Sequence Analysis of the Mitochondrial Genomes from Dutch Pedigrees with Leber Hereditary Optic Neuropathy

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The complete mitochondrial DNA (mtDNA) sequences for 63 Dutch pedigrees with Leber hereditary optic neuropathy (LHON) were determined, 56 of which carried one of the classic LHON mutations at nucleotide (nt) 3460, 11778, or 14484. Analysis of these sequences indicated that there were several instances in which the mtDNAs were either identical or related by descent. The most striking example was a haplogroup J mtDNA that carried the 14484 LHON mutation. Four different but related mitochondrial genotypes were identified in seven of the Dutch pedigrees with LHON, including six of those described by van Senus. The control region of the founder sequence for these Dutch pedigrees with LHON matches the control-region sequence that Macmillan and colleagues identified in the founder mtDNA of French Canadian pedigrees with LHON. In addition, we obtained a perfect match between the Dutch 14484 founder sequence and the complete mtDNA sequences of two Canadian pedigrees with LHON. Those results indicate that these Dutch and French Canadian 14484 pedigrees with LHON share a common ancestor, that the single origin of the 14484 mutation in this megalineage occurred before the year 1600, and that there is a 14484/haplogroup J founder effect. We estimate that this lineage—including the 14484 LHON mutation—arose 900–1,800 years ago. Overall, the phylogenetic analyses of these mtDNA sequences conservatively indicate that a LHON mutation has arisen at least 42 times in the Dutch population. Finally, analysis of the mtDNA sequences from those pedigrees that did not carry classic LHON mutations suggested candidate pathogenic mutations at nts 9804, 13051, and 14325.

Introduction

Forty years ago, van Senus (1963) published his tour de force study of Leber hereditary optic neuropathy (LHON [MIM 535000]) in the Netherlands. One explicit purpose of his investigation was "The formation of archives containing all the data about the Leber patients and their families in the Netherlands. By this means, possibly future investigators may be able to build further on the material collected. In this way we hope to be able to collect material by means of which new light may be thrown on the hereditary problems of Leber's disease" (van Senus 1963, p. 1). Although it was not recognized at the time of van Senus's (1963) study, it is now understood that the unusual pattern of maternal inheritance in pedigrees with LHON reflects a complex etiology in which the primary

event is a mutation in the mitochondrial genome (reviewed by Howell 1997, 1998). Mutations at nucleotides (nts) 3460, 11778, and 14484, which occur in mitochondrial genes that encode subunits of respiratory chain complex I, account for ~95% of all pedigrees with LHON in populations of European descent (Mackey et al. 1996), although rare LHON mutations continue to be identified (e.g., see Chinnery et al. 2001; Brown et al. 2002; Valentino et al. 2002). The available evidence supports a complex I–mediated pathogenesis of LHON with apoptotic death of retinal ganglion cells and optic-nerve degeneration (Howell 1997, 1998; Carelli et al. 2002; Wong et al. 2002).

The Dutch pedigrees with LHON, subsequent to their analysis by van Senus (1963), have been used for a number of studies, including the identification of the primary LHON mutations, the correlation of those LHON mutations with the ophthalmological abnormalities, the evaluation of the role of secondary mtDNA polymorphisms in the etiology, and the testing of whether a simple X-linked modifier locus determines the prevalence of the optic neuropathy among males (Oostra et al. 1994*a*, 1994*b*, 1996; Mackey et al. 1996). It is the aim of the

analyses reported here to use the Dutch pedigrees with LHON for another type of mitochondrial genetic investigation.

In his extensive analysis of Dutch families with LHON, van Senus (1963) was able to "connect" many of the original set of 46 matrilineal pedigrees through extensive genealogical investigations, and he obtained a final total of 27 maternal lineages with LHON. In the present study, we have determined the complete mtDNA sequences for a total of 63 Dutch pedigrees with LHON. We then used these sequences to derive information about the origin of LHON mutations within the Netherlands, and we were able to connect a number of these matrilineal pedigrees. In addition, our results reveal an unanticipated "connection" between Dutch and French Canadian pedigrees with LHON, as well as other instances of pedigrees with LHON that are related by descent.

Material and Methods

DNA Samples and Numbering of Dutch Pedigrees with LHON

A total of 64 DNA samples from 63 different Dutch pedigrees with LHON, as well as 1 from an apparently sporadic case, were included in this study. Twenty-three of these samples were from the 27 pedigrees reported by van Senus (1963). Two of those pedigrees (van Senus numbers S002 and S024) had apparently died out by the early 1990s, and no mtDNA sequence information is available. Another pedigree (van Senus number S028) is now known to be affected with autosomal dominant optic atrophy rather than LHON, and this family has not been analyzed here. An additional five pedigrees with LHON were added by L. N. Went, and these were given van Senus numbers S040–S044. During the period from 1992 to 1995 at the Netherlands Ophthalmic Research Institute, additional patients with optic atrophy who were suspected to have LHON were assigned van Senus numbers S050 *et seq.* Unless noted otherwise, the information provided for the pedigrees analyzed here was that available in 1995. In addition, the mtDNA from a subject with an apparently sporadic case of bilateral optic atrophy has since been found to carry a LHON mutation, and it has been assigned a van Senus number of S999 in this study. There are additional patients with LHON who were identified after 1995, but they were not included in the present study.

As a general procedure for pedigrees S050 *et seq.*, the maternal genealogies of patients with LHON were traced back through five generations (i.e., the earliest known maternal ancestor would generally have been born between 1800 and 1850). If this pedigree did not show genealogical linkage to a previously identified Dutch pedigree with LHON, it was assigned a unique identifier number. Therefore, if the mtDNA sequencing results (see below) indicate coalescence of two or more pedigrees, it is reasonable to assume that their common maternal ancestor was born before this period.

