marked changes in the results. No new regions in the genome showed Zlr of more than 2. Zlr scores at 11 regions (excluding 2 regions in which Zlr dropped after removing 11 errors from Merlin) showing Zlr > 2 in the analysis with marker allele frequencies were compared with those from using the new frequencies. The scores changed no more than 15%.

The same nonparametric linkage analysis was also done on the originally estimated marker allele frequencies from the founder (without adding any allele counts) and the result compared with the main analysis (adding 0.5 count). Similarly, Zlr at the 11 peak regions changed no more than 20% (Table 12). However, a new region showed Zlr score of more than 2 in several allele sharing models at marker D4S1534 (93 cM). Further investigation found that this signal was largely come from F30. By looking at inferred haplotypes from Allegro, the affected people in F30 shared an allele whose frequency was set to very rare (0.000001) in the originally estimated marker allele frequencies, making it more likely that

they all received the allele from the same founder haplotype (share the allele IBD). When the frequency of this allele was increased (by adding 0.5 count), Allegro computed that those affected people received the allele from different haplotypes and the linkage signal lost.

In conclusion, our results did not depend much on the marker allele frequencies. The 13 peak regions, including the 6 promising candidates, were fairly robust to changing of marker allele frequencies.

Table 12. Sensitivity of Zlr scores in the regions showing Zlr >2.

Chromosome	Marker	Zlr		
		Marker allele frequency		
		original	+0.5 count	+3 counts
1	**D1S207[a]**	**2.333**	**2.317**	**2.280**
2	D2S162[b]	1.966	2.035	2.137
3	**D3S1565**	**2.458**	**2.468**	**2.499**
4	D4S406[c]	2.072	2.075	2.081
4	*D4S1534[d]*	*2.281*	*1.000*	*0.885*
7	D7S507[e]	2.022	2.039	2.063
12	**D12S352**	**2.454**	**2.161**	**2.102**
13	**D13S1265**	**2.184**	**2.403**	**2.466**
14	D14S70[c]	2.074	2.022	1.962
15	D15S1007[f]	2.251	2.259	2.275
18	**D18S68**	**2.396**	**2.400**	**2.415**
X	**DXS1227[g]**	**2.366**	**2.205**	**2.009**

The data were from exp pairs equal model otherwise indicated in superscripts; a, exp all 0.5; b, lin all equal; c, lin robdom equal; d, exp pairs 0.5; e, lin pairs equal; f, lin all 0.5; g, lin mnallele 0.5. Six interesting candidate regions are in bold. The additional Zlr > 2 which were seen only in the analysis using original marker allele frequencies is in italics.

วิจารณ์

Like in most countries worldwide, the G11778A mutation is the most prevalent LHON mutation in Thailand. Currently, 31 LHON families have been identified in Thailand; 97% (30 families in the present study) of them carry the G11778A mutation and 3% (1 family) carry the T14484C mutation. So far, the 3460 mutation has never been reported in Thai as well as in Southeast Asian. The prevalence of these mutations in Thai LHON is consistent with most of LHON families from multiple Asian countries. In Japan [Mashima et al., 1998; Oguchi, 2001], Taiwan [Yen et al., 2002], Chinese [Feng et al., 2001] and Indonesia [Sudoyo et al., 2002], the prevalence of LHON mutations was 87-100% for the 11778 mutation, 0-8% for the 3460 mutation and 0-11% for the 14484 mutation. However, a recent report of sixty Korean LHON probands showed the prevalence of 76% for the 11778 mutation, 2% for the 3460 mutation and 22% for the 14484 mutation [Kim et al., 2003]. In contrast, among most Caucasian LHON pedigrees, the prevalence is lower for the 11778 and higher for the 3460 mutation and the14484 mutation when compared with Asian LHON families. In Australia, Denmark, Finland, Italy, the Netherlands, the United Kingdom and North America [Harding et al., 1995; Howell et al., 2003; Johns et al., 1993; Lamminen et al., 1997; Mackey et al., 1996; Man et al., 2003; Riordan-Eva et al., 1995; Torroni et al., 1997], the prevalence is 60-79% for the 11778 mutation, 9-21% for the 3460 mutation and 0-25% for the 14484 mutation. In French Canadian LHON, however, the 14484 mutation is the most prevalent accounting for 82% of pedigrees, while the 11778 mutation and the 3460 mutation were found in 14% and 4% of families, respectively [Macmillan et al., 1998]. This predominance of the 14484 mutation in French Canadian LHON families has been showed to be owing to a founder effect [Macmillan et al., 2000]. The marked difference in the prevalence of each of the classical LHON mutations between Asian and Caucasian LHON families might reflect the effects of different genetic backgrounds (nuclear and/or mitochondrial) on the generation and clinical expression of these LHON mutations.

In the present study, the estimated overall penetrance of our 11778 LHON population was 37% for males, and 13% for females. These figures were comparable to those in 11778 Finnish LHON (39% for males and 14% for females) [Nikoskelainen et al.,

1996] but were different from 11778 British LHON (51% for males and 8.5% females) [Man et al., 2003]. When each pedigree was considered separately, the penetrance was widely varied from 7% to 77% with the mean of 26 ± 15%. To avoid the effects of the pedigree size and degree of ascertainment on the disease penetrance, only large pedigrees were considered and the disease penetrance varied from 9% to 45% with the mean of 19 ± 11%. This broad variation is also described in Europeans that penetrance in LHON families from different northern European countries can vary more than twofold [Howell, 1998]. Even within a single pedigree, there are matrilineal branches in which the penetrance has dropped [Howell and Mackey, 1998]. Actually, penetrance is difficult to measure accurately because of the variable and unpredictable age at onset, and different studies use different kinds of pedigrees for calculating penetrance; some include several pedigrees with isolated cases while some rely on large pedigrees with a clear maternal transmission. In Caucasian, as a rule of thumb, ~50% of males and 10% of females in LHON families lost vision [Howell, 1997; Howell, 1998; Man et al., 2002; Newman, 1993]. However, more extensive data regarding the penetrance are needed for Asian LHON.

In a pedigree analysis of combined 3460, 11778, and 14484 LHON families, it is found that affected mothers are more likely to have affected children than are unaffected mothers [Harding et al., 1995]. However, another analysis of the 14484 mutation fails to demonstrate the above correlation [Macmillan et al., 1998]. The analysis of our 11778 LHON pedigrees provided supporting evidence that affected mothers seemed to be more likely to have affected children, either daughters or sons, than were unaffected mothers.

Comparison of several features of our 11778 LHON pedigrees with those of other 11778 LHON pedigrees in the literature revealed no marked difference in several features (Table 13). These features included the proportion of cases with positive family history and the average age of onset of 11778 LHON patients. The higher average age of onset in female patients compared with in the male group are observed in several 11778 LHON pedigree series. This observation was also found in our 11778 LHON pedigrees in this study. However, there are some studies which report no difference in the average age of onset between male and female 11778 LHON patients [Man et al.,

2003; Riordan-Eva et al., 1995]. The sex bias in LHON resulting in a small number of female patients might have some effect on the above inconsistency.

However, some pedigree features in our series were different from most 11778 LHON in the literature (Table 13). The most striking was the high prevalence of blood leukocyte heteroplasmy of the 11778 mutation in Thailand. Thirty-seven percents (11/30) of our 30 LHON pedigrees contained at least one individual heteroplasmic for the mutation, while such a proportion is generally considered to be 15% in most studies [Chinnery et al., 2001]. Moreover, the proportion of our heteroplasmic families might be higher if additional blood samples of maternal relatives could be collected, as described in the Results section. If heteroplasmy reflects a recent muational event [Savontaus, 1995], it is interesting how recent mutational events could occur in our population between 10 years (1994-2003) of our sample collection in such a high incidence. A recent epidemiological study in the North East of England also shows a high proportion (33%) of heteroplasmic families than the general figure of 15%.

Another different feature of our 11778 LHON patients was that the male to female ratio (2.6:1 or 72%) appeared to be smaller than that of most 11778 LHON patient series worldwide (Table 13). We also observed that affected females tended to cluster in a few pedigrees; in 3 pedigrees, each one consisted of at least 4 affected females, and those affected females constituted 72% (13/18) of all affected females in our pedigree series. It was possible that those pedigrees might contain some pedigree specific genetic and/or environmental factors that increased the risk of LHON expression in usually low risk females.

Table 13. Comparison of 11778 LHON pedigree characteristics of the present study with previous reports.

	Asian			Caucasian				
	Present study	Hotta et al., 1995	Yen et al., 1999	Newman et al., 1991	Smith et al., 1993	Oostra et al. , 1994	Harding et al., 1995	Man et al., 2003
No. of families	30	79	17	49	68	15	66	9
No. of affected cases	65	90	24	72	75	146	109	49
Cases with positive family history (%)	50%	62%	-	43%	-	-	56%	-
Male patients (%)	72%	92.1%	88%	82%	77%	88%	79%	84%
Heteroplasmy								
Heteroplasmic families (%)	37%	-	0%	14%	18%	13%	7.6%	33%
Heteroplasmic persons (%)	28%	19%	0%	-	14%	-	-	-
Heteroplasmic patients (%)	14%	14%	0%	-	6.7%	-	0%	16%
Average age at onset (years)								
Males	20.7 (6-44)	-	21.85 (10-39)	26.2 (8-60)	-	28.55 (6-61)	21.0 (6-62)	-
Females	28.6 (10-53)	-	13, 56 [a]	34.0 (9-54)	-	31.47 (8-69)	28.0 (10-58)	-
Both	22.6 (6-53)	23.4 (7-59)	20.52 (10-56)	27.6 (8-60)	-	28.87 (6-69)	24.0 (6-62)	-

Figures within the brackets represent ranges of the age at onset; -, not available.

[a] Only two female in their series with the age at onset of 13 and 56 years old.

Our analyses of heteroplasmy supported the belief that heteroplasmy influences the expression of LHON as described in several study [Chinnery et al., 2001; Harding et al., 1995; Smith et al., 1993]. However, there was no correlation between higher mutation load and younger age of onset, similar to a previous study [Smith et al., 1993].

Several secondary LHON mutations have been found [Wallace and Lott, 2003]; however, in most cases, their pathogenicity is still uncertain and several studies showed conflicting evidences of the roles of secondary mutations [Brown et al., 2002; Howell, 1997; Howell et al., 1995; Lodi et al., 2000; Oostra et al., 1994]. Two secondary LHON mutations (G3316A and C3497T) were found in the screening of 27 LHON associated mutations in our 17778 LHON pedigree series. Whether the secondary LHON mutations, G3316A and C3497T have any effect to the 11778 mutation in precipitating the onset of the disease needs more cases and further analyses.

Anecdotal evidence suggests that environments can precipitate the onset of blindness in LHON [Cullom et al., 1993; DuBois and Feldon, 1992; Hwang and Park, 1996; Riordan-Eva et al., 1995]. Smoking, alcohol consumption, and head injuries are of much interest among suspected environmental factors but several case-control studies show conflicting results [Charlmers and Harding, 1996; Kerrison et al., 2000; Sadun et al., 2002; Tsao et al., 1999]. We retrospectively analysed those factors between affected patients and unaffected maternal relatives, all carrying a homoplasmic 11778 mutation. Consideration of exposure before the onset of visual loss was used in the analysis. Even though the present study was a large scale study of LHON in Thailand, the rareness of LHON limited the sample size in our environmental factor analyses. In addition, using a stringent inclusion criterion of molecularly confirmed homoplasmic persons also further reduced the sample size. In fact, a significant proportion (almost 50 samples) of our Thai 11778 LHON blood samples were heteroplasmic and were not included in the analyses.

From our limited sample size, we observed some non-significant evidence that smoking and alcohol consumption might increase the risk of 11778 LHON disease expression in persons aging $\leq$30 years, which is the most critical period where the onset of LHON usually occur, but did not increase the risk after persons had already passed through this most susceptible period. We also observed that the frequencies of

head injuries were higher in the affected group and the difference was slightly more evident in old age (<30) group compared with the younger group. It might be possible that the effect of head injuries on precipitating the 11778 LHON expression might accumulate as people get older. Larger sample sizes would be required to test this hypothesis.

It is clear that there have to be factors other than the primary LHON mutations responsible for the LHON features not able to be explained by the mitochondrial inheritance. These features include the incomplete penetrance, male predominance and optic nerve specific disease expression. Currently, genetic backgrounds in the mitochondria and/or in the nucleus are strongly suggested to play a role in the disease expression [Brown et al., 2002; Brown et al., 2000; Carelli et al., 2003; Cock et al., 1998; Howell et al., 2003; Qi et al., 2003; Sadun et al., 2002; Sudoyo et al., 2002]. Despite the different genetic backgrounds, most of the LHON features that constitute the picture of LHON are quite common in different population, however, there seemed to be a few ethnic-specific differences. Deep looking into these differences may provide some clues to the discovery of other factors modifying the disease, its pathophysiology and eventually an effective therapeutic intervention for this devastating disease.

This project is also the first genome scan in LHON. Although no significant linkage was found in this study, our results did show some interesting regions (Zlr>2, corresponding to $p<0.05$) that were worth further studying. The p-value for significant linkage for a genome scan depends on mapping methods and relationship in the pedigrees and it falls in the order of 10^{-4}-10^{-5} as proposed by Lander and Kruglyak, 1995 [Lander and Kruglyak, 1995]. However, those authors also supported the reporting of regions with p-value 0.05 in a complete genome scan. The most promising would be the 6 regions on chromosome 1, 3, 12, 13, 18, and X as indicated more precisely in Table 11.

The ~35 nuclear genes encoding subunits of complex I would be reasonable candidates for nuclear modifier(s) in LHON since all of the 3 most common LHON primary mutations are located in mitochondrial subunits of complex I as well. Thus LHON mutations might interact with variant(s) in the nuclear-encoded subunit, giving rise to different activity of the complex and ultimately different disease expression.

Remarkably, two of the six markers at the linkage peaks in this study were quite close to two of the nuclear subunit genes. The first marker, D3S1565, located at 175 Mb in chromosome 3, was 4.9 Mb away from *NDUFB5* (179.9 Mb) nuclear subunit gene. The second marker was D12S352 (7.6 Mb), which was 2.95 Mb from the nuclear subunit gene *NDUFB1* (4.65 Mb). These two regions were suggestively worth fine mapping.

The ~35 nuclear genes are pretty scattered across the genome (Table 14). It may well be reasonable that the 2 out of 6 markers happened to be closed to the nuclear subunit genes by chance rather than biological reasons. We observed that 11 of them are clustered (say within about 10 Mb) in 4 regions, which reduced the probability of this observation happening by chance. Given the total length of the nuclear genome was 3,300 cM, imagine that the genome was divided into 330 fragments of 10 cM long (as corresponding to 10 cM scan), 28 intervals would contain the 35 nuclear subunits genes (some genes are clustered). The probability of finding an interval with nuclear subunit gene(s) would be about 1 in 12 (28/330) whereas in our case, we found the nuclear subunit genes in 2 out of 6 peak regions (1 in 3). Therefore, our result was about 3-4 folds more likely to happen just only by random chance. Anyway, the two regions were worth studying further.