DNA Sequencing

A description of our DNA sequencing approach has been published elsewhere (Herrnstadt et al. 2002). In brief, the entire mtDNA was PCR amplified as a set of 68 overlapping fragments, each ~550 bp in length. Sequencing reactions were performed with BigDye Terminator chemistry (PE Applied Biosystems), and electrophoresis and "base calling" were performed with a 3700 DNA Analyzer (PE Applied Biosystems). The PCR primers were designed to provide ~50% sequence overlap between adjacent segments of the mtDNA. In addition, both strands of the mtDNA were sequenced, so that each nucleotide position was sequenced up to a total of four times. The mtDNAs of all 63 pedigrees were assessed for heteroplasmy of the 3460, 11778, and 14484 LHON mutations by visual inspection of the sequencing electropherograms. This approach is sufficiently sensitive that we can reliably detect heteroplasmy if the minority allele is present in at least 15%–20% of the mtDNA molecules.

Efforts to ensure mtDNA sequence accuracy extended beyond the use overlapping primers. Thus, two samples from van Senus pedigree S022 were obtained and sequenced in a "blinded" fashion. The two mtDNA sequences matched perfectly. Furthermore, one sample was chosen for complete reanalysis, and—again—perfect agreement was obtained. In multiple instances, we found pedigrees with LHON whose mtDNA sequences differed at one or two sites. These sites were reanalyzed to verify the sequence difference. Finally, the 1.1-kb noncoding control-region sequences for 22 samples were independently obtained with a manual sequencing approach at a different site (University of Texas Medical Branch; see the article by Howell and Smejkal [2000] for methods). Perfect agreement between the two approaches was obtained.

mtDNA sequences are presented as differences from the revised Cambridge reference sequence (Andrews et al. 1999). Only single-base-pair substitutions are reported here, and we did not analyze expansions/contractions of simple repeat sequences. The mtDNA sequences of the Dutch pedigrees with LHON are provided in a supplementary table that can be downloaded from the MitoKor Web site.

Results

Preliminary Analysis

We determined the complete mtDNA sequences for 63 Dutch pedigrees with LHON. It was found that 7 of

these did not carry one of the three "classic" LHON mutations, whereas 56 (89%) carried a LHON mutation at nt 3460, 11778, or 14484. A breakdown of these pedigrees by LHON mutation and by European haplogroup is given in table 1. The relative prevalence of the three LHON mutations is in accord with previous studies of pedigrees with LHON in populations of European descent (e.g., Mackey et al. 1996). The 11778 mutation is the most prevalent and was found in ~60% of the Dutch pedigrees that carried a classic LHON mutation. The 3460 and 14484 mutations each occur in ~20% of these families. In contrast to the 3460 and 11778 pedigrees with LHON, the mtDNAs from the 14484 Dutch pedigrees with LHON show a preferential association with haplogroup J (10 of 12, or ~80%), a result that has been reported for other European and North American populations (Brown et al. 1997; Torroni et al. 1997).

In five of the pedigrees (S061J/11778, S064H/3460, S073H/11778, S083H/3460, and S188H/3460), the family member analyzed was heteroplasmic for the LHON mutation. This heteroplasmy suggests a relatively recent origin of the LHON mutation, and it is therefore not surprising that none of these pedigrees were those assembled by van Senus (1963), all of which involve maternal ancestors that can be traced to the 17th or early 18th centuries (see his fig. 7).

14484 Pedigrees with LHON

There were 10 14484/haplogroup J and 2 14484/haplogroup K Dutch families with LHON (table 1). However, detailed analysis indicated that these 12 families actually represent no more than four independent mtDNA lineages. The most striking finding was that the mtDNA sequences of seven of the 14484/haplogroup J families with LHON form a group of four closely related genotypes. In his genealogical analysis of the original 46 pedigrees, van Senus (1963) found that 10 pedigrees could be traced back to a common female ancestor who was born sometime around 1613 in the town of Rhenen (see his fig. 7), and this constituted his largest pedigree (S001). Our results show that the mtDNA of the S008

family with LHON has a sequence identical to that of S001. Pedigree S008 was traced to a woman born in 1690 in Utrecht, a city that is ~35 km from Rhenen. The perfect identity of the mtDNA sequences for these two pedigrees and the close geographical proximity of the last known maternal founders, indicate a single matrilineal pedigree with LHON that accounts for a substantial proportion of the family members with LHON in the Netherlands. However, the situation was more complicated than this, and the mtDNAs of five other families with LHON were very similar to that of the S001/S008 lineage.

Pedigree S005 was traced genealogically to a woman born in ~1655 in Papendrecht. In addition, the mtDNA sequences of families S011 and S079 are identical to that of S005. Family S011 could be traced to a woman born in ~1636 in Rotterdam (van Senus 1963), whereas pedigree S079 is a small family with four affected members. Moreover, the mtDNA sequences from these three pedigrees are identical to that of "superpedigree" S001/S008, except for the absence of the nonsynonymous polymorphism at nt 9682 in the COX3 gene (data summarized in fig. 1). It was also observed that the mtDNA from van Senus pedigree S023 was identical to the mtDNA from van Senus pedigrees S005/S011/S079, except for the presence of a polymorphism at nt 195 in the noncoding control region or D-loop (table 1). Pedigree S023 was traced by van Senus (1963) to a woman born in the year ~1745 in Rhenen; that is, the mtDNAs from pedigrees S001/S008 and from pedigree S023 differ at two nucleotide positions but trace genealogically to the same small town in the Netherlands. Finally, the mtDNA sequence of family S013 differed from that of S005/S011/S079 at two sites, one in the coding region (nt 15514) and one in the noncoding control region (nt 16189). The members of family S013 could be traced to a woman born in ~1678 in Almkerk. The six families with LHON in this group that were studied by van Senus (1963) thus originate within 75 km of one another (see his fig. 7).