Table 14. The nuclear Complex I subunit genes and their location in the genome.

Chromosome	Gene	Physical location (Mb)
1	*NDUFS5*	39.2
	NDUFS2	158
2	*NDUFB3*	201.8
	NDUFS1	206.8
	NDUFA10	240.6
3	*NDUFB4*	121.8
	NDUFB5	**179.9**
4	*NDUFC1*	140.6
5	*NDUFS4*	53
	NDUFA2	140
	NDUFS6	1.9
7	*NDUFA4*	10.75
	NDUFA5	122.8
	NDUFB2	139.9
8	*NDUFB9*	125.6
9	*NDUFB6*	32.5
	NDUFA8	122
10	*NDUFB8*	102.3
11	*NDUFS3*	47.6
	NDUFV1	67.1
	NDUFS8	67.6
	NDUFC2	77.5
12	***NDUFA9***	**4.7**
14	*NDUFB1*	91.7
16	*NDUFB10*	1.95
	NDUFAB1	23.5
18	*NDUFV2*	9.1
19	*NDUFS7*	1.3
	NDUFA11	5.9
	NDUFA7	8.3
	NDUFB7	14.4
	NDUFA3	59.3
21	*NDUFV3*	43.2
22	*NDUFA6*	40.8
X	*NDUFA1*	118

Genes that lies close together are shaded. The two genes close to the linkage peaks in this study are in bold. (data from http://www.ncbi.nlm.nih.gov/)

It would be interesting to investigate haplotypes in families with lots of affected people contributing to the Zlr scores in the 6 interesting regions to see what was causing the linkage signal in those regions. Although the nonparametric linkage analysis only looked for allele sharing in affected people, haplotype analysis would allow us to employ information from unaffected people as well by comparing haplotypes between the affected and unaffected people in the same family. In this sense, F11 would be quite interesting to look at because of a unique pedigree structure of this family. This family had one sibship with five siblings typed, all of which were homoplasmic male. Notably, in the five siblings, only one was unaffected whereas their mother was affected as well. This unaffected person was 33 years old and should not have great chance of developing LHON later in life. Given that they were of the same sex and have the same homoplasmic G11778A mutation (and also the same mtDNA background as their mother), the difference in nuclear gene(s) might explain why he was the only one who could escape the disease. F11 contributed to the linkage peaks in chromosome 3, 12, 13, and 18 (Table 11) and the haplotypes around these peak regions were investigated. It was interesting that in 3 out of the 4 peak regions, this unaffected person did not share the haplotypes shared by most affected people (Table 15 and Figure 6). However, not all affecteds shared all haplotypes and there was also one haplotype shared by the unaffected person. If these haplotypes really carried the disease susceptibility genes, these gene might have synergistically to increase the risk of LHON and carrying only one or few of those genes would not be enough to cross the threshold of disease expression.

This study provided some candidate regions for nuclear modifier(s) in LHON. It would be interesting to further fine map in those regions with more microsatellite markers and in more samples. The information in Table 11 could be use as a guideline to choose which families in which regions for further fine mapping. It would also be useful to genotype a number of unrelated individuals for markers in these regions to estimate the marker allele frequency more precisely in Thai population.

Table 15. The presence/absence of the haplotypes shared by most affected people in F11 under the interesting regions in chromosome 3, 12, 13 and 18, in which the family contributed to the peaks.

ID	Sex	Affection status	Marker at the linkage peak			
			D3S1565	D12S352	D13S1265	D18S68
F11.II3	F	A	√	√	√	√
F11.II6	M	A	□	□	□	□
F11.III2	M	A	□	□	□	□
F11.III4	M	A	□	-	□	□
F11.III7	M	A	□	□	□	□
F11.III9	M	A	-	□	□	□
F11.III6	M	U	-	-	-	□

M, male; F, female; A, affected; U, unaffected; □, presence; -, absence.

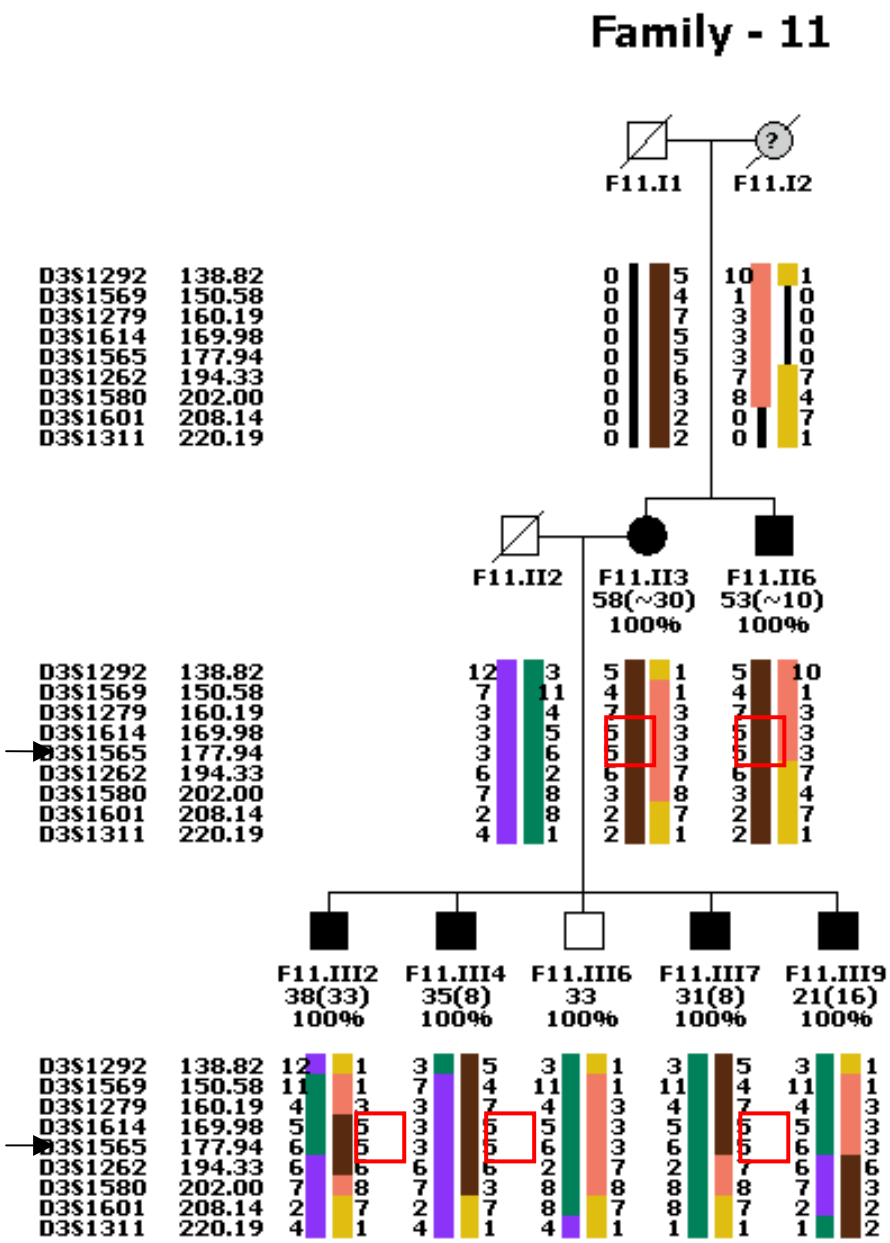

Figure 6. Haplotype analysis in of F11 under the interesting regions in which the family contributed to the linkage peaks. The arrow indicates the marker at the peak. The haplotype shared by most of the affected people is highlighted by the red box. The numbers next to the marker names are genetic distances of the markers.

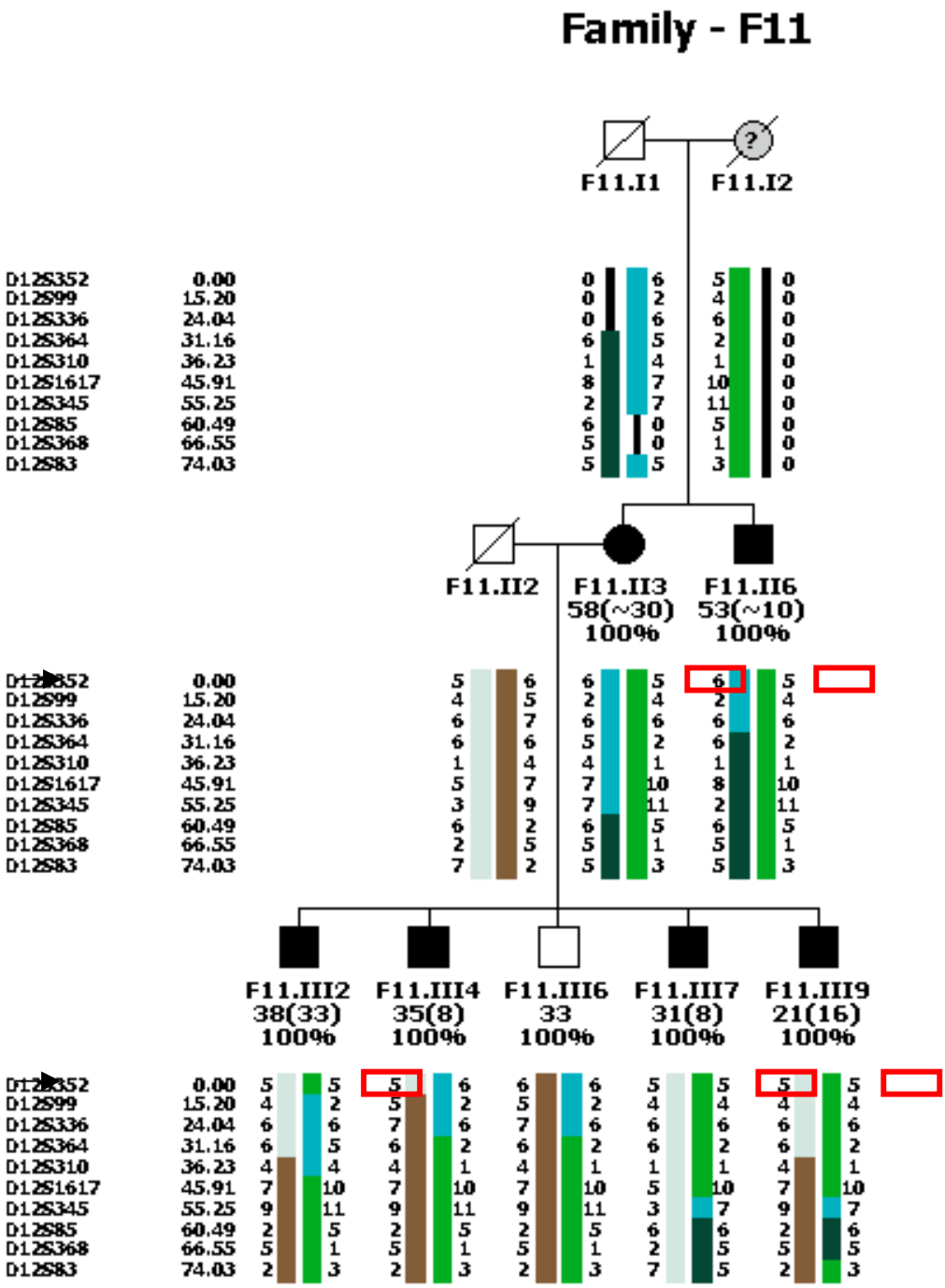

Figure 6. Haplotype analysis in of F11…(cont.)

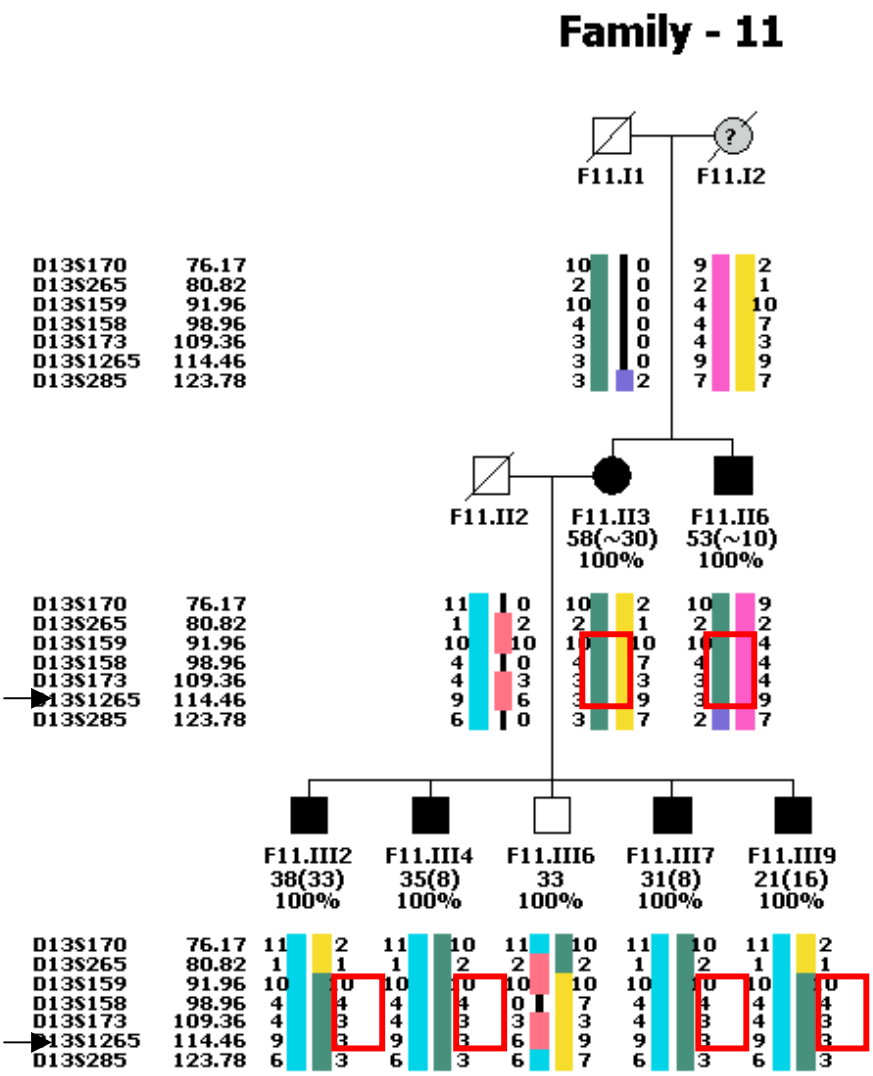

Figure 6. Haplotype analysis in of F11…(cont.)

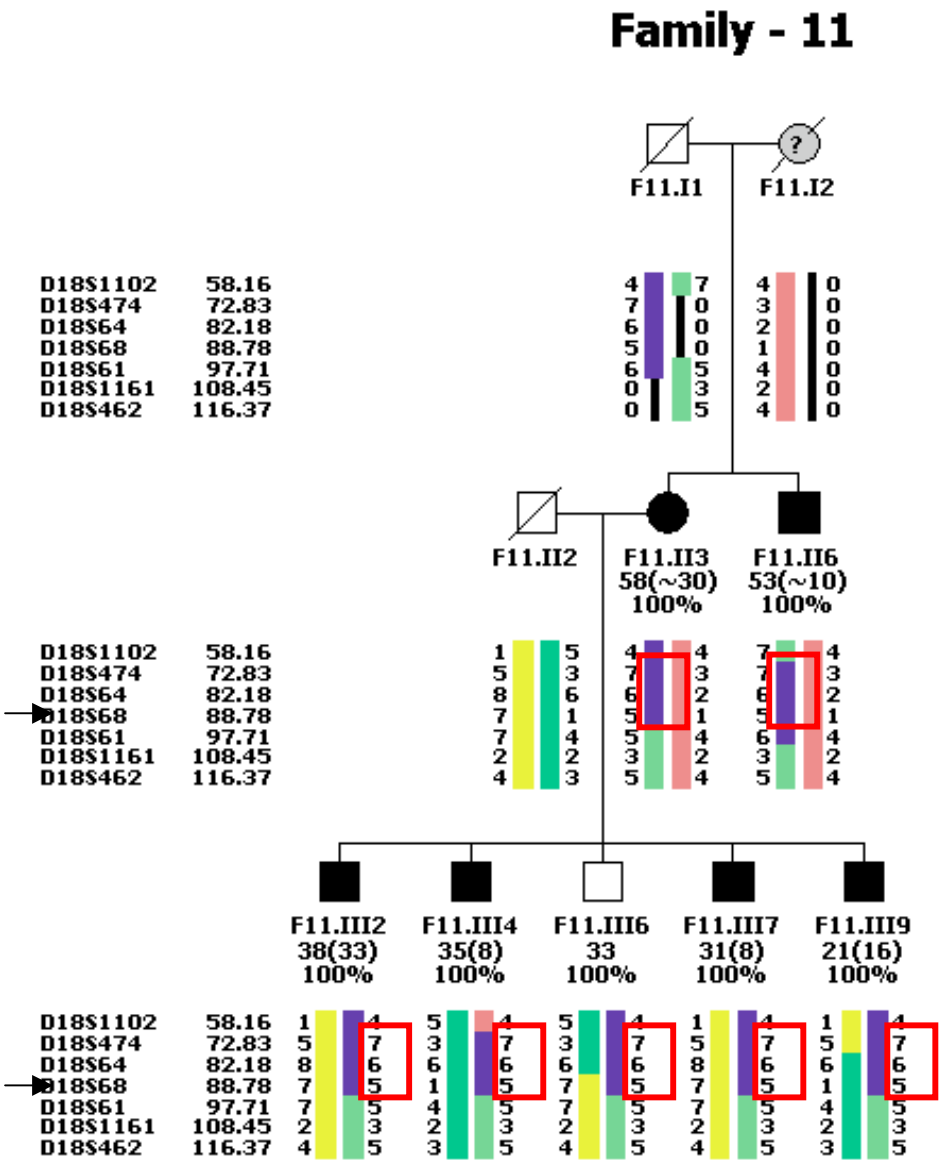

Figure 6. Haplotype analysis in of F11…(cont.)

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งานที่จะทำต่อในอนาคต

Since we can identify 6 candidate regions that could possibly be the regions (in the nuclear genome) carrying LHON modifying gene(s), in our LHON pedigrees. We are interested to examine these regions closely in order to find the modifying gene(s). First, we would like to do the fine mapping of these regions using a set of markers covering these areas. The gene(s) in the promising area(s) of the genome will be identified using the combination of the following methods, either *ab initio* program to look for the special signatures of genes in genomic sequences and to distinguish genes from intergenic sequences or database similarity search techniques or comparative genomic alignment. Once the genes (estimated to be 15-20 per Mb) have been identified, they will be examined based on their function. Each potential candidate gene will be screened in the families.

ผลงานวิจัยที่ตีพิมพ์และอยู่ในระหว่างดำเนินการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

1. Chuenkongkaew WL, Suphavilai R, Vaeusorn L, Phasukkijwatana N, Lertrit P and Suktitipat B. Proportion of 11778 mutant mitochondrial DNA and clinical expression in a Thai population with Leber's hereditary optic neuropathy. J Neuro-Ophthalmol,2005; 25:173-5

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3. Phasukkijwatana N, Chuenkongkaew WL, Suphavilai R, Suktitipat B, Pingsuthiwong S, Ruangvaravate N, Atchaneeyasakul L, Warrasak S, Poonyathalang A, Sura Tand **Lertrit P**. The Unique Characteristics of Thai Leber Hereditary Optic Neuropathy: Analysis of 30 G11778A Pedigrees. J Hum Genet; during submission.

4. Tharaphan P, Chuenkongkaew WL, Luangtrakool K, Sanpachudayan T, Suktitipat B, Suphavilai R, Srisawat C, Sura T and **Lertrit P**. Mitochondrial DNA haplogroup distribution in Pedigrees of Southeast Asian G11778A Leber Hereditary Optic Neuropathy. Am J Hum Biol, During submission

5. **Lertrit P**, Phasukijwattana N, Chuenkongkaew WL, Bahlo M, Stankovich J and Sura T. Nonparametric linkage analysis of genome-wide scan reveals 6 candidate regions as the nuclear modifier (s) for Leber hereditary optic neuropathy in Thai families. Manuscript in preparation

6. Phasukijwattana N, Chuenkongkaew WL, Khunhapan B, Luangtrakool K and **Lertrit P**. A large pedigree of G11778A shows an opposite segregation of the mutation in different brunch. Manuscript in preparation

Proportion of 11778 Mutant Mitochondrial DNA and Clinical Expression in a Thai Population With Leber Hereditary Optic Neuropathy

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Background: The proportion of mutant mtDNA in blood has been found to correlate with the frequency of visual loss in cases with mtDNA mutations associated with Leber hereditary optic neuropathy (LHON), especially in men. We sought to determine this correlation in a Thai population of LHON.

Methods: Densitometric quantification of blood mtDNA with the 11778 LHON mutation in 137 symptomatic cases and their asymptomatic maternal relatives in 30 Asian pedigree families was performed. Asymptomatic maternal relatives under the age of 16 years were excluded. The visual outcome in symptomatic cases with homoplasmy and heteroplasmy was compared.

Results: Heteroplasmy was detected in eight (12.9%) symptomatic and 30 (40%) asymptomatic individuals. The quantification of blood mutant mtDNA in the eight symptomatic cases ranged from 44% to 93% (mean = 75%). The visual outcome of the cases with heteroplasmy was not different from that of cases with homoplasmy. There was a correlation between the proportion of mutant mtDNA and the likelihood of visual loss.

Conclusions: The prevalence of heteroplasmy among pedigrees of the 11778 LHON mutation in Thailand was similar to that of other Asian populations and may be greater than in 11778 LHON pedigrees from white backgrounds. The proportion of mutated mtDNA correlated with visual loss, but the effect of heteroplasmy on clinical expression seemed not to relate to gender.

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Leber hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by the sudden onset of loss of central vision, usually in early adulthood. More than 50% of all cases with LHON carry a mitochondrial DNA (mtDNA) mutation at nucleotide position 11778. This nucleotide change converts a highly conserved arginine residue to histidine at codon 340 in the NADH-ubiquinone oxidoreductase subunit 4 (ND4) gene of mtDNA. This mutation is present in all mtDNA molecules of the individuals within the maternal lineage (homoplasmy). However, some pedigrees harbor both mutant and wild-type mtDNA (heteroplasmy). The proportion of the 11778 mutant mtDNA is an important risk factor, particularly in affected males, for the development of blindness in LHON (1).

We studied the correlation between the proportion of blood mutant mtDNA in pedigrees of Asian 11778 LHON cases and its clinical expression.

METHODS

The cases enrolled in this study included individuals from Thai (27), Chinese (1), Chinese-Thai (1), and Indian (1) pedigree families who developed the typical clinical neuro-ophthalmic features of LHON and showed the 11778 LHON mutation in peripheral blood (symptomatic group) as well as their visually intact maternal relatives aged 16 or older (asymptomatic group). Younger asymptomatic maternal relatives were excluded as a result of the possibility of developing visual loss later in life.

Whole blood samples obtained with informed consent were quantified by the restriction fragment length polymorphism (RFLP) technique to estimate the degree of heteroplasmy, the proportion of mutant to total (both wild-type and mutant) mtDNA in each individual. Homoplasmy was defined as mutant mtDNA of more than 95%.

Data collected included age, age at onset, gender, maternal relation, and best-corrected visual acuity (BCVA). For statistical analysis, BCVA was converted to logMAR (logMAR = log 1/Snellen visual acuity). The unmeasured visual acuities were at the following logMAR values: counting

fingers corresponding to Snellen visual acuity 6/600 (logMAR = 2.0) and hand motion corresponding to Snellen visual acuity 6/6000 (logMAR = 3.0).

RESULTS

Of the 30 pedigrees, 11 (37%) were heteroplasmic. In 14 pedigrees, at least 80% of the maternal relatives at risk were examined. Of 137 individuals, 62 (45.3%) were symptomatic and 75 (54.7%) were asymptomatic; 73 (53.3%) were male and 64 (46.7%) were female. In the symptomatic group, the male to female ratio was 3.1:1; in the asymptomatic group, it was 0.5:1.

The age of onset of LHON in symptomatic individuals ranged from 8 to 68 years (mean = 37.3 years); the age of asymptomatic individuals ranged from 18 to 90 years (mean = 41.8 years).There was no statistical significant between the mean age of symptomatic and asymptomatic groups (P = 0.10, T test). The age at onset of visual loss ranged from 6 to 44 years (mean = 20.6 years) in men and from 10 to 53 years (mean = 27.2 years) in women.

Of 62 symptomatic individuals, eight (12.9%) were heteroplasmic and 54 (87.1%) were homoplasmic. Of 75 asymptomatic individuals, 30 (40%) were heteroplasmic and 45 (60%) were homoplasmic.

The age at onset ranged from 10 to 42 years (mean = 21.1 years) in symptomatic heteroplasmic individuals and from 6 to 53 years (mean = 22.2 years) in homoplasmic individuals. There was no statistical difference between the age at onset in symptomatic heteroplasmic and homoplasmic groups (P = 0.68, Spearman's rho). The age of asymptomatic heteroplasmic individuals ranged from 18 to 90 years (mean = 46.9 years) and from 18 to 70 years (mean = 38.5 years) in homoplasmic individuals. There was no statistical significance between the age of asymptomatic heteroplasmic and homoplasmic groups (P = 0.95, Pearson correlation).

The prevalence of heteroplasmy in the 137 individuals is shown in Table 1. The proportion of mutant DNA in symptomatic heteroplasmic individuals ranged from 44% to 93% (mean = 75%) and from 18% to 94% (mean = 62%) in asymptomatic heteroplasmic individuals.

The BCVA ranged from 0.2 to 3.0 logMAR (mean = 1.7 logMAR) (approximate Snellen visual acuity = 20/1000) in heteroplasmic individuals and from 0.3 to 3.0 logMAR (mean =1.8 logMAR) (approximate Snellen visual acuity = 20/1250) in homoplasmic individuals. The visual outcome of the cases with heteroplasmy was not different from that of cases with homoplasmy (P = 0.74, Mann-Whitney tests). There was statistical significance between the proportion of mutant mtDNA and the expression of visual loss ($P = 0.001$; chi-square tests) (male group, $P = 0.011$; female group, $P = 0.05$; chi-square tests).

TABLE 1. ***Prevalence of heteroplasmy in 137 cases with the 11778 mutation of Leber hereditary optic neuropathy***

	Symptomatic (%)	Asymptomatic (%)	Total (%)
Males			
Heteroplasmy	6 (12.8)	10 (38.5)	16 (21.9)
Homoplasmy	41 (87.2)	16 (61.5)	57 (78.1)
Total	47 (100)	26 (100)	73 (100)
Females			
Heteroplasmy	2 (13.3)	20 (40.8)	22 (34.4)
Homoplasmy	13 (86.7)	29 (59.2)	42 (65.6)
Total	15 (100)	49 (100)	64 (100)
Total	62 (45.3)	75 (54.7)	137 (100)

Of 75 asymptomatic maternal relatives, 24 with homoplasmy and 11 with heteroplasmy were in the same generation as the proband of the family; 19 with homoplasmy and 19 with heteroplasmy were from the preceding generation; one with homoplasmy and one with heteroplasmy were from subsequent generations.

DISCUSSION

In our maternal pedigrees harboring the 11778 LHON mutation, heteroplasmy for the mutation in peripheral blood leukocytes was detected in 37% of pedigrees, whereas in previous studies of maternal pedigrees harboring this mutation, heteroplasmy was documented in at least one family member in approximately 15% of pedigrees (1–3). Most of these pedigrees likely represented white individuals.

In the study of Smith et al (2), heteroplasmy was found in 7% of symptomatic individuals and in 19% of asymptomatic individuals. In our study, heteroplasmy was detected in approximately 13% and 40% of symptomatic and asymptomatic individuals, respectively, which is similar to the 14% and 44% of symptomatic and asymptomatic Japanese 11778 LHON cases (4). Although one cannot draw definitive conclusions without a systematic screening of all maternal relatives within these pedigrees, the suggested higher prevalence of heteroplasmy reported in these two studies of Asian individuals might be the result of the different mtDNA haplotype backgrounds found in Asians and whites.

To clearly classify affected and unaffected groups of our cases, asymptomatic individuals under the age of 16 years of age were excluded. Only one case with homoplasmy and one case with heteroplasmy were from subsequent generations. Therefore, the unaffected maternal relatives who were recruited for this study were less likely to develop subsequent visual loss.

TABLE 2. ***Comparison of the quantity of 11778 mutant mtDNA in the study of Smith et al (2) and the current study***

	No. of cases with 11778 mutant mt DNA (%)			
	Smith et al (2)		Current study	
Mutant mtDNA	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
0–25%	0 (0)	2 (2)	0 (0)	2 (3)
26–50%	0 (0)	2 (2)	0 (0)	16 (21)
51–75%	0 (0)	4 (4)	3 (5)	7 (9)
76–90%	5 (7)	11 (11)	3 (5)	5 (7)
>90%	70 (93)	82 (81)	56 (90)	45 (60)
Total	75	101	62	75

Our symptomatic cases in both heteroplasmic and homoplasmic groups developed loss of vision at the same age and had the same visual outcome. We found a correlation between the proportion of mutant mtDNA and the likelihood of having visual loss; this finding agrees with that of a previous study (1).