The most parsimonious explanation for the close similarity of these mtDNA sequences is that they are related

Table 1

LHON Mutations and mtDNA Haplogroups in Dutch Pedigrees

Mutation	N	Pedigrees (Haplogroup)
3460	11	S017 (H), S063 (H), S064 (H), S069 (H), S083 (H), S166 (H), S188 (H), S016 (J), S027 (J), S196 (J), and S044 (K)
11778	33	S003 (H), S004 (H), S009 (H), S010 (H), S012 (H), S014 (H), S015 (H), S022 (H), S025 (H), S040 (H), S060 (H), S062 (H), S065 (H), S073 (H), S080 (H), S130 (H), S136 (H), S138 (H), S007 (U), S042 (U), S066 (U), S072 (U), S020 (T), S041 (T), S050 (T), S055 (T), S172 (T), S192 (T), S189 (V), S026 (J), S061 (J), S999 (J), and S067 (K)
14484	12	S001 (J), S005 (J), S008 (J), S011 (J), S013 (J), S019 (J), S023 (J), S056 (J), S068 (J), S079 (J), S081 (K), and S088 (K)
None[a]	7	S006 (H), S051 (H), S077 (H), S078 (H), S092 (H), S089 (T), and S084 (J)

[a] "None" indicates that the 3460, 11778, and 14484 mutations are not present; our analyses indicate that some of these pedigrees carry LHON mutations at other sites.
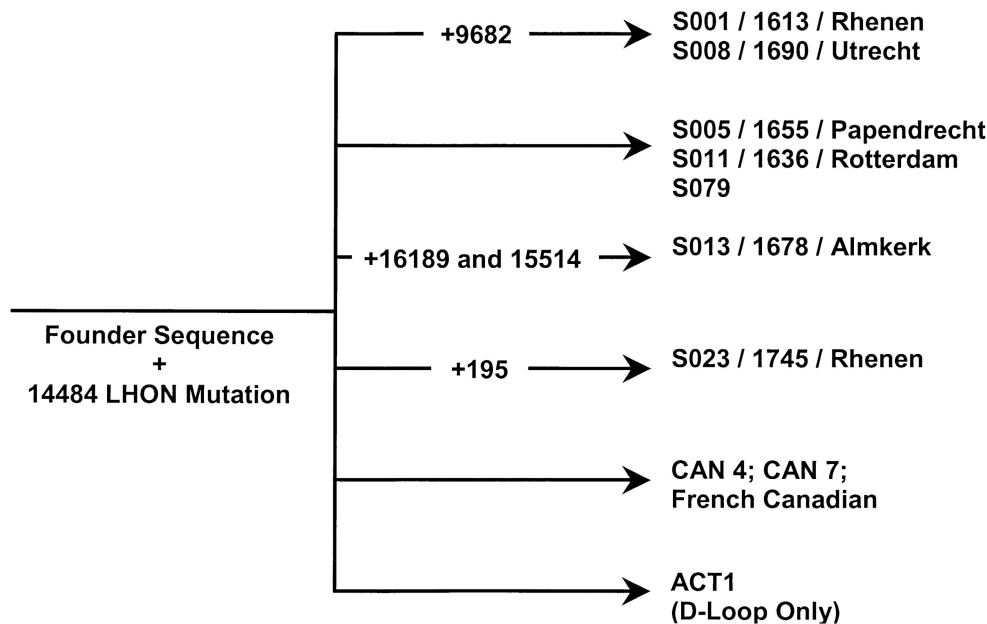
Figure 1   Simplified cladogram showing the relationship, at the level of mtDNA sequences, among a cluster of 14484 pedigrees with LHON. The founder sequence, shown in terms of sequence differences from the revised Cambridge reference sequence, is listed in a table available from the MitoKor Web site as that of Dutch LHON pedigrees S005, S011, and S079.

by descent to a common ancestral sequence, and a simplified cladogram is shown in figure 1. In this scheme, we envision that the maternal founder carried the 14484 LHON mutation within a haplotype that we designate the "founder" sequence. This founder sequence was transmitted, without further sequence changes, to the maternal descendants that comprise pedigrees S005/S011/S079. The mtDNA sequence in the branch that leads to van Senus (1963) pedigree S023 gained the polymorphism at nt 195; the third branch, leading to pedigrees S001/S008, gained the nt 9682 polymorphism; and the fourth branch, leading to family S013, gained two sequence changes.

The evolutionary scenario shown in figure 1—specifically, the single origin of the 14484 LHON mutation in these six pedigrees—is supported by other results. We note, however, that it is extremely difficult to prove the single occurrence of an mtDNA mutation in two closely related sequences. The probability of a single mutation is a complex function of multiple factors, including the frequency of the LHON mutation and the frequency of the background mtDNA type in the population. As an extreme example, if the population contains only a single mtDNA subhaplogroup, then it would be virtually impossible to determine whether only a single LHON mutation had occurred or multiple mutations had arisen.

We have begun an extensive phylogenetic analysis of haplogroup J mtDNAs, and the first results have been published (Herrnstadt et al. 2002). Of ~100 haplogroup

J mtDNAs in our current database, there are a total of 16 that belong to the subcluster defined by the presence of a polymorphism at nt 3394 (data not shown). Of these 16 mtDNA sequences, 7 represent this cluster of Dutch pedigrees with LHON. Another three sequences also carried the 14484 LHON mutation (the two Canadian families with LHON described below and the VIC11 Australian family with LHON that can be traced back to Ireland), whereas two carried the 11778 LHON mutation. Of the remaining four non-LHON mtDNA sequences, the one that was most similar to the founder sequence differed at one position in the coding region (not counting the LHON mutation) and at four positions in the control region (data not shown). That is, there is no evidence that the founder sequence lacking the 14484 LHON mutation occurs in a measurable proportion of haplogroup J mtDNAs, and it may be unique in the human population.