In contrast to cases with mutant mtDNA of less than 75% in the study of Smith et al (2), 5% of our cases who had blood mutant mtDNA of less than 75% developed visual loss (Table 2). The percentage of cases with mutant mtDNA less than 75% who had visual loss was not statistically significantly different from that of cases who had blood mtDNA of more than 75% and had visual loss ($P < 0.01$, chi-square tests). However, when analyzing results using an odds ratio calculation, the cases who had blood mutant mtDNA of greater than 75% were more likely to develop blindness than those who had mutant mtDNA of less than 75% (odds ratio = 6.41 [2.52, 16.78]).

Interestingly, the frequency of visual loss has been documented to have a relationship to the mutation load of mtDNA in the peripheral blood of male cases with LHON but not to that of female cases (1). By contrast, our study did not show any gender difference. Also interesting is that in whites, the mtDNA haplogroup J has preferential correlation to the LHON mutation, whereas in Asians, the haplogroups B and BM have a close relationship to the 11778 LHON mutation (5). The mtDNA haplotype background may influence disease expression.

We conclude that the prevalence of heteroplasmy among pedigrees of 11778 LHON in Thailand is similar to that of other Asian populations (4) and may be more frequent than in 11778 LHON pedigrees from white backgrounds (2). Similar to previous studies (1,2), the proportion of mutated mtDNA in Thai individuals with 11778 LHON correlated with the clinical expression of visual loss. However, in contrast to one previous study of English LHON pedigrees (1), the effect of heteroplasmy on clinical expression in our cases seemed not to relate to gender.

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Mitochondrial DNA haplotype analysis in Thai population

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Abstract

One hundred samples of normal healthy Thai were drawn from 4 major parts, covering the whole country of Thailand, namely: northern, central, northeastern and southern parts, for the study of their mitochondrial DNA polymorphisms. A total of 126 high resolution RFLP polymorphisms and the corresponding 86 different haplotypes were detected and reported. Base sequencing of the hypervariable segment (HVS-1; 16024-16383) of the control region (CR) was also performed, yielding 100 polymorhisms of 90 different haplotypes as reported. The frequencies of 9 bp deletion in the COII/tRNA^{Lys}, T16189C and length heteroplasmy were detected to be 23%, 34% and 28% in these samples respectively. The mitochondrial haplotypes were subjected to either the standard RFLP or HVS-1 haplogroup designation as applicable to the case, as well as to the phylogenetic analysis using PAUP program. The clustering patterns of haplotypes in the RFLP and HVS-1 phylogenetic trees agreed well with each other and with the haplogroups designated under standard criterion. It was found that the haplogroup M at the frequencies of 43/35% (RFLP/HVS-1) was the most common in our studied population, in agreement with previous reports for other Asian populations. Haplogroups F and B/B* were the second and third most common in our samples, at the frequencies of 18/20% (RFLP/HVS-1) and 20/15% (RFLP/HVS-1) respectively. When comparing with the native Thai populations from previous study, our result showed that this data set can be used to represent the present-day mixed Thai population

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Introduction

The majority of mitochondrial haplogroups have been shown to be continent-specific. In Africa, haplogroup L encompasses between 70% and 100% of the sub-Saharan mtDNAs (Chen et al. 1995). In Asia, 55% of East Asian and Siberian mtDNAs are members of macro haplogroup M which can be subdivided into smaller subhaplogroups designated C, D, G and E. (Ballinger et al. 1992; Chen et al. 1995; Torroni et al. 1994; Torroni et al. 1993a; Wallace 1995). Most of the remaining Asian mtDNAs are encompassed by haplogroups A, B and F (Torroni and Wallace 1994). Among Native Americans, only four Asian haplogroups (A, B, C and D) are observed. In addition to the specific haplogroups, a 9-bp deletion in the mitochondrial genome has been useful for examining genetic relationships among human populations (Redd et al. 1995; Soodyall et al. 1996). The specific haplogroups and a 9-bp deletion have been previously reported in North, East, South and some area in Southeast Asia. However no such data have been reported from Thai population in Thailand situated in the mainland of the Southeast Asia.

รหัสเขตข้อมูลที่ถูกเปลี่ยนแปลง
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จัดรูปแบบ: ฝรั่งเศส ฝรั่งเศส

In this study, we determined the mitochondrial DNA haplogroup in the Thai population who are present-day Thais living in the city of central, Northeastern, Northern and Southern part of Thailand using high-resolution RFLP (18 restriction endonucleases) and nucleotide sequences in the hypervariable segment 1 (HVS-1) (16024 to 16383) as well as the 9 bp deletion in the COII/tRNA^{Lys} in order to provide the information for Thai population which would serve as an important database for the study of Medicine, Anthropology and Archaeology at the molecular level in the future.

Materials and Methods

Subjects

The 100 healthy individuals recruited in this study were from the city area in four part of Thailand including central, Northeastern, Northern and Southern. Khmer population in this study was from Thailand-Cambodia border in Chantaburi province. Five ml of venous blood were drawn from each individual with written informed consent. Their blood chemistry and physical examination were within normal limit. Genomic DNA was extracted from venous blood samples using standard phenol-chloroform protocol.

Determination of mtDNA haplotypes by Restriction Fragment Length Polymorphism (RFLP)

The mitochondrial DNA haplotypes determined by Restriction Fragment Length Polymorphism (RFLP) was carried out in all samples. The mtDNA was amplified, using the oligonucleotide primers, into 9 overlapping regions covering the whole mitochondrial genome. The PCR product from each primer pair was precipitated and then digested with 18 restriction endonucleases: *Alu*I, *Ava*II, *Bam*HI, *Dde*I, *Hae*II, *Hae*III, *Hha*I, *Hin*cII, *Hin*fI, *Hpa*I, *Hpa*II, *Mbo*I, *Pst*I, *Pvu*II, *Rsa*I, *Taq*I, *Xba*I and *Xho*II. The pattern of the digested fragments was resolved either by the 2%-4% agarose gel or 8%-12% polyacrylamide gel electrophoresis (Ballinger et al. 1992).

Detection of the 9 bp COII/tRNA^{Lys} deletion

The 9 bp COII/tRNA^{Lys} deletion was determined in these 100 normal control individuals. The amplification of 101 bp of mtDNA from position 8211 to 8311 using primer L8211 and H8311 was carried out. The PCR product was electrophoresed on 4% Nusieve 3:1 agarose gel for 120 minutes at 60 volt. The 9 bp COII/tRNA^{Lys} deletion yields the PCR product of 92 bp in size whereas the 101 bp in size was detected in non deleted samples.

Determination of the mitochondrial control region (HVS-I)

The mitochondrial DNA covering the hypervariable segment 1 (HVS-1) from nucleotide position 16024 to 16383 in the mitochondrial control region was determined. The HVS-1 was amplified using either L15904 and H16417 or L15790 and H731 primers. The PCR product was purified and sequenced using H16417 as the primer for the DNA sequencing and either L15904 or L15790 as the primer for the sequencing reaction when the sequence had the length heteroplasmy.

Mitochondrial haplogroup determination and Phylogenetic analysis

The mitochondrial haplogroup of all 100 samples was determined both by the presence or absence of the recognition site of the restriction endonucleases used and 9 bp deletion in COII/tRNA^{Lys}, and the nucleotide variant within the HVS-1 region, whereas mitochondrial haplogroup from the native Thais (Fucharoen et al. 2001; Yao et al. 2001) was determined by the nucleotide variant in HVS-1 region only. The detail of haplogroup determination using RFLP and HVS-1 polymorphisms was shown in Table 1. The phylogenetic tree was constructed using the computer program PAUP* (Phylogenetic Analysis Using Parsimony and Other Methods) 4.0 beta version (Swofford 2002). The phylogenetic relationships among 100 Thai individuals were constructed from both the restriction site polymorphisms including 9 bp deletion data and the nucleotide polymorphisms of the HVS-1 in the control region. Population tree of 10 native Thai populations and our present-day Thais was constructed based on interpopulation distance matrix calculated from haplogroup frequencies using Arlequin version 2.000 (downloaded from http://lgb.unige.ch/arlequin/software) and run on Molecular Evolutionary Genetics Analysis program version 3.0) MEGA3).

Table 1

Table 1. RFLP and HVS-1 polymorphisms used to identify mtDNA haplogroups in this study (Stoneking et al. (1990), Ballinger et al. (1992), Torroni et al. (1994), Schurr et al. (2000),and Schurr and Wallace (2002)

Results and discussion

Mitochondrial haplogroup determined by RFLP in 100 Thais

A total of 126 RFLP polymorphisms and 86 haplotypes were detected in our study. The RFLP mitochondrial haplogroup in our population was determined according to the criteria in Table 1. The RFLP haplogroup of each individual were in Table 2. Mitochondrial macrohaplogroup M including haplogroup C, D, G, was the most common haplogroup in our population (46%) (Table 3). The macrohaplogroup M occurs in all Southeast Asian populations at various frequencies 30-61.1% (Figure 1) (Schurr and Wallace 2002). The haplogroup D was found 19.5% of Amerrinds (Torroni et al. 1992), 23.1% of Koreans, 14.3% of Chinese Han and 10.0% of Taiwanese Han but was not found in Vietnamese (Ballinger et al. 1992) which is possible to be found only 1% in Thais. This frequency shows the reduction of this haplogroup as moving downward from North to South.

Figure 1

Figure 1. Distribution of major mitochondrial DNA haplogroup M, B/B* and F in some Asian populations (Stoneking et al. (1990), Ballinger et al. (1992), Torroni et al. (1994), Schurr et al. (2000),and Schurr and Wallace (2002)).

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Table 2

Table 2. The definite haplogroup determined by at least 3 of 4 criteria; 1) polymorphisms in RFLP and 9 bp deletion 2) RFLP phylogenetic tree 3) HVS-1 sequence polymorphisms and 4) HVS-1 phylogenetic tree in the 100 normal individuals including the presence or absence of 9 bp deletion, T16189C and length heteroplasmy in each individuals.

Table 3

Table 3. The frequency of each mitochondrial haplogroup determined by either high resolution RFLP or nucleotide variation in HV 1 region in 100 normal Thai individuals in this study.

Haplogroup F, the second most haplogroup found in present-day Thai population (15%), was also commonly seen in Southeast Asian, particularly in Vietnamese (32.1%) and Malaysian aborigines populations (Orang Asli, 27.3 %,) and Tibetans 14.8% (Figure 1) (Ballinger et al. 1992). It was also found in East Asian population; Japanese, Koreans, and Ainu and also in coastal Papua New Guinean (1%). The frequency distribution of this mtDNA lineage shows that this haplogroup is found mostly in Southeast and East Asian. It is being seen as far north as central Siberia (Evenks) (Torroni et al. 1993b) and as far south as Borneo (Kadazan).

Haplogroup B was found in 11% of Thai population whereas haplogroup B* frequency in our population was 9%. The 9 bp deletion in the COII/tRNA^{Lys} gene was found in a high frequency in our population (23%). This marker defines haplogroup B and B*, which is present at significant frequencies in mtDNAs from all East and Southeast Asian population. The frequency ranges from 3% to 18% from Japan to mainland Asia to Malaysian peninsular; from 8% to 42% in islands in Asia (with the exception of the Negritos of Aeta in the Philippines) and along the coast of Papua New Guinea; and from 77% to 100% in Polynesia (Ballinger et al. 1992; Harihara et al. 1992; Horai et al. 1987; Horai et al. 1991; Lum et al.

รหัสเขตข้อมูลที่ถูกเปลี่ยนแปลง

จัดรูปแบบ: ฝรั่งเศส ฝรั่งเศส

1994; Stoneking et al. 1986). Our data confirm the southward migration of this marker.

จัดรูปแบบ: ฝรั่งเศส ฝรั่งเศส

Haplogroup A was found in 3% of our population, A1 (1%) and A9 (2%). The RFLP haplogroup was not able to be classified in 15 individuals using standard RFLP criteria. The detail of RFLP polymorphisms was available on line of this Journal.

Mitochondrial haplogroup determined by HVS-1 sequence in 100 Thais

The total of 100 polymorphisms and 90 haplotypes in the HVS-1 region (16024-16383) was found in our studied population. The polymorphism in the HVS-1 sequence of each samples were deposited in the GenBank databases accession number DQ 145827-145926). Mitochondrial DNA haplogroup M determined by C16223T, was the most common haplogroup found in our studied samples (50%). This macrohaplogroup (50%) included haplogroup M only (35%), haplogroup C (2%) and haplogroup D (13%).

Haplogroup F was seen in 20% of our studied population (Table 3). Fourteen of them (14%) carried the same haplogroup F as defined by RFLP criteria. The mitochondrial haplogroup A, B and B* were also found in our population. The frequency of the HVS-1 haplogroup A, B and B* were 1% (C37), 10%, and 8% respectively (Table 3). Six individuals (6%) were not able to be classified using HVS-1 criteria.

Since our studied samples were healthy individuals from the city area, their original habitat, in some cases, could not be ascertained. In the past few decades, there were several migrations of the population from all over the country to the big cities in various part of Thailand. They have migrated and/or married with the local population, hence they become mixed-urban population. The haplogroup frequency of those from four parts of the country in our studied samples then could not be compared properly. However, this dataset should be able to represent the present-day Thai population which has been migrated and mixed in the country.

Phylogenetic trees constructed from 100 healthy present-day Thai individuals

Phylogenetic trees of 100 Thai individuals in this study was constructed from the polymorphism of the total mitochondrial genome derived from RFLP analyses (Figure 2) and from nucleotide sequence of HVS-1 region (16024-16383) (Figure 3) using Khwe, http://www.genpat.uu.se/mtDB/sequences.html (GenBank no. AY195777) and rCRS (Andrews et al. 1999) as the non Asian sequences. All individuals in the tree are clustered into different branches of the tree according to their haplogroups (Figure 2, 3).

Figure 2

Figure 2. Unrooted phylogenetic tree constructed from 100 healthy Thai individuals using the high resolution RFLP information.

Figure 3

Figure 3. Unrooted phylogenetic tree constructed from 100 healthy Thai individuals using the nucleotide variants in HVS-1 region of mitochondrial genome.

Most of the mitochondrial DNA haplogroup of 6 individuals of unclassified HVS-1 haplogroup, and 15 unclassified RFLP haplogroup could be determined according to their location in the cluster of the trees (Figure 2, 3 and Table 2).

The T16189C variant was found in 34% of our population whereas the length heteroplasmy between nucleotide 16184-16193 was found in 28%. The association of length heteroplasmy and T16189C is of statistical significant ($p<0.001$). The 9 bp deletion was also found in most of T16189C individual with length heteroplasmy. The T16189C with length heteroplasmy and 9 bp deletion was detected in 19/33 of individuals only carrying T16189C (58%) (Figure 4).

Figure 4

Figure 4. The number of individuals carrying T16189C alone, length heteroplasmy alone, 9bp deletion alone, T16189C + length heterplasmy, T16189C + 9bp deletion, length heteroplasmy + 9 bp deletion and T16189C + length heteroplasmy + 9 bp deletion in this study.

Population tree and haplogroup analysis between present-day Thai and native Thai populations

In order to examine whether our data set of present-day Thai population can be used as the representative of the Thai population as a whole, the genetic difference between this data set was compared with 10 native Thai populations previously reported (Fucharoen et al. 2001; Yao et al. 2002). The mitochondrial DNA haplogroup frequency of each native population, except for Sakai, is very close to those in the present day Thai population (Table 4). Population tree constructed from the haplogroup frequencies diversity between populations showed 2 main branches. One branch contained only Sakai population and the other branch contained the rest of the groups (Figure 5). These results confirmed that Sakai was the isolated population and have their own and unique genetic background. The rest of the populations resided in the other branch of the tree indicated that native populations in this study are genetically closely related to each other. The present-day Thai population was closer to the Thai populations from various parts of Thailand more than native populations who have migration history from Vietnam, Lao and Southern part of China. This information indicated that our present-day Thai data set which is the admixture Thai population can be used as the representative of the Thai population in the country as a whole, except for some unique population such as Sakai, for example.

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Table 4

Table 4. Mitochondrial DNA haplogroup frequency in 10 native Thai populations (Fucharoen et al. 2001; Yoa et al. 2002) and our present-day Thais determined by nucleotide polymorphisms in the HVS-1 region.

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Figure 5

Figure 4. Population tree constructed from 10 native Thai populations and our present-day Thais data set.

In conclusion, mitochondrial DNA haplogroup determination using RFLP and HVS-1 criteria in our studied samples yields 78% similar result. RFLP criteria are more stringent and more accurate than HVS-1 criteria. However the HVS-1 haplogrouping using the nucleotide variants in the hypervariable segment 1 of mitochondrial genome gave a reasonably good and reliable result and mostly in agreement with the RFLP haplogroup criteria. Mitochondrial DNA haplogroup of almost all samples could be identified by phylogenetic trees. Thus, phylogenetic trees, constructed from either information are very useful in the haplogroup identification of these samples.

The analysis of mitochondrial DNA haplogroup in the present-day Thai corresponded well with the result from pervious published studies from Southeast Asians. Haplogroup M was found at the highest frequency in our population. Haplogroup F, B and B* were also found to be the major mitochondrial haplogroup in Thai population. Haplogroup frequency and population tree constructed from our present-day Thai and 10 native Thai populations confirmed that our data set represent the present-day admixture Thai population.

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Table 1. RFLP and HVS-1 polymorphisms used to identify mtDNA haplogroups in this study (Stoneking et al. (1990), Ballinger et al. (1992), Torroni et al. (1994), Schurr et al. (2000),and Schurr and Wallace (2002)

Haplogroup	Characteristic restriction sites*	Characteristic HV I nucleotide positions
A		T16362C, G16319A, C16290T, C16223T
A1	+ 633 *Hae*III, -10394 *Dde*I, -10397 *Alu*I	
A9	+ 633 HaeIII, +16517 *Hae*III, -10394 *Dde*I, -10397 *Alu*I	
B	9-bp deletion, +16517 *Hae*III, -10394 *Dde*I, -10397 *Alu*I	T16189C, T16217C, T16519C
B*	9-bp deletion, +16517 *Hae*III, +10394 *Dde* I, -3534 *Dde*I , -3537 *Alu*I, -15234 *Hinf*I, +15235 *Mbo*I	T16140C, T16189C, T16519C
C	-13259 *Hinc*II, +13262 *Alu*I, +10394 *Dde*I, +10397 *Alu*I	C16327T, T16298C, C16223T
D	-5176 *Alu*I, +10394 *Dde*I, +10397 *Alu*I	T16326C, C16223T
E	-7598 *Hha*I, +16389 *Hinf*I, -16390 *Ava*II, +10394 DdeI, +10397 AluI	
F	-12406 *Hinc*II, -12406 *Hpa*I, -10394 *Dde*I, -10397 *Alu*I	T16304C
G	+ 4830 *Hae*II, +4831 *Hha*I, +10394 *Dde*I, +10397 *Alu*I	
H	-7052 *Alu*I, - 14766 *Mse*I, -10394 *Dde*I	
I	-1715 *Dde*I, -4529 *Hae*II, +8249 *Ava*II/ -8250 *Hae*III, +10028 *Alu*I, +10394 *Dde*I, +16389 *BamH*I, -16390 *Ava*II	
J	-13704 *BstO*I, +10394 *Dde*I, -16065 *Hin*fI	
K	+12308 *Hinf*I, -9052 *Hae*II/-9053 *Hha*I, +10394 *Dde*I	
L1	+3592 *Hpa*I,+10806 *Hinf*I, +10394 *Dde*I	
L2	+3592 *Hpa*I, +16389 *Hin*fI, -16390 *Ava*II, +10394 *Dde*I	
L3a	-3592 HpaI, + 3249 MboI,	
L3b	-3592 *Hpa*I, - 8616 *Mbo*I	
L3c	-3592 *Hpa*I, + 10084 *Taq*I	
L3d	-3592 *Hpa*I, -10394 *Dde*I	
M	+10394 *DdeI*, +10397 *Alu*I	C16223T
P	+207 *Hinc*II, +207 *Hpa*I, +15606 *Alu*I, -10394 *Dde*I, -10397 *Alu*I	T16357C
Q	+10394 *Dde*I, +10397 AluI, +16178 *Taq*I	G16129A, T16144C, C161481, C16223T A16241G, A16265C, T16311C, A16343G
W	+8249 *Ava*II/-8250 *Hae*III, -8994 *Hae*III, -16223 *Hae*III, -16278 *Hae*III, -10394 *Dde*I	
X	+14465 *Ava*II, +16223, *Ava*II, +16278 *Ava*II, -10394 *Dde*I , -1715 *Dde*I	T16189C, C16223T, C162781
Y	+10394 *Dde*I, -10397 *Alu*I, +7933 *Mbo*I, -8391 *Hae*III, +16517 *Hae*III	
Z	+10394 *Dde*I, +10397 *Alu*I, +11074 *Dde*I, +16517 *Hae*III	

จัดรูปแบบ: ฝรั่งเศส ฝรั่งเศส

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จัดรูปแบบ: ฝรั่งเศส ฝรั่งเศส

Table 2. The definite haplogroup determined by at least 3 of 4 criteria; 1) polymorphisms in RFLP and 9 bp deletion 2) RFLP phylogenetic tree 3) HVS-1 sequence polymorphisms and 4) HVS-1 phylogenetic tree in the 100 normal individuals including the presence or absence of 9 bp deletion, T16189C and length heteroplasmy in each individuals.(Blank = Unclassified)

Sample	9bp	T 16 189 C	Length Hetero-plasmy	RFLP haplogroup		HVS-1 haplogroup		Definite haplogroup
				From cirteria	From phylogenetic tree	From cirteria	From phylogenetic tree	
C1	+	+	+	B*	B*	B*	B*	B*
C2	-	-	-	A9	A	M	M	
C3	+	+	+	B	B	B	B	B
C4	+	+	+	B*	B*	B*	B*	B*
C5	-	-	-	F	F	F	F	F
C6	-	-	-	M	M	M	M	M
C7	-	-	-				M	
C8	-	-	-	F	F	F	F	F
C9	-	-	-	F	F	F	F	F
C10	-	-	-	M	M	D	M	M
C11	+	+	+	B	B	B,B*	B	B
C12	-	-	-			D	M	
C13	-	-	-			F	F	
C14	-	-	-	M	M	D	M	M
C15	-	-	-			M	M	
C16	+	+	+	B*	B*	B*	B*	B*
C17	-	-	-			M	M	
C18	-	-	-	M	M	D	M	M
C19	-	-	-	M	M	M	M	M
C20	-	-	-	F	F	F	F	F
C21	+	+	+	B	B	B	B	B
C22	-	+	+			D	M	
C23	+	-	-	M	M	C	M	M
C24	+	+	+	B	B	B	B	B
C25	-	-	-	M	M	M	M	M
C26	+	+	+	B*	B*	B*	B*	B*
C27	-	+	+	M	M	M	M	M
C28	+	-	-	A9,B	A	M	M	
C29	-	-	-	F	F	F	F	F
C30	-	-	-	M	M	M	M	M
C31	+	+	+	B*	B*	B*	B*	B*
C32	-	-	-	M	M	M	M	M
C33	-	+	+	M	M	M	M	M

Sample	9bp	T 16 189 C	Length Hetero-plasmy	RFLP haplogroup		HVS-1 haplogroup		Definite haplogroup
				From cirteria	From phylogenetic tree	From cirteria	From phylogenetic tree	
C34	-	-	-	M	M	M	M	M
C35	-	-	-				M	
C36	+	+	+		B*	B*	B*	B*
C37	-	-	-	A1	A	A	M	A
C38	-	-	-	M	M	D	M	M
C39	-	-	-	M	M	M	M	M
C40	-	-	-	M	M	F, M	M	M
C41	+	+	+	B	B	B,B*	B	B
C42	-	-	-	F	F		F	F
C43	-	-	-	F	F	F	F	F
C44	-	+	-	M	M	M	M	M
C45	-	-	-	F	F	F	F	F
C46	-	+	-	M	M	M	M	M
C47	-	+	+			F		
C48	-	-	-	M	M	M, P	M	M
C49	-	-	-	M	M	D	M	M
C50	-	+	-	M	M	M	M	M
C51	-	-	-	A9	A	M	M	
C52	-	+	+	M	M	M	M	M
C53	-	-	-	M	M	D	M	M
C54	-	-	-	F	F	F	F	F
C55	-	-	-	M	M	M	M	M
C56	-	-	-	M	M	M	M	M
C57	+	+	+	B	B	B	B	B
C58	-	-	-	M	M	D	M	M
C59	-	-	-			F	F	
C61	-	-	-	M	M	M	M	M
C62	-	-	-	M	M	F, M	M	M
C63	-	-	-	M	M	M	M	M
C64	-	+	+	M	M	D	M	M
C65	+	+	+	B	B			
C66	+	+	+	B*	B*	B*	B*	B*
C67	-	-	-	M	M	M	M	M
C68	-	+	-	M	M	M	M	M
C69	-	+	+	M	M	M	M	M
C70	-	-	-	M	M	F, M	M	M
C71	-	-	-	M	M	M	M	M
C72	-	-	-	F	F	F	F	F
C73	-	-	-	F	F	F	F	F
C74	-	-	-	M	M	D	M	M
C75	-	-	-			M, P	M	M
C76	-	-	-	G	M	D	M	
C77	-	-	-	M	M	M	M	M
C79	+	+	-	B	B	B	B	B
C80	-	-	-	M	M	M	M	M
C81	-	-	-	M	M	M	M	M

Table 2. Continued

Sample	9bp	T 16 189 C	Length Hetero-plasmy	RFLP haplogroup		HVS-1 haplogroup		Definite haplogroup
				From cirteria	From phylogenetic tree	From cirteria	From phylogenetic tree	
C84	+	+	+	B*	B*	B*	B*	B*
C85	-	-	-			F	F	
C86	+	+	+	B	B	B	B	B
C87	-	+	+	F	F	F		F
C88	-	-	-	M	M	M	M	M
C89	-	-	-		B	M	M	
C90	+	+	+	B	B	B	B	B
C91	+	+	+	B*	B*			
C92	-	-	-	M	M	M	M	M
C93	-	-	-	D	M	D	M	
C94	-	-	-	M	M	M	M	M
C95	-	-	-	C	M	C	M	
C96	-	+	+			F	F	
C97	-	-	-	F	F	F	F	F
C98	-	-	-	M	M	M	M	M
C99	-	-	-	F	F	F	F	F
C100	-	-	-	M	M	M	M	M
C101	+	+	-	B*	B*			
C102	-	-	-			F	F	
C104	-	-	-	F	F	F	F	F
C105	+	+	+	B	B	B	B	B