The VIC11 mtDNA sequence, relative to the Dutch 14484 founder sequence (S005/S011/S079), differed at one site in the coding region and at three sites in the control region. We cannot rule out that VIC11 is an "ancient" member of the Dutch LHON "superlineage," but this is unlikely for the following reason. If we take the four non-LHON sequences and the two 11778 LHON mtDNAs in this subclade of haplogroup J, the mean pairwise difference between any two of them is 6.7 substitutions. In contrast, the value for the six Dutch 14484 mtDNAs is only 1.5 substitutions (the difference

between the two distributions is highly significant; $P < .001$). In other words, the Dutch 14484 mtDNAs are much more closely related than a random set of mtDNAs from this haplogroup J subclade. The four differences between the VIC11 sequence and that of the Dutch founder sequence more likely indicate an independent origin of the 14484 mutation in the former. More importantly, these results support the evolutionary model in figure 1, in which there has been a single occurrence of the 14484 LHON mutation within the Dutch megalineage.

The mtDNA sequences of 14484/haplogroup J pedigrees S019 and S056 were identical, but this sequence clearly differed from that of the "superlineage." No genealogical connection between these pedigrees has been established. Pedigree S019 was traced to a woman born ~1657 in Klein-Ammers, whereas S056 is a small family with 10 affected members. In contrast, the sequence of the S068 pedigree was clearly different from the other two groups of genotypes. There were 17 site differences between the founder sequence (S005/S011/S079) and the S019/S056 sequence, whereas there were 25 between S005/S011/S079 and S068 and 30 site differences between S019/S079 and S068. Therefore, one may safely conclude that the 14484 LHON mutation occurred independently in these three groups of sequences. As a corollary, the 10 14484/haplogroup J Dutch families with LHON represent only *three* origins of the LHON mutation.

It is our intention to analyze a large collection of pedigrees with LHON from other countries to determine whether there have been other such founder events. A comprehensive comparison of the mtDNA sequences from the Dutch pedigrees with LHON to those from other countries is underway. For example, one of the Australian 14484 pedigrees with LHON (designated "ACT1") has a perfect sequence match for the control region to that of the Dutch founder sequence; a partial sequence of the coding region was also identical. This finding was not surprising, because the members of this family are recent immigrants from the Netherlands, and the available genealogical information indicates linkage to Dutch pedigree S001 (D.A.M., unpublished data). As a final piece of evidence, we have determined that the ACT1 mtDNA sequence carries the substitution at nt 9682 that is a specific marker for the S001/S008 lineage (fig. 1).

Thus far, we have not yet "connected" the large cluster of 14484 Dutch LHON mtDNA sequences to any of those from the United Kingdom (data not shown), but an analysis of the available sequence information for French Canadian families with LHON revealed a striking and unanticipated result. Macmillan et al. (2000) have shown that the predominance of the 14484 LHON mutation in French Canadian families is due to a strong founder effect. They reported that 26 of 27 14484 families carried mtDNA with an identical sequence in the noncoding control region and carried the following polymorphisms: C16069T, T16126C, G16213A, A73G, G185A, G228A, A263G, and C295T. This is the same control-region sequence that occurs in the van Senus (1963) S001, S005, S011, S008, and S079 pedigrees. The French Canadian and Dutch mtDNAs also share the 3394 polymorphism that signifies a subcluster of haplogroup J (Macmillan et al. 2000; see also the haplogroup J network in fig. 4 of the article by Herrnstadt et al. [2002]). We have obtained additional evidence for a connection between Dutch and the French Canadian families with LHON, from mtDNA sequence analyses of two apparently unrelated 14484 families with LHON, designated "CAN4" and "CAN7," that were referred to us by physicians in Ontario and in British Columbia, respectively. The complete mtDNA sequence from both probands was identical to that of the Dutch LHON founder sequence. Furthermore, the family with LHON from British Columbia also has a genealogical connection to the French Canadian population. The mother of the affected CAN7 proband has a sister who lives in Montreal and who also has a visually affected son. These results indicate that the cluster of Dutch families with LHON and the cohort of French Canadian families with LHON share the same founder sequence.

The French Canadian population has attracted a great deal of interest, because of the numerous instances of founder effects for rare inherited disorders (de Braekeleer and Dao 1994; Heyer and Tremblay 1995; Heyer et al. 1997; Labuda et al. 1997). It has been estimated that the French Canadian population of Quebec descends from ~1,600 women and ~6,850 men who settled in this region during the 17th century, and it is not surprising that there would be a particularly strong founder effect for LHON, a maternally transmitted disorder. Analysis indicated that >90% of the founding population in this region of Canada came from France, particularly from the northwestern region of that country (e.g., see table 2 of Heyer et al. [1997]). Although the French Canadian and the Dutch 14484 pedigrees with LHON are "branches" of the same lineage, the geographical origin of the common maternal ancestor can only be the subject of speculation at the present time. One clear implication of this model, however, is that the 14484 LHON mutation must have arisen before the year 1600. On the basis of the two coding-region substitutions that have arisen in the Dutch/French Canadian LHON lineage and with a pedigree rate of coding-region divergence of 0.15 mutations/bp/million years in families with LHON (Howell et al. 2003), the "age" of this lineage is ~900 years. Alternative calculations with a control-region LHON pedigree divergence rate of 1.0 mutation/bp/million years (Howell et al. 2003) and with the observation of two control-region substitutions in

this lineage (fig. 1) provide an estimated age of ~1,800 years. In other words, this lineage and the 14484 LHON mutation appear to have originated 500–1,400 years before the maternal ancestor of the Dutch pedigrees with LHON was born. In view of the uncertainty in these divergence-rate estimates, the twofold disparity in calculated age of the lineage is not surprising.