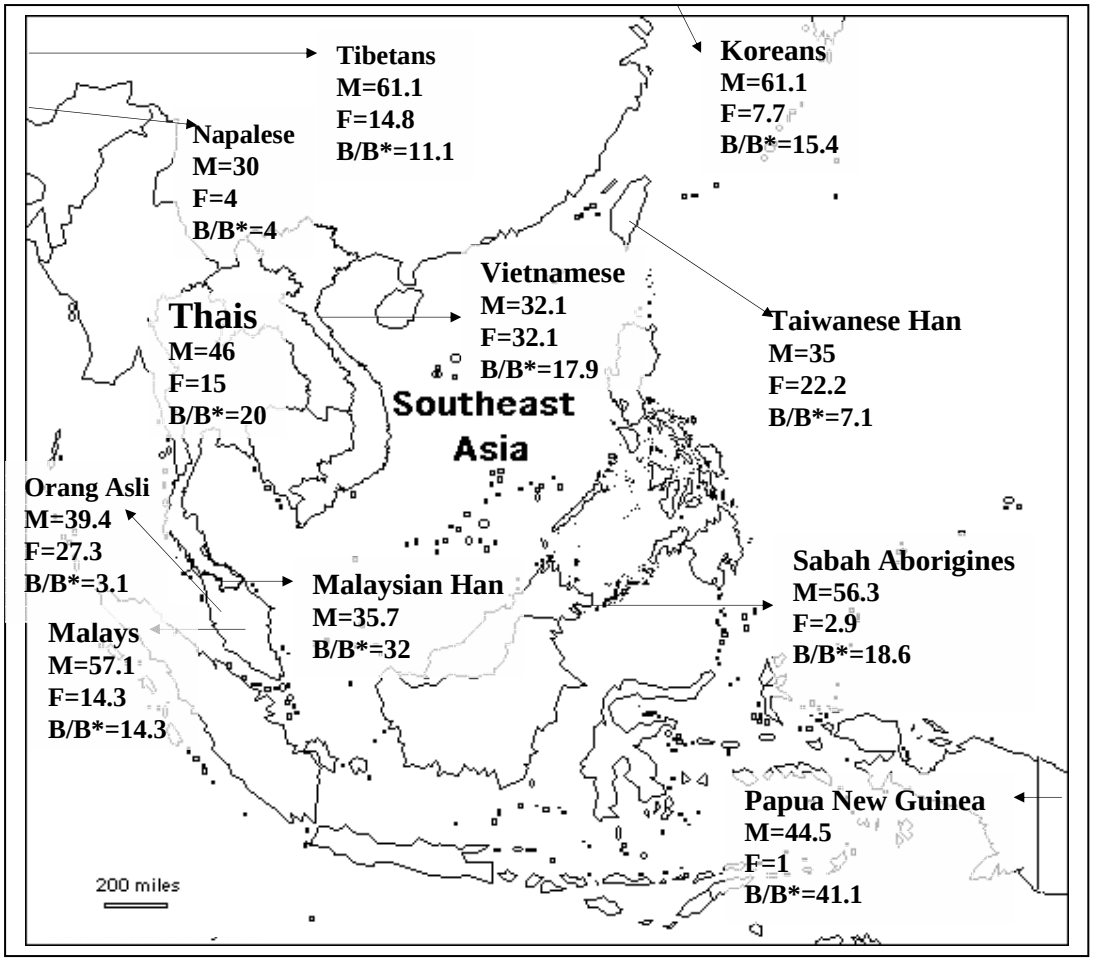
Table 2. Continue

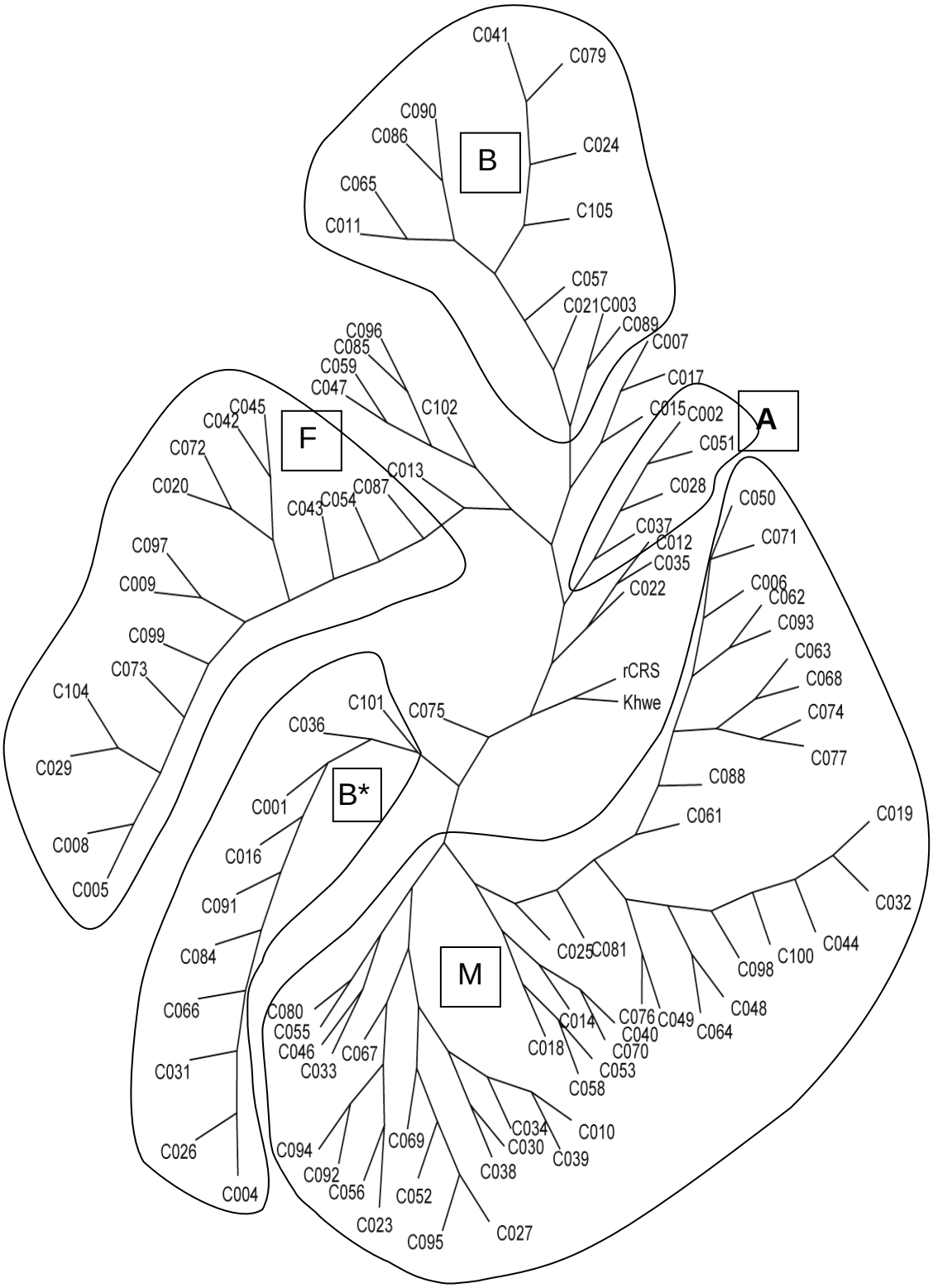
Table 3. The frequency of each mitochondrial haplogroup determined by either high resolution RFLP or nucleotide variation in HV 1 region in 100 normal Thai individuals in this study.

Haplogroup	RFLP criteria	HVS-1criteria
A1	1 (1%)	1 (1%)
A9	2 (2%)	
B	11 (11%)	8 (8%)
B*	9 (9%)	8 (8%)
B/B*	-	2 (2%)
C	1 (1%)	2 (2%)
D	1 (1%)	13(13%)
F	15 (15%)	20 (20%)
G	1 (1%)	-
M	43 (43%)	35(35%)
M/F	-	3 (3%)
M/P	-	2 (2%)
A9/B	1 (1%)	-
Unclassified	15 (15%)	6 (6%)
Total	100 (100%)	100 (100%)

Table 4. Mitochondrial DNA haplogroup frequency in 8 native Thai populations (Fucharoen et al. 2001) determined by nucleotide polymorphisms in the HVS-1 region.

Population	Total number	Mitochondrial haplogroup						
		A	B	B*	C	F	M	?
Present THAI	100	1 (1%)	10 (10%)	8 (8%)	2 (2%)	21 (21%)	55 (55%)	3 (3%)
Phuthai	25	0	5 (20%)	0	0	8 (32%)	12 (48%)	0
Lao Song	25	0	4 (16%)	5 (20%)	0	4 (16%)	12 (48%)	0
Lisu	25	0	4 (16%)	1 (4%)	0	6 (24%)	14 (56%)	0
Mussur	21	0	1 (5%)	0	0	8 (38%)	12 (57%)	0
Chiang Mai	30	0	5 (17%)	2 (7%)	0	9 (29%)	14 (47%)	0
Khon Kaen	44	0	4 (9%)	5 (11%)	0	11 (25%)	24 (55%)	0
Chong	25	0	2 (8%)	8 (32%)	0	2 (8%)	13 (52%)	0
Sakai	20	0	0	0	0	0	20 (100%)	0
Khmer	22	0	2 (9%)	2 (9%)	0	11 (50%)	7 (32%)	0
northern Thai	32	0	1 (3%)	3 (9.5%)	0	9 (28%)	19 (59.5%)	0


Tibetans
M=61.1
F=14.8
B/B*=11.1
Koreans
M=61.1
F=7.7
B/B*=15.4
Napalese
M=30
F=4
B/B*=4
Vietnamese
M=32.1
F=32.1
B/B*=17.9
Thais
M=46
F=15
B/B*=20
Taiwanese Han
M=35
F=22.2
B/B*=7.1
Southeast Asia
Orang Asli
M=39.4
F=27.3
B/B*=3.1
Malaysian Han
M=35.7
B/B*=32
Sabah Aborigines
M=56.3
F=2.9
B/B*=18.6
Malays
M=57.1
F=14.3
B/B*=14.3
Papua New Guinea
M=44.5
F=1
B/B*=41.1
200 miles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C090
C086
B
C024
C065
C105
C011
C057
C021
C003
C089
C007
C096
C085
C059
C047
C102
C017
C045
C042
F
C015
C002
A
C072
C051
C020
C013
C028
C087
C043
C054
C050
C037
C071
C097
C012
C035
C009
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C062
C099
C093
C073
C063
rCRS
C068
C104
C101
C075
Khwe
C074
C036
C029
C077
C088
B*
C001
C061
C019
C008
C016
C032
C005
C091
C025
C081
C098
C100
C044
C084
M
C080
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C056
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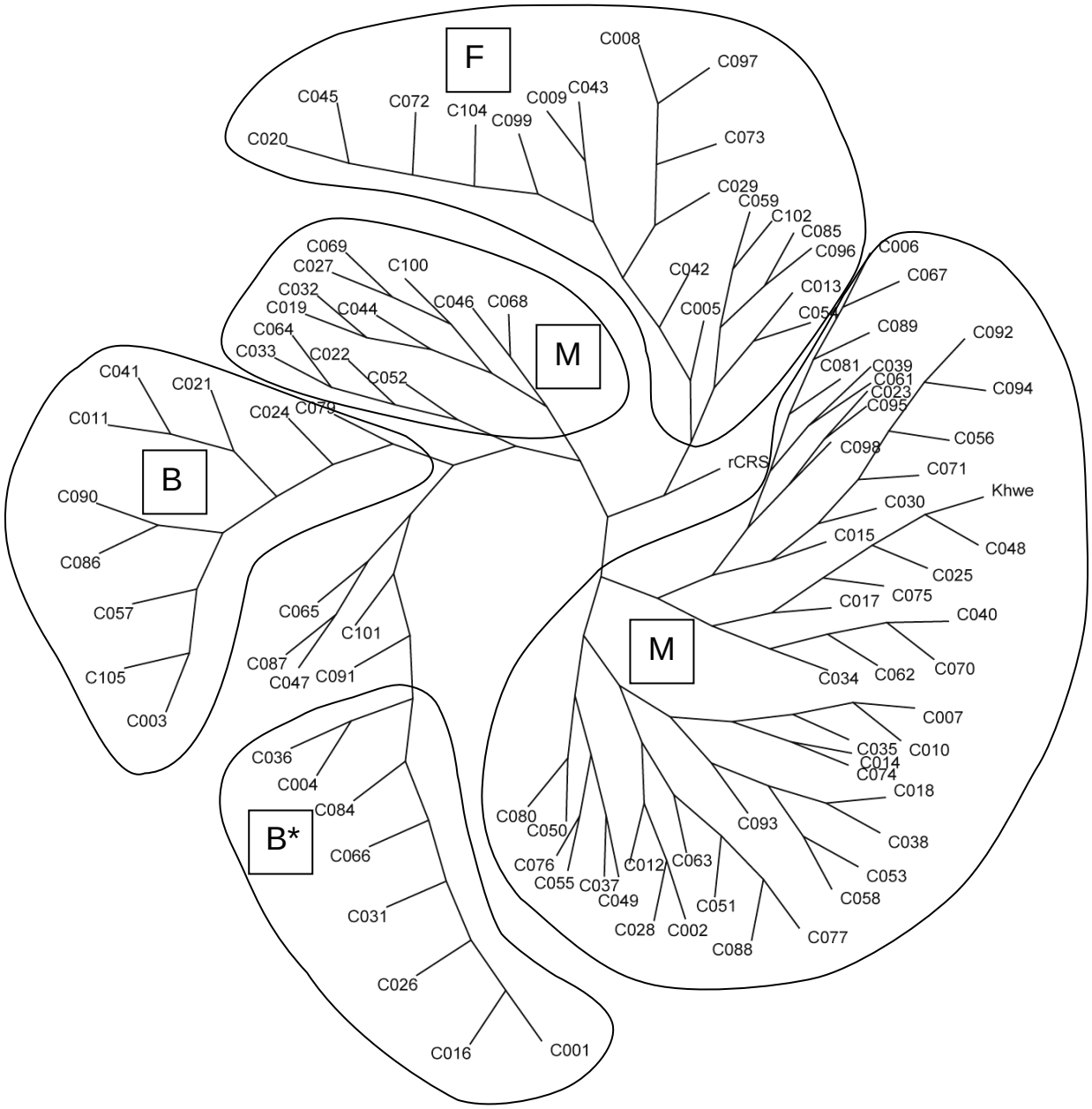
C095
C027


F
M
B
B*
M
rCRS
Khwe
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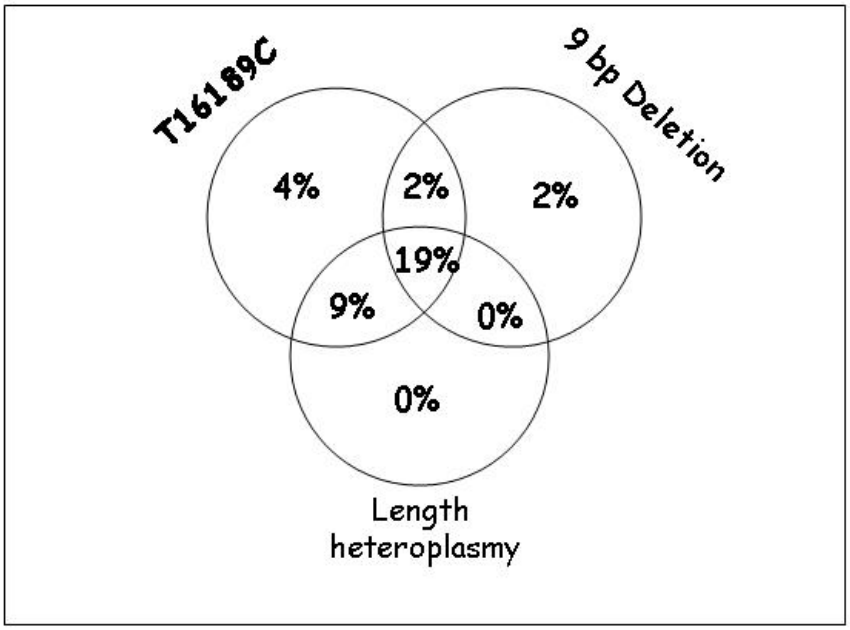
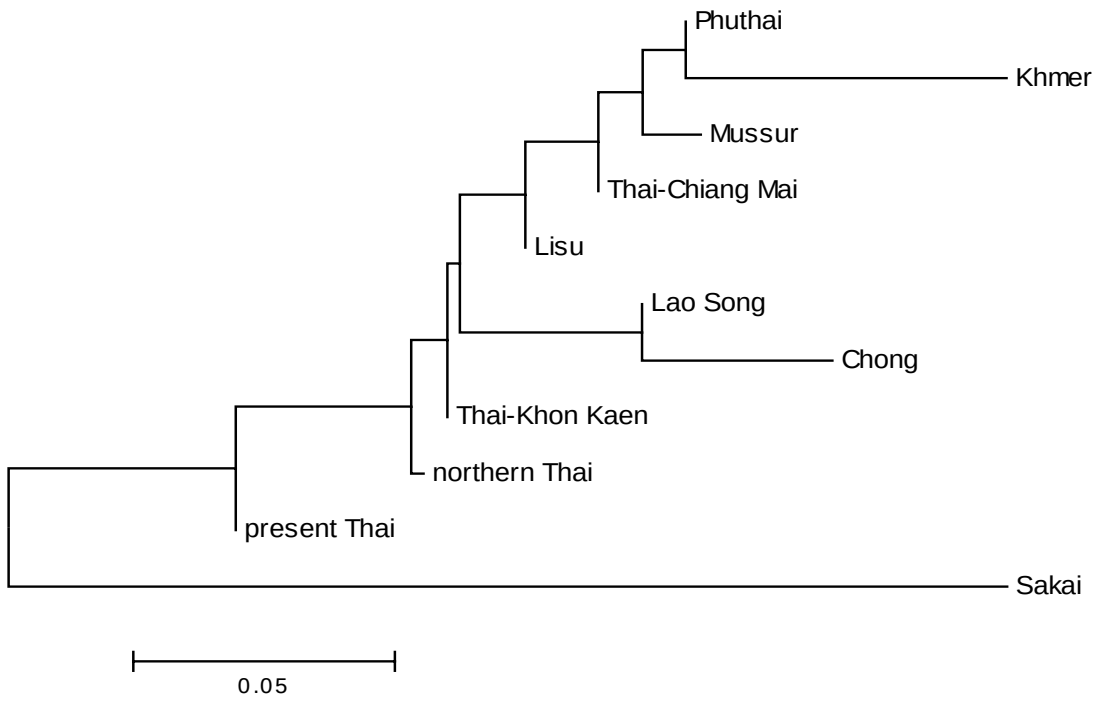