In addition to the 14484/haplogroup J Dutch families with LHON, there were two 14484/haplogroup K pedigrees (table 1). These two sequences are identical except for the presence of a substitution at nt 16293 (control region) in the mtDNA of the S081 pedigree, which was lacking in the S088 pedigree. Both pedigrees are small, with two and one affected family members, respectively. In view of the paucity of 14484/haplogroup K pedigrees with LHON and the shared presence of rare substitutions at nts 1694, 12795, and 16400 in the two mtDNAs, we conclude that these two pedigrees with LHON arose from a common maternal ancestor who carried the 14484 mutation. This conclusion is further supported by the genealogical information. Pedigree S081 can be traced to a woman born in ~1760 in Leeuwarden. The members of pedigree S088 currently live Groningen, which is ~40 miles from Leeuwarden. However, the genealogy can be traced to a woman born in ~1815 in Leeuwarden itself. Although a definitive genealogical linkage between the two pedigrees has not yet been established, their common origin is beyond question in our view.

3460 LHON mtDNAs

A total of 11 Dutch pedigrees carry the LHON mutation at nt 3460 (table 1). The haplogroup distribution comprises one haplogroup K, three haplogroup J, and seven haplogroup H mtDNAs. There is no evidence that any of these mtDNAs are related at the sequence level. For example, the average number of pairwise site differences between 3460/haplogroup H mtDNAs is 10.5, and the smallest number of site differences is 5. In a similar fashion, the 3460/haplogroup J mtDNAs differ from one another at 16–29 sites. Therefore, these 11 pedigrees with LHON, in all likelihood, represent independent origins of the 3460 mutation.

11778 LHON mtDNAs

The 11778 mtDNAs from the 33 Dutch pedigrees with LHON are distributed among six different haplogroups, and more than half of these pedigrees (18 of 33, or 55%) are members of haplogroup H, the most common among European populations (e.g., Brown et al. 1997; Torroni et al. 1997). Inspection of these 18 haplogroup H mtDNAs revealed that four of the pedigrees had identical sequences (S014, S060, S062, and S080), with the exception that the mtDNA from pedigree S014 lacked the private polymorphism at nt 9750. Pedigree S014 was traced to a woman born ~1633 in Sprundel (van Senus 1963). Pedigrees S060, S062, and S080 are relatively small, with two or three affected males each and no affected females. These pedigrees can be traced to the same subregion of the Netherlands, but no closer geographical or genealogical relationship can be established.

There was an initial concern that haplogroup H mtDNAs may not have diverged to a degree sufficient to allow firm conclusions about shared ancestry. However, when we analyzed the 15 mtDNA sequences ("counting" the S014/S060/S062/S080 sequence only once), the average number of site differences between any two of the mtDNAs was 11.1 (similar to the average for the 3460/haplogroup H mtDNAs), with 3 being the smallest number of differences. Therefore, the probability of the 11778 mutation arising separately in four founders within the same haplogroup H mtDNA sequence is vanishingly small, and we conclude that there has been a single 11778 maternal ancestor to these four pedigrees. We also note that the mtDNA sequences of the S003 and S136 pedigrees differ at three sites in the control region. Because these two mtDNAs are so similar, relative to the average divergence between any two of these haplogroup H mtDNAs, the question of a single origin of the LHON mutation in these two families must be considered. These two LHON mtDNAs both carry a rare polymorphism at nt 3333 in the coding region, a finding that suggests that these sequences are members of the same small subclade within haplogroup H. We have found two non-LHON haplogroup H mtDNAs in the MitoKor database that carry the same polymorphism, and these two mtDNAs differ from each other at two sites in the control region and at one site in the coding region (data not shown). Thus, when we consider the specific subclade of haplogroup H rather than the haplogroup as a whole, the number of sequence differences between the S003 and S136 mtDNAs is typical for members of the subclade. Independent origins of the LHON mutations thus remains the most likely explanation. It is noteworthy, in comparison to the cluster of 14484 sequences (fig. 1), that this is an example of how analysis of closely related mtDNA sequences supports independent origins of the LHON mutation.

It was also found that the mtDNA sequences of the S007 and S066 11778/haplogroup U pedigrees with LHON were identical. The S007 pedigree was traced to a woman born in ~1631 in Groningen (van Senus 1963). Pedigree S066 is very small, with only two affected family members, and it has been traced back no further at this point than a woman born in ~1843 in Leiden. In addition, the 11778/haplogroup J mtDNA sequences from the S026 pedigree and from patient S999 are identical. Pedigree S026 could be traced to a woman born ~1736 in Wijnaldum (van Senus 1963), whereas patient

S999 had no known family history of optic neuropathy and was initially thought to have LHON of sporadic origin.

There were no other instances of shared ancestry among Dutch pedigrees with LHON that could be identified among the remaining 11778 mtDNA sequences. For example, the six 11778/haplogroup T mtDNAs are sufficiently diverged (an average of 13.8 site differences between a pair of sequences) that independent origins of the LHON mutation is the only reasonable conclusion.

Dutch Pedigrees with LHON That Lack One of the Three Classic LHON Mutations

A total of seven Dutch pedigrees with LHON lack the classic LHON mutations at nts 3460, 11778, and 14484: S006, S051, S077, S078, S084, S089, and S092. We inspected the electropherograms to ensure that a heteroplasmic LHON mutation had not been missed by the sequence analysis software.

Pedigree S006 was traced to a woman born ~1679 in Nootdorp (van Senus 1963). In contrast to the other Dutch pedigrees with LHON, the maternally related members of this family were affected by severe neurological abnormalities, particularly dystonia, in addition to the optic neuropathy. De Vries et al. (1996) have sequenced parts of the mtDNA of this pedigree, in an effort to identify the pathogenic mtDNA mutations. They identified a heteroplasmic mutation at nt 11696 and a homoplasmic mutation at nt 14596, both of which alter the amino acid sequence of respiratory chain complex I subunits. It was not clear which of these mutations caused the clinical abnormalities in this family or if they both did. Inspection of the complete mtDNA sequence (see table 1 at the MitoKor Web site) does not reveal any other candidate mutations. The 11696 sequence change also occurs in the S061 11778 pedigree with LHON, in which only the typical optic neuropathy occurs and in which the mutation is homoplasmic. Furthermore, this homoplasmic substitution also occurs in two unrelated but clinically normal individuals whose mtDNA sequences are in the MitoKor database (>1,200 complete mtDNA sequences; data not shown). Overall, these results suggest that the nt 11696 mutation may not be pathogenic.