T16189C
9 bp Deletion
4%
2%
2%
19%
9%
0%
0%
Length
heteroplasmy


Phuthai
Khmer
Mussur
Thai-Chiang Mai
Lisu
Lao Song
Chong
Thai-Khon Kaen
northern Thai
present Thai
Sakai
0.05

To be submitted to J Hum Genet

The Unique Characteristics of Thai Leber Hereditary Optic Neuropathy: Analysis of 30 G11778A Pedigrees

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Abstract

Leber hereditary optic neuropathy (LHON) is characterized by acute or subacute bilateral visual loss mostly in young males. The most common mitochondrial DNA mutation responsible for LHON worldwide is G11778A. Despite different genetic backgrounds, which are believed to influence the disease expression, most of the LHON features are quite common in different populations. However, there seem to be a few ethnic-specific differences. The analyses of our 30 G11778A LHON pedigrees in Thailand showed some characteristics different from those of Caucasians and Japanese. In particular, our pedigrees showed lower male to female ratio of affected persons (2.6:1) and much higher prevalence of G11778A blood heteroplasmy (37% of the pedigrees contained at least one heteroplasmic G11778A individual). Heteroplasmicity seemed to influence the disease manifestation in our patients but did not appear to alter the onset of the disease. In our pedigrees, the mean age of onset in females was higher than in males. The disease penetration varied from 9-45% between pedigrees and varied between different branches of the same large pedigree. The affected mothers from our pedigrees were more likely to have affected children than did the unaffected mothers no matter what gender of their children was. Survival analysis showed that the secondary LHON mutations G3316A and C3497T have synergistic deleterious effect with the 11778 mutation and accelerating the onset of the disease in our patients.

Keywords: mitochondria; mitochondrial DNA (mtDNA); mitochondrial genetics; mitochondrial disease; Leber Hereditary Optic Neuropathy (LHON)

Introduction

Leber hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by acute or subacute bilateral painless loss of central vision resulting from optic atrophy (Nikoskelainen EK et al. 1987). The three most common mtDNA mutations responsible for >95% of LHON pedigrees worldwide are G3460A, G11778A and T14484C (Mackey DA et al. 1996; Man PY et al. 2002). Of these, the G11778A is the most common worldwide, however, the frequency of each of these 3 mutations varies markedly in different populations.

Only ~50% of males and ~10% of females harboring LHON mutation develop optic neuropathy (Harding AE et al. 1995; Riordan-Eva P et al. 1995). In addition, about 80% of affected individuals are males (Nikoskelainen EK et al. 1987). The incomplete penetrance as well as the male preponderance indicates that, apart from mtDNA mutations, there must be other unknown factors responsible for the disease manifestation.

In Southeast Asia, including Thailand, where the genetic background is different, only a few reports of LHON families have been published (Chuenkongkaew WL et al. 2001; Sudoyo H et al. 1998; Sudoyo H et al. 2002). We report here the analyses of our 30 unrelated G11778A LHON pedigrees. The purposes of this study were to define the mitochondrial genetics and the pedigree characteristics in multiple Thai G11778A LHON families. The analyses of LHON in the Southeast Asia together with other regions around the world will have implications regarding how distantly related genetic backgrounds both in the mitochondrion and the nucleus might contribute to the phenotypic expression and the complexity of LHON.

Materials and methods

Pedigrees and Sample Collection

Blood samples of patients clinically similar to LHON were sent to our laboratory following informed consent. Pedigree information of the patients who were positive for the mutation was investigated and blood samples from their family members were also collected with informed consent.

In each pedigree, the clinical data were obtained by direct examination by the ophthalmologists, or indirectly, by interviews with one or more of the family members. Affected status in unseen maternal relatives was based on a history of acute visual loss without other known causes.

Mitochondrial Genetics Analysis

Total leukocyte DNA was extracted from at least 5 ml of whole blood sample containing EDTA or ACD-A using the standard phenol/chloroform method. The 11778 mutation was tested in all available family members. One sample in the maternal lineage of each family was tested for other primary and secondary mutations (nt 3316, 3394, 3460, 3496, 3497, 3635, 4136, 4160, 4171, 4261, 4917, 5244, 7444, 9738, 9804, 13708, 13730, 14459, 14482, 14484, 14495, 14498, 14568, 14596, 15257, and 15812) by either Restriction Fragment Length Polymorphism (RFLP) analyses or direct sequencing of the mtDNA as detailed in Lertrit P et al. 1998 and Sudoyo H et al. 2002, respectively. Degrees of heteroplasmy of the G11778A mutation were quantitated using radioactive restriction analysis modified from Moraes (Moraes CT et al. 1992). In order to be certain that all 30 pedigrees are genetically unrelated, the hypervariable region I (HV I) in the mtDNA D-loop (nt 16024 to nt 16383) from the proband of each family was sequenced.

To avoid confounding effects from multiple risk factors on one another, we studied the effects of sex, secondary mutation and degree of heteroplasmy simultaneously on the age-dependent penetrance of the G11778A mutation. From the 166 samples positive for the G11778A mutation, 13 samples with only one blood sample per family were taken out to reduce ascertainment bias, resulting in 152 samples with known phenotype and mtDNA profile. We did a survival analysis using Cox's proportional hazards model to fit our data. This model assumes that for all individuals the hazard function *h(t)* (the probability that a person gets LHON at a particular age *t*) has the same basic shape, but that certain factors (sex, secondary mutation, and degree of heteroplasmy) may change the risk of LHON by multiplying *h(t)* by a fixed factor. The analysis was performed using R v1.8.1 statistical software (R Development Core Team 2003).

Results

Pedigrees

Thirty G11778A LHON pedigrees were identified in the study. All the pedigrees are of Thai or Chinese ethnic origins except for one pedigree with Indian ethnic origin. Six of them were large pedigrees comprising 4-7 generations. From these 30 families, 27 HVS-1 mitochondrial haplotypes were detected. However, when the mitochondrial genome was subject to high resolution screening of polymorphic restriction sites and screen for 9-bp deletion, they all carried distinct mtDNA haplotypes. Therefore these 30 families were not closely genetically related.

From these pedigree, 166 samples (81 males and 85 females) were positive for the G11778A mutation consisting of 65 affected, 2 possibly affected (the affected status was difficult to be assigned owing to cataract both eyes), and 99 unaffected individuals.

One family was found to have two genetic diseases simultaneously: LHON, a mitochondrial disease and facioscapulohumeral dystrophy (FSHD), an autosomal dominant disorder (Chuenkongkaew WL et al. 2005).

Age of Onset and Male:Female Ratio

The 65 affected patients consisted of 47 males and 18 females, and the male:female ratio was 2.6:1. In other words, 72% of patients were male. In affected persons directly evaluated, 58 were documented with their age of onset. The mean age of onset was 22.6 ± 11.7 years (range: 6-53, median: 20 years) in all the patients, 20.7 ± 10.0 years (n = 44, range: 6-44, median: 19 years) in males, and 28.6 ± 14.6 (n = 14, range: 10-53, median: 30 years) in females. It appeared that the mean age of onset in female was higher than in male in our patients. The difference was almost statistically significant (p=0.073; Mann-Whitney U test).

Disease Penetration

Excluding the 2 possibly affected persons, the directly evaluated 164 samples harbouring the 11778 mutation consisted of 40% (65/164) affected and 60% (99/164) unaffected individuals. When male and female groups were analysed separately, 58% (47/81) of males and 22% (18/83) of females carrying the mutation expressed the disease. It should be noted that 34% of the currently unaffected persons who were directly evaluated aged less than 24 years (the average age of onset of LHON in Thailand) and some of them might become affected later. When consider only 10 large pedigrees with more than 10 maternal relatives spanning at least 3 generations, the disease penetrance varied from 9% to 45% with the mean of 19 ± 11%. In addition, our preliminary observation showed that the proportion also varied between different branches of the same large pedigree.

With this criterion that all the unaffected persons were at least 24 years of age, 19 sibships (and their mothers) were identified, comprising 13 sibships with unaffected mothers and 6 sibships with affected mothers. The affected mothers seemed to be more likely to have affected children than the unaffected mothers (OR=2.51, p=0.12; Chi-square test) independent of gender of their children; 56% (9/16) of males born to affected mothers became affected, compared with 34% (10/29) of those born to unaffected mothers; whereas 33% (3/9) of females born to affected mothers developed optic neuropathy, compared with only 17% (4/23) of those born to unaffected mothers.

Heteroplasmy of the G17778A Mutation

Eleven (37%) of our 30 LHON pedigrees contained at least one individual with the heteroplasmic 11778 mutation (heteroplasmic pedigree). The number of such pedigrees might be underestimated owing to the fact that in 11 of our 19 homoplasmic pedigrees, only blood samples from probands were obtained. Therefore, other family members whose blood samples were not available could be heteroplasmic for the mutation if they were tested. Of the 166 individuals positive for the 11778 mutation, 28% (46/166) were heteroplasmic and 72% (120/166) were homoplasmic. Considering only the patients (affected persons), only 14% (9/65) were heteroplasmic, while in the unaffected group, 35% (35/99) were heteroplasmic. It was found that 20% (9/44) of heteroplasmic persons manifested the disease, compared with 47% (56/120) of the homoplasmic group (OR=3.40 p=0.004; Chi-square test). When sex was considered in the analysis, similar results were obtained. Our results supported the belief that heteroplasmy influences the expression of LHON and the prevalence of heteroplasmy was higher in the unaffected group compared with the affected group.

It should be noted that in 2 of our heteroplasmic families, 8 samples of maternal lineages were found to be negative for the 11778 mutation. This provided evidence that the heteroplasmic 11778 mutation could segregate to pure wild type. This supports the important of molecular mtDNA testing in family members seeking genetic counseling as suggested by Man et al., 2003 (Man PY et al. 2003).

Other Primary and Secondary LHON Mutations in the 11778 LHON Pedigrees

Other 27 LHON secondary mutations were screened and two families were found to possess mutations other than the G11778A; one (F11) carried a C3497T and the other (F19) carried the G3316A mutation. The mean age of onset in the patients carrying 11778 mutation plus the secondary mutation (n = 10) was 16.4 ± 8.9 years (range: 8-33, median = 14.5 years), while in the patients carrying only G11778A (n = 43), the mean age of onset was 23.5 ± 11.8 years (range: 6-53, median = 20 years). The result indicated that the secondary mutations, G3316A and C3497T, seemed to have synergistic deleterious effect with the 11778 mutation, accelerating the onset of the disease ($p<0.05$; Mann-Whitney U test).

From the survival analysis using Cox's proportional hazard model, we found that male sex, secondary mutation, and high mutation load each had a significant effect on the age-dependent penetrance of LHON. The model predicted that males were 2.8 times more likely than females to develop LHON (P=0.00062). People with secondary mutations (G3316A or C3497T) were 3.5 times more likely to express the disease than people without (P=0.0069). Moreover, the model predicted that each 1% drop in degree of heteroplasmy reduced the rate of getting LHON by a factor of 0.97 (P=0.0053). We also tested for interactions between these three risk factors but none were significant. Examples of survival curves for 6 individuals using this fitted model are plotted in Figure 1.

Figure 1

Figure 1. Survival curves fitted using Cox's proportional hazards model from 152 samples positive for the G11778A LHON mutation in Thailand. The curves represent 6 samples with different sex, secondary LHON mutation status, or mutation load. S(t) = probability of a person being unaffected at age t.

Discussion

Like in most countries worldwide, the G11778A mutation is the most prevalent LHON mutation in Thailand. So far, the 3460 mutation has never been reported in Thai as well as in Southeast Asian. The prevalence of these mutations in Thai LHON is consistent with most of LHON families from multiple Asian countries (Table 1). In contrast, among most Caucasian LHON pedigrees, the prevalence is lower for the 11778 and higher for the 3460 and the14484 mutation when compared with Asian LHON families (Table 1). The marked difference in the prevalence of each of the classical LHON mutations between Asian and Caucasian LHON families might reflect the effects of different genetic backgrounds (nuclear and/or mitochondrial) on the generation and clinical expression of these LHON mutations.

Table 1

Table 1. Comparison of 11778 LHON pedigree characteristics of the present study with previous reports.

In the present study, the estimated overall penetrance of our 11778 LHON population was 37% for males, and 13% for females. These figures were comparable to those in 11778 Finnish LHON (39% for males and 14% for females, Newman NJ 1993) but were different from 11778 British LHON (51% for males and 8.5% females, Man PY et al. 2003). In Caucasian, as a rule of thumb, ~50% of males and 10% of females in LHON families lost vision (Man PY et al. 2002; Newman NJ 1993; Howell N 1997; Howell N 1998). However, more extensive data regarding penetrance are needed for Asian

LHON. The analysis of our 11778 LHON pedigrees also provided supporting evidence that affected mothers were more likely to have affected children, either daughters or sons, than were unaffected mothers.

However, some pedigree features in our series were different from most 11778 LHON in the literature. The most striking was the high prevalence of blood leukocyte heteroplasmy of the 11778 mutation in Thailand. Thirty-seven percents (11/30) of our 30 LHON pedigrees contained at least one individual heteroplasmic for the mutation, while such a proportion is generally considered to be 15% in most studies (Chinnery PF et al. 2001). Moreover, the proportion of our heteroplasmic families might be higher if additional blood samples of maternal relatives could be collected, as described in the Results section. If heteroplasmy reflects a recent muational event (Savontaus ML 1995), it is interesting how recent mutational events could occur in our population between 10 years (1994-2003) of our sample collection in such a high incidence. A recent epidemiological study in the North East of England also shows a high proportion (33%) of heteroplasmic families than the general figure of 15%. Our analyses of heteroplasmy supported the belief that heteroplasmy influences the expression of LHON.

Another different feature of our 11778 LHON patients was that the male to female ratio (2.6:1 or 72%) appeared to be smaller than that of most 11778 LHON patient series worldwide (Table 1).