The other six pedigrees were classified as having LHON on the basis of the optic neuropathy, and it is thus possible that some of these cases do not involve a pathogenic mtDNA mutation. For multiple reasons, it is difficult to identify with confidence rare LHON mutations (Howell 1994), but one reasonable criterion is to consider only those candidate mutations that alter subunits of respiratory chain complex I. For example, the complete mtDNA sequences from S078 and S084 do not harbor substitutions that can be pathogenic; that is, the mtDNA carries only polymorphic changes that have been observed in European population surveys and that are haplogroup-associated (Herrnstadt et al. 2002). In addition, both S078 and S084 are pedigrees with only single affected individuals, and we conclude that these are not true LHON pedigrees. In a similar fashion, pedigree S089 carries a polymorphism at nt 3338 that results in the substitution of Ala for Val at position 11 of the ND1 subunit of complex I. This is a poorly conserved region of the protein; it is unlikely that the nt 3338 polymorphism is pathogenic.

The S077 mtDNA carries a transition at nt 14325, which changes the Asn residue at position 117 of the ND6 subunit to Asp. This sequence change does not occur in any of the >1,200 mtDNA sequences in the MitoKor database. LHON mutations in the ND6 gene are predicted to localize to a specific region (see fig. 4 of the article by Chinnery et al. [2001]). The Asn residue at position 117 does not map to this "pathogenic" domain. Furthermore, the S077 pedigree contains only a single affected individual. The 14325 substitution is a candidate LHON mutation, but the available evidence is weak at this point. Pedigree S051, which includes two affected brothers and their visually affected maternal aunt, carries a mutation at nt 13051 that results in the substitution of Ser for Gly at position 239 in the ND5 subunit and that does not occur among the other >1,200 mtDNA sequences in the MitoKor database. Mutations at nts 13513 and 13514 cause an LHON/MELAS (mitochondrial myopathy, encephalopathy, lactacidosis, and stroke) overlap syndrome (see, e.g., Corona et al. 2001). More recently, Liolitsa et al. (2003) identified a MELAS mutation at nt 13045, which changes the Met residue at position 237 to Leu. They noted that this patient was also affected with an LHON-like optic neuropathy. The proximity of the nt 13045 and nt 13051 mutations is striking, as is the stringent evolutionary conservation of this region of the ND5 subunit (see fig. 3 of the article by Liolitsa et al. [2003]).

The mtDNA from pedigree S092 did not carry any suspect substitutions in the genes that encode complex I subunits, but it did have a candidate mutation at nt 9804 that results in a substitution of Thr for the Ala at position 200 of the CO3 subunit of cytochrome oxidase. Johns and Neufeld (1993) were the first to propose that this mutation causes LHON. In our previous analyses, the nt 9804 mutation appeared as a polymorphism associated with a small cluster of haplogroup H sequences (see fig. 3 of the article by Herrnstadt et al. [2002]), including the S166 3460 pedigree with LHON. In the MitoKor database, the nt 9804 mutation has been found in five other haplogroup H mtDNAs, none of which is from a clinically normal individual. These five patients included two patients with Alzheimer disease, two with

insulin-dependent diabetes, and an individual affected with progressive supranuclear palsy. In addition, we have also observed the mutation in a patient with optic atrophy (data not shown). In contrast to the other mtDNAs, that from this last patient belongs to African haplogroup L2b. Therefore, this nt 9804 mutation represents an independent mutational event that also results in an optic nerve disorder. Taken together, these results suggest that the nt 9804 mutation may be pathogenic, but the diversity of associated clinical abnormalities confounds any simple interpretation at this time.

Discussion

The major finding from this study is that there was a founder event for the Dutch 14484 pedigrees with LHON, and we were able to "link up" seven of the Dutch pedigrees with LHON that had been first assembled by van Senus (1963). Some of these pedigrees have accumulated additional sequence changes, a phenomenon that has been observed elsewhere (e.g., Heyer et al. 2001; Howell et al. 2003). Furthermore, the founder mtDNA for the Dutch 14484 pedigrees with LHON was also identified as the source of the founder effect previously observed for French Canadian pedigrees with LHON (Macmillan et al. 2000). Because the maternal origin of the van Senus S001 pedigree can be traced to ~1613 and because French Canadian settlement first dates to the early 17th century, the 14484 LHON mutation arose some time before 1600. We estimate here that the mutation arose 900–1,800 years ago. The geographical origin of the common 14484 mutational event is not known, but it is hoped that these results will catalyze further studies of the population history of LHON mutations.

There were five other instances in which we could identify a relation by descent among different Dutch pedigrees with LHON. Taking these results into consideration, we make the conservative estimate that one of the three classical LHON mutations has arisen 42 times in the Dutch population. In other words, our sequencing analyses identify a total of 42 different mtDNA lineages that carry an LHON mutation at nt 3460, 11778, or 14484. In these pedigrees, the genealogical studies of van Senus (1963) could trace a maternal ancestor to the 17th or early 18th centuries, a result that suggests an even earlier origin of the LHON mutation for these Dutch pedigrees with LHON.

Of the six instances in which we could connect families with LHON through their mtDNA sequences, three involve additional substitutions that have arisen in a branch of the lineage. That result supports our previous suggestion that divergence in the mtDNA coding region is substantially higher at the level of the pedigree than the divergence rate estimated with phylogenetic or population-based approaches (Howell et al. 1996, 2003).