Several secondary LHON mutations have been found (Wallace DC and Lott MT 2003); however, in most cases, their pathogenicity is still uncertain and several studies showed conflicting evidences of the roles of secondary mutations (Howell N 1997; Howell N et al. 1995; Brown MD et al. 2002; Oostra RJ et al. 1994; Lodi R et al. 2002). Two secondary LHON mutations (G3316A and C3497T) were found one each in 2 pedigrees. Our analysis of the age at onset supported the synergistic role of the

secondary LHON mutations, G3316A and C3497T, with the 11778 mutation in precipitating the onset of the disease.

The G3316A mutation changes a nonpolar alanine to a polar threonine at the fourth amino acid in ND1 protein. Although definite conclusion still could not be drawn regarding pathogenicity of the 3316 mutation, these evidences in several independent studies indicate that the mutation might cause a mild defect on mitochondrial function and thus, precipitating type 2 diabetes as well as LHON in appropriate genetic backgrounds. For our "11778 plus 3316" LHON pedigree, it was difficult to make interpretation regarding the contribution of the 3316 mutation to the manifestation of the 11778 mutation since this family also suffered from FSHD, which might confound the expression of the mitochondrial disease. At least there is evidence indicating that FSHD is associated with a deficiency of the mitochondrial respiratory chain complex III (Slipetz DM et al. 1991).

The C3497T changes an alanine to a valine at the 64th amino acid of the ND1 protein. It was proposed by Mutsumoto M et al. 1999 to be a secondary LHON mutation since it is found in 5% (1/19) of Japanese LHON patients and 1.9% (2/108) of Japanese normal controls. We observed that our "11778 plus 3497" LHON family displayed the highest proportion of affected individuals (77%) in our pedigree series, which could possibly be partly due to the effect of the 3497 mutation. Note that, while the proportion of men with LHON was about 50%, which is similar to other studies (Man PY et al. 2003), the 'life-time' risk of LHON for homoplasmic men without secondary mutations as predicted by this model is around 80%. A long-term prospective cohort study is required to verify this life-time risk.

It is clear that there have to be factors other than the primary LHON mutations responsible for the LHON features not able to be explained by the mitochondrial inheritance. These features include the incomplete penetrance, male predominance and optic nerve specific disease expression. Currently,

genetic backgrounds in the mitochondria and/or in the nucleus are strongly suggested to play a role in the disease expression (Sudoyo H et al. 2002; Brown MD et al. 2000; Brown MD et al. 2002; Howell N et al. 2003; Sadun AA et al. 2002; Cock HR et al. 1998; Qi X et al. 2003; Carelli V et al. 2003). Despite the different genetic backgrounds, most of the LHON features that constitute the picture of LHON are quite common in different population, however, there seemed to be a few ethnic-specific differences. Deep looking into these differences may provide some clues to the discovery of other factors modifying the disease, its pathophysiology and eventually an effective therapeutic intervention for this devastating disease.

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Table 1. Comparison of 11778 LHON pedigree characteristics of the present study with previous reports.

	Asian			Caucasian				
	Present study	Hotta et al. 1995	Yen et al. 1999	Newman et al. 1991	Smith et al. 1993	Oostra et al. 1994	Harding et al. 1995	Man et al. 2003
No. of families	30	79	17	49	68	15	66	9
No. of affected cases	65	90	24	72	75	146	109	49
Cases with positive family history (%)	50%	62%	-	43%	-	-	56%	-
Male patients (%)	72%	92.1%	88%	82%	77%	88%	79%	84%
Heteroplasmy								
Heteroplasmic families (%)	37%	-	0%	14%	18%	13%	7.6%	33%
Heteroplasmic persons (%)	28%	19%	0%	-	14%	-	-	-
Heteroplasmic patients (%)	14%	14%	0%	-	6.7%	-	0%	16%
Average age at onset (years)								
Males	20.7 (6-44)	-	21.85 (10-39)	26.2 (8-60)	-	28.55 (6-61)	21.0 (6-62)	-
Females	28.6 (10-53)	-	13, 56 [a]	34.0 (9-54)	-	31.47 (8-69)	28.0 (10-58)	-
Both	22.6 (6-53)	23.4 (7-59)	20.52 (10-56)	27.6 (8-60)	-	28.87 (6-69)	24.0 (6-62)	-

Figures within the brackets represent ranges of the age at onset; -, not available.

[a] Only two female in their series with the age at onset of 13 and 56 years old.

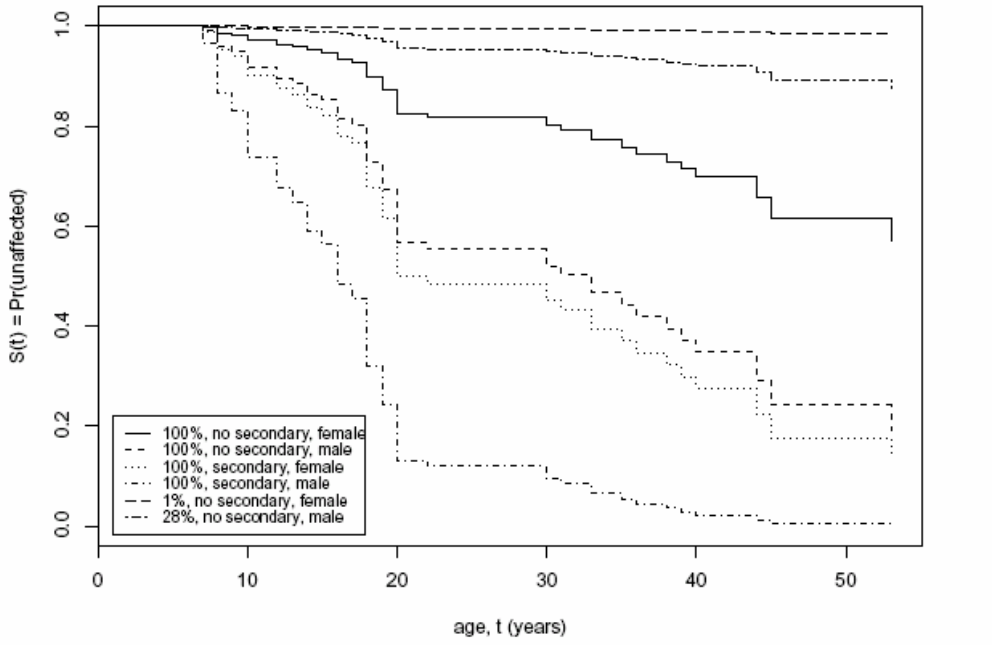

Figure 1. Survival curves fitted using Cox's proportional hazards model from 152 samples positive for the G11778A LHON mutation in Thailand. The curves represent 6 samples with different sex, secondary LHON mutation status, or mutation load. S(t) = probability of a person being unaffected at age t.

Mitochondrial DNA haplogroup distribution in Pedigrees of Southeast Asian G11778A Leber Hereditary Optic Neuropathy

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ABSTRACT

In order to investigate the association of mtDNA haplogroup and LHON in the Southeast Asian which is of distinct mitochondrial background from European and North American, mtDNA haplogroup determined by high-resolution restriction fragment length polymorphism (RFLP) was performed in 42 LHON individuals carrying G11778A mutation. They are of Thai, Thai-Chinese and Indian origin. Three mtDNA haplogroups, M, B* and B, were found in a similar frequency to normal population. MtDNA haplogroup F was found in none of our patients although it was the second most common haplogroup in our population. No specific mtDNA haplotype was associated with these LHON patients.

INTRODUCTION

Leber Hereditary Optic Neuropathy (LHON) is a mitochondrial disease characterized by late-onset visual failure resulting from bilateral optic nerve atrophy. The disease is associated with mitochondrial DNA mutation. Three of which are primary mutations, G11778A (Wallace et al., 1988), T14484C (Johns et al., 1992; Mackey and Howell, 1992) and G3460A (Howell et al., 1991; Huoponen et al., 1991), in the ND4, ND6 and ND1 gene respectively. However, less than 50% of male and 10% of female LHON carriers will develop the optic neuropathy. This marked incomplete penetrance and gender bias indicates that additional genetic and/or environmental factors are required for the phenotypic expression of the pathogenic mtDNA mutations in LHON

In European LHON, the G11778A and T14484C mutations show an association with the European-specific mtDNA haplogroup J (Brown et al., 1997; Brown et al., 2002; Howell et al., 1995; Torroni et al., 1997; Man et al., 2004). This leads to the suggestion that a combination of polymorphisms specific to this haplogroup increase the penetrance of these two primary mutations (Torroni et al., 1997).

Although mtDNA haplogroup have been performed in LHON from European, Scandinavian, and North American populations, few studies have been carried out on LHON in other parts of the world. The information of Asian LHON, especially when compared with those of European, Scandinavian, and North American LHON, could facilitate the understanding of molecular mechanism and the pathogenesis of LHON in general. In order to investigate the role of mitochondrial background in the expression of LHON, the mtDNA haplogroup analysis was performed in 42 LHON individuals of Southeast Asian origin.

PATIENTS AND METHODS

Patients

All 42 LHON individuals were from the Department of Ophthalmology, Faculty of Medicine at Siriraj Hospital, Ramathibodhi Hospital, and Chulalongkorn Hospital, Bangkok, Thailand. All of them, either Thai or Chinese-Thai or Indian origin, were born in Thailand. Five ml venous blood sample of each individual was obtained with informed consent.

Detection of G11778A LHON mutation

DNA was extracted from venous blood sample using standard phenol-chloroform extraction. The G11778A mutation was detected by mismatched PCR-RFLP method as previously described.

MtDNA haplogroup analysis

The mtDNA haplogroup was determined by high resolution restriction fragment length polymorphisms (RFLP) as details in (Ballinger et al., 1992).

Statistical analysis

Chi-square test or Fisher Exact test were used to compare the difference in haplogroup frequency between healthy Thais and LHON patients.

RESULTS

For our 42 G11778A LHON individuals, they were from provinces located in the central, northern, northeastern, and southern part of Thailand. These 42 individuals are not relative as confirm by the RFLP in the D-loop region**.**

For the RFLP analysis, mtDNA haplogroup M, B* and B were identified in our 42 G11778A LHON patients. Twenty two of them were classified to be haplogroup M (22/40=55%) whereas 8 and 4 are haplogroup B* (8/40=20%) and B (5/40=12.5%) respectively (Table 2). MtDNA haplogroup could not be classified in 5 patients (5/40=12.5%). In the present-day Thai population (Luangtrakool K, personal communication), the frequency of mtDNA haplogroup are 43%, 11%, 9% for haplogroup M, B, and B* respectively. Haplogroup F was not detected in any of our patients although it was found in 15% of the present-day Thai population.

To test whether the mtDNA haplogroups distribution in the G11778A LHON individuals and normal controls are significantly different, the Chi-square test was performed. The p value of the frequency difference among the LHON individuals and the normal controls of the mtDNA haplogroup M, B*, B, and F were 0.272, 0.130, 0.967, and 0.022 respectively. The results indicated no significant difference in the frequency of haplogroup M, B, and B* between the LHON samples and the normal controls ($p>0.05$) (Table 1). However, the frequency difference of haplogroup F in normal controls and LHON individuals was significant ($p=0.022$).

Table 1

Discussion

The preferential association of LHON with haplogroup J in Caucasian patients suggested a pathogenic role of the subset of mtDNA variants associated with this haplogroup in promoting the clinical expression of the primary LHON mutations. However, the combination of polymorphisms within haplogroup J that increases the risk of LHON expression is not yet identified. Haplogroup J is one of nine common European specific haplogroups, therefore it would also be expected that LHON should be more prevalent in Europeans. This is not the case since these two primary mutations, G11778A and T14484C were also found in distantly related ethnic population. Our 40 independent LHON pedigrees whose ethnic origin are of Southeast Asian carry G11778A mutation, the mutation which found in most LHON patients in all other parts of the world including Europe, North America and Scandinavia (Chinnery et al., 2001; Hofmann et al., 1997; Huoponen et al., 1990; Wallace et al., 1999). In our patients, only mitochondrial DNA haplogroup M, B* and B were found. None belongs to mitochondrial DNA haplogroup J. These three haplogroups are the Asian major haplogroups (Ballinger et al., 1992; Schurr and Wallace, 2002; Wallace and Lott, 2003). Most of our G11778A LHON patients were classified to be haplogroup M (22/40=55%), which is not significantly different from the frequency of this haplogroup in healthy present-day Thai population which is 43% (p value = 0.272). The other 5 and 8 patients are haplogroup B (5/40=12.5%) and B* (8/40=20%) respectively. The frequency of these two haplogroups in the LHON patients and normal control was not significantly different either (p value = 0.967 and 0.130 respectively). Thus, the mitochondrial DNA haplogroup distribution of our LHON patients was not significantly different from those of present-day Thai population (Table 1). Our result also showed that the G11778A mutation must have arisen in our population independently from this mutation in Caucasian.

Interestingly, there was one mitochondrial haplogroup, haplogroup F, which was found at a high frequency in present-day healthy Thai population but not in our LHON patients. Haplogroup F was found at 15% of our normal population but was found in none of our LHON patients, neither G11778A nor T14484C. We have also looked at mitochondrial DNA haplogroup in the other G11778A LHON patient who was Indian in origin and two of our T14484C LHON individuals. These patients were classified in haplogroup M, or B*, not haplogroup F. Only macrohaplogroup M, haplogroup B, B* and BM were reported in Asian LHON so far (Sudoyo et al., 2002; Nishioka et al., 2003). Until now there was no report of mitochondrial DNA haplogroup F in LHON patients of Asian origin. However, whether the mitochondrial haplogroup F carries the variant(s) that can negatively effect or prevent the occurrence of mutations in this disorder needs more cases and further investigation.

In conclusion, our study showed no association between specific mitochondrial DNA haplotype and the expression of G11778A mutation in our 40 LHON individuals. However, one of the major mtDNA haplogroup found in 15% of our normal population was not detected at all in our 41 LHON pedigrees with G11778A and 2 LHON pedigrees with T14484C. None of the previously reported Asian LHON belongs to this haplogroup.

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Table 1. The distribution of the mtDNA haplogroups in 100 normal controls (Luangtrakool K, personal communication) and 40 LHON patients determined by RFLP polymorphisms in this study.

Haplogroup	**N**	**A**	**B**	**B***	**F**	**M**	**C**	**D**	**G**	**Others**	**Unclassified**
Controls (N,%)	100	3 (3%)	11 (11%)	9 (9%)	15 (15%)	43 (43%)	1 (1%)	1 (1%)	1 (1%)	1 (1%)	15 (15%)
LHON (N,%)	40	0 (0%)	5 (12.5%)	8 (20%)	0 (0%)	22 (55%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (12.5%)
p-value	-	0.645	0.967	0.130	**0.022**	0.272	0.634	0.634	0.634	0.634	0.901