Previous investigations have identified an association of the 14484 LHON mutation with haplogroup J, and this association was interpreted to mean that there was a higher penetrance of the mutation when it arose in this mtDNA background (Brown et al. 1997; Torroni et al. 1997). It is worthwhile to consider whether the strong founder effects in the Dutch and French Canadian populations have contributed to this identification of this association. The prevalence of the 14484 LHON mutation is actually quite low in Denmark, Finland, and the United Kingdom (e.g., see table 1 of the article by Mackey et al. [1996]) and is apparently low in the Italian population (Torroni et al. [1997] identify two families). It appears that the 14484/haplogroup J association has relied heavily on analysis of families with LHON from North America (e.g., Johns et al. 1993; Brown et al. 1995). We therefore considered the possibility that the 14484/haplogroup J association detected in North American families with LHON may have been inflated by "overcounting" pedigrees that carry the Dutch/French Canadian founder sequence (e.g., see Johns et al. 1993; Brown et al. 1995). However, Brown et al. (2002) performed a phylogenetic analysis of mtDNA sequences from 18 haplogroup J pedigrees with LHON (see their fig. 3). Only two of the seven 14484 mtDNAs belong to the 3394 subclade, thus setting an upper limit on bias due to Dutch LHON founder effects.

As noted above, we are analyzing the LHON mtDNA sequences from a number of populations of European descent. Although these analyses are not yet complete, our results for the Australian population are relevant. Thus far, we have mtDNA sequences for 46 Australian pedigrees with LHON, 10 (22%) of which carry the 14484 mutation (1 of these 10 also carries the 11778 LHON mutation; Howell et al. 2002). Of these 10, 4 (40%) mtDNAs belong to haplogroup J, and the LHON mutations represent independent events (N.H. and D.A.M., unpublished data), a proportion that is lower than the reported association of ~75%. In summary, these various results indicate that there is a positive association of the 14484 mutation with haplogroup J, although the association may not be as marked as observed in the initial studies. Although the Dutch LHON founder sequence may have caused some overestimation of the association, the relatively large number of independent 14484 mutational events in different populations of European descent provides convincing evidence that there is a positive haplogroup J association with the 14484 mutation.

Of additional interest, only 1 (10%) of the 10 Australian 14484 mtDNAs belongs to haplogroup H, a result that agrees with results from the Dutch pedigrees

(table 1). Therefore, our results indicate that the penetrance of the 14484 LHON mutation is lowered in a haplogroup H background (see the article by Howell et al. [in press] for details). That is, there also appears to be a negative association of this LHON mutation with haplogroup H.

A 11778/haplogroup J association has also been suggested (Brown et al. 1997; Torroni et al. 1997), and our preliminary results indicate such an association among the Australian pedigrees with LHON (N.H. and D.A.M., unpublished data). However, there is no such association among the Dutch pedigrees with LHON, and only ~10% of these pedigrees carry a haplogroup J mtDNA (table 1), a value in accord with the frequency of this haplogroup in the European population. More recently, Brown et al. (2002) have found an association between a LHON mutation at nt 10663 and haplogroup J, although none of the Dutch pedigrees with LHON carry this mutation. How the mtDNA background determines or modifies the phenotypic and clinical expression of pathogenic mutations is just one of the questions that further analysis of pedigrees with LHON will help to answer. There is much to be gained from the meta-analysis of large numbers of pedigrees for which mtDNA sequence information is available.

Note added in proof.— Since the submission and review of this article, we have completed the sequencing of the mtDNAs from pedigrees S018 and S021, both of which carry the 11778 LHON mutation. The mtDNA from pedigree S018 does not match that from any other Dutch pedigree with LHON. The mtDNA from pedigree S021 (haplogroup T) matches perfectly that from pedigree S041. Pedigree S021 was traced to a woman born in ~1724 in Scharmer (van Senus 1963), whereas pedigree S041 can be traced to a woman born in 1749 in Spijk, a town that is ~35 km from Scharmer. These sequences have been included in the Dutch LHON database.

Acknowledgments

Electronic-Database Information

The URLs for data presented herein are as follows:

MitoKor Web site, http://www.mitokor.com/science/ dutchLHONsequences.php (for the mtDNA sequences of the Dutch pedigrees with LHON)

Online Mendelian Inheritance in Man (OMIM), http://www .ncbi.nlm.nih.gov/Omim/ (for LHON)

References

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 23:147

Brown MD, Starikovskaya E, Derbeneva O, Hosseini S, Allen JC, Mikhailovskaya IE, Sukernik RI, Wallace DC (2002) The role of mtDNA background in disease expression: a new primary LHON mutation associated with Western Eurasian haplogroup J. Hum Genet 110:130–138

Brown MD, Sun F, Wallace DC (1997) Clustering of Caucasian Leber hereditary optic neuropathy patients containing the 11778 and 14484 mutations on an mtDNA lineage. Am J Hum Genet 60:381–387

Brown MD, Torroni A, Reckord CL, Wallace DC (1995) Phylogenetic analysis of Leber's hereditary optic neuropathy mitochondrial DNA's indicates multiple independent occurrences of the common mutations. Hum Mutat 6:311–325

Carelli V, Ross-Cisneros FN, Sadun AA (2002) Optic nerve degeneration and mitochondrial dysfunction: genetic and acquired optic neuropathies. Neurochem Int 40:573–584

Chinnery PF, Brown DT, Andrews RM, Singh-Kler R, Riordan-Eva P, Lindley J, Appelgarth DA, Turnbull DM, Howell N (2001) The mitochondrial *ND6* gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy. Brain 124:209–218

Corona P, Antozzi C, Carrara F, D'Incerti L, Lamantea E, Tiranti V, Zeviani M (2001) A novel mtDNA mutation in the ND5 subunit of complex I in two MELAS patients. Ann Neurol 49:106–110

De Braekeleer M, Dao T-N (1994) Hereditary disorders in the French Canadian population of Quebec. I. In search of founders. Hum Biol 66:205–223

De Vries DD, Went LN, Bruyn GW, Scholte HR, Hofstra RMW, Bolhuis PA, van Oost BA (1996) Genetic and biochemical impairment of mitochondrial complex I activity in a family with Leber hereditary optic neuropathy and hereditary spastic dystonia. Am J Hum Genet 58:703–711

Herrnstadt C, Elson JL, Fahy E, Preston G, Turnbull DM, Anderson C, Ghosh SS, Olefsky JM, Beal MF, Davis RE, Howell N (2002) Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups. Am J Hum Genet 70:1152–1171

Heyer E, Tremblay M (1995) Variability of the genetic contribution of Quebec population founders associated to some deleterious genes. Am J Hum Genet 56:970–978

Heyer E, Tremblay M, Desjardins B (1997) Seventeenth-century European origins of hereditary diseases in the Saguenay population (Quebec, Canada). Hum Biol 69:209–225

Heyer E, Zietkiewicz E, Rochowski A, Yotova V, Puymirat J, Labuda D (2001) Phylogenetic and familial estimates of mitochondrial substitution rates: study of control region mutations in deep-rooting pedigrees. Am J Hum Genet 69:1113–1126

Howell N (1994) Mitochondrial gene mutations and human diseases: a prolegomenon. Am J Hum Genet 55:219–224

——— (1997) Leber hereditary optic neuropathy: mitochondrial mutations and degeneration of the optic nerve. Vision Res 37:3495–3507

——— (1998) Leber hereditary optic neuropathy: respiratory chain dysfunction and degeneration of the optic nerve. Vision Res 38:1495–1504

Howell N, Herrnstadt C, Shults C, Mackey DA. Low penetrance of the 14484 LHON mutation when it arises in a non-haplogroup J mtDNA background. Am J Med Genet (in press)

Howell N, Kubacka I, Mackey DA (1996) How rapidly does the human mitochondrial genome evolve? Am J Hum Genet 59:501–509

Howell N, Miller NR, Mackey DA, Arnold A, Herrnstadt C, Williams IM, Kubacka I (2002) Lightning strikes twice: Leber hereditary optic neuropathy families with two pathogenic mtDNA mutations. J Neuroophthalmol 22:262–269

Howell N, Smejkal CB (2000) Persistent heteroplasmy of a mutation in the human mtDNA control region: hypermutation as an apparent consequence of simple-repeat expansion/contraction. Am J Hum Genet 66:1589–1598

Howell N, Smejkal CB, Mackey DA, Chinnery PF, Turnbull DM, Herrnstadt C (2003) The pedigree rate of sequence divergence in the human mitochondrial genome: there is a difference between phylogenetic and pedigree rates. Am J Hum Genet 72:659–670

Johns DR, Heher KL, Miller NR, Smith KH (1993) Leber's hereditary optic neuropathy: clinical manifestations of the 14484 mutation. Arch Ophthalmol 111:495–498

Johns DR, Neufeld MJ (1993) Cytochrome c oxidase mutations in Leber hereditary optic neuropathy. Biochem Biophys Res Comm 196:810–815

Labuda D, Zietkiewicz E, Labuda M (1997) The genetic clock and the age of the founder effect in growing populations: a lesson from French Canadians and Ashkenazim. Am J Hum Genet 61:768–771

Liolitsa D, Rahman S, Benton S, Carr LJ, Hanna MG (2003) Is the mitochondrial complex I ND5 gene a hot-spot for MELAS causing mutations? Ann Neurol 53:128–132

Mackey DA, Oostra R-J, Rosenberg T, Nikoskelainen E, Bronte-Stewart J, Poulton J, Harding AE, Govan G, Bolhuis PA, Norby S, Bleeker-Wagemakers EM, Savontaus M-L, Chan C, Howell N (1996) Primary pathogenic mitochondrial DNA mutations in multigeneration pedigrees with Leber hereditary optic neuropathy. Am J Hum Genet 59:481–485

Macmillan C, Johns TA, Fu K, Shoubridge EA (2000) Predominance of the T14484C mutation in French-Canadian families with Leber hereditary optic neuropathy is due to a founder effect. Am J Hum Genet 66:332–335

Oostra R-J, Bolhuis PA, Wijburg FA, Zorn-Ende G, Bleeker-Wagemakers EM (1994a) Leber's hereditary optic neuropathy: correlations between mitochondrial genotype and visual outcome. J Med Genet 31:280–286

Oostra R-J, Bolhuis PA, Zorn-Ende I, de Kok-Nazaruk MM, Bleeker-Wagemakers EM (1994b) Leber's hereditary optic neuropathy: no significant evidence for primary and secondary pathogenicity of the 15257 mutation. Hum Genet 94:265–270

Oostra R-J, Kemp S, Bolhuis PA, Bleeker-Wagemakers EM (1996) No evidence for "skewed" inactivation of the X-chromosome as cause of Leber's hereditary optic neuropathy in female carriers. Hum Genet 97:500–505

Torroni A, Petrozzi M, D'Urbano L, Sellitto D, Zeviani M, Carrara F, Carducci C, Leuzzi V, Carelli V, Barboni P, De Negri A, Scozzari R (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing penetrance of the primary mutations 11778 and 14484. Am J Hum Genet 60:1107–1121

Valentino ML, Avoni P, Barboni P, Pallotti F, Rengo C, Torroni A, Bellan M, Baruzzi A, Carelli V (2002) Mitochondrial DNA nucleotide changes C14482G and C14482A in the ND6 gene are pathogenic for Leber's hereditary optic neuropathy. Ann Neurol 51:774–778

van Senus AHC (1963) Leber's disease in the Netherlands. Doc Ophthalmol 17:1–163

Wong A, Cavelier L, Collins-Schramm HE, Seldin MF, McGrogan M, Savontaus M-L, Cortopassi GA (2002) Differentiation-specific effects of LHON mutations introduced into neuronal cells. Hum Mol Genet 11:431–438