Chapter 19

NEANDERTAL EVOLUTION AND HUMAN ORIGINS: A TALE FROM THE SCLADINA I-4A CHILD

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Michel Toussaint & Dominique Bonjean (eds.), 2014.
The Scladina I-4A Juvenile Neandertal (Andenne, Belgium),
Palaeoanthropology and Context
Études et Recherches Archéologiques de l'Université de Liège, 134: 379-394.

Foreword

The following article was written soon after the publication of the first genetic analysis of the Scladina Neandertal Child (Orlando et al., 2006). By then — it was in 2006 — sequencing even short DNA pieces from ancient material represented a big challenge. It is still true to some extent as ancient DNA molecules are nowhere near the long stretches of millions of As, Cs, Gs and Ts (nucleotides) that constitute our chromosomes. Those molecules are rather extremely fragmented into 50-100 nucleotide chunks at best and are also chemically degraded, which makes their analysis particularly difficult (Paabo et al., 2004). However, the recent years have experienced a massive technological revolution. First, so-called next-generation sequencing platforms can now generate millions to billions of sequences in a cost- and time- effective manner. This, coupled with sound bioinformatic procedures, makes it possible to identify the minority of sequences that come from the Neandertal individual as opposed to the majority of those originating from environmental microbes that colonized its bones and teeth after death (Schubert et al., 2012, 2014; Der Sarkissian et al., 2014). Second, novel methods have been developed in ancient DNA research facilitating access to (Dabney et al., 2013; Meyer et al., 2014), and possibly even enriching for ancient molecules (Briggs et al., 2009; Burbano et al., 2010). This resulted in an unexpected blossoming characterization of ancient genomes (Rasmussen et al., 2010, 2011, 2014; Keller et al., 2012; Raghavan et al., 2014) that recently culminated with the characterization of the complete genome from a horse that lived 700 ka BP ago, and for Neandertals, the complete genome sequence of no more than several individuals from Croatia (Green et al., 2010), the Caucasus and the Altai (Prüfer et al., 2014). We know today the genome of Neandertals, as well as that of other archaic hominins, called Denisovans (Reich et al., 2010; Meyer et al., 2012). The sequencing of the genome of the pre-Neandertal *Homo heidelbergensis* is on its way (Meyer et al., 2014; Orlando, 2014). At the same time, our knowledge and understanding of the genetic diversity present amongst modern human populations has improved (The 1000 Genomes Project Consortium 2012). Therefore, the sequence information available now is order of magnitude greater than what we knew back in 2006. The 123 nucleotides characterized from the mitochondrial DNA of the Scladina Child represent only 0.000004% of the information present in its genome! Therefore, and not surprisingly, what we know today from our relationships with Neandertals goes well beyond the conclusions that we could draw in 2006. In particular, we know now that Neandertals and non-African modern humans share an excess of derived (non-chimp-like) mutations (Green et al., 2010; Prüfer et al., 2014). Although alternative models could explain this pattern (Yang et al., 2012; Eriksson and Manica, 2014), this probably indicates that Neandertal and modern human population outside of Africa admixed, most likely around 50 thousand years ago (Sankararaman et al., 2012). This contradicts the picture as depicted by mitochondrial DNA alone, where Neandertals and modern humans appeared as two distinct entities. This is because mitochondrial DNA can only track one single genealogy

where the genome reflects a large number of independent genealogies as recombination makes every single gene/locus independent. We can even scan our own genome for segments of Neandertal origin (Sankararaman et al., 2014; Vernot & Akey, 2014)! We also know, as hypothesized following the genetic analysis of the Scladina Child, that Neandertal populations were limited demographically and harbored only low levels of genetic diversity (Prüfer et al., 2014). Therefore, the value of the following article is mostly historical, as it ultimately illustrates that knowledge is a moving target and thereby showcases the profound changes in paradigm that we experienced over the last few years. There are still many things left to discover about our past and our relationships with Neandertals: there is no doubt that the Scladina Child, who lived in Belgian at a time when modern humans did not yet discover the European continent, could potentially help solve some of the final pieces of the puzzle!

Ludovic Orlando, April 2014.

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1. Introduction

In Europe as a whole, Neandertals and modern humans have coexisted from 42-28 ka BP (Mellars, 2006; Finlayson, et al., 2006). Though the coexistence took place only in some parts of the continent, it might have provided enough time for interbreeding (Smith et al. 1999; Hublin, 2000). Between 1997 and 2006, sequences of mtDNA hypervariable regions (HVR) have been reported from ten Neandertal specimens dated 42-28 ka BP from Feldhofer (Germany), Mezmaïskaya (northern Caucasus), Vindija (Croatia), Engis (Belgium), La Chapelle-aux-Saints (France), Rochers-de-Villeneuve (France) and El Sidron (Spain) (Krings et al., 1997; Krings et al. 1999; Ovchinnikov et al., 2000; Krings et al., 2000; Schmitz et al., 2002; Lalueza-Fox et al., 2005). Comparison with modern humans and chimpanzees revealed that Neandertal haplotypes were more similar to humans than chimpanzees but fall outside the range of modern human genetic diversity, making any suggestion that Neandertals contributed to the mitochondrial gene pool of contemporary humans through interbreeding highly unlikely (Currat & Excoffier, 2004). Furthermore, the facts that *(i)* Neandertal sequences show no preferential regional affinity with Europeans and *(ii)* that the divergence between Neandertals and modern humans well predated the origin of the current mitochondrial diversity of modern humans have been taken as key arguments for the validity of the Rapid Replacement Model (also called 'Out of Africa').

These interpretations have been criticized though. For instance, Relethford (2001[ab]) has convincingly demonstrated that the lack of regional affinity between Neandertals and modern Europeans does not preclude multiregionalism as multiregional evolution is *not* independent from regional evolution. Archaic human populations (including the European Neandertals) remained rather interconnected by gene flow across the Old World (Relethford, 1999). And actually, even low levels of continued gene flows result in equivalent accumulated Neandertal ancestry for any kind of modern human population according to migration matrix theory (Relethford, 2001[a, b]). Another critic relied on the fact that Neandertals have been compared with current (and not ancient) modern humans, leaving open the possibility for recent elimination of Neandertal-like sequences from the modern human gene pool as a consequence of demographic events (Nordborg, 1998) or selective sweeps (Hawks & Wolpoff, 2001). Methodological concerns have also been raised since the models used for reconstructing phylogenies did not take into account possible homoplasic effects occurring on numerous mutational hotspots described in mtDNA hypervariable regions. Yet a reanalysis of the sequence data under appropriate models has surprisingly suggested that Neandertals "would be more akin to modern humans than what recent claims suggest" (Guttierez et al., 2002).

Hence, at this point, it was clear that more data were needed for the debate to progress. Albeit they came from dispersed locations over the whole Neandertal geographic range (west to east from Spain to the Caucasus, and north to south from Belgium to Croatia), all the Neandertal specimens that delivered genetic information belonged to the Oxygen Isotopic Stage 3. Older Neandertal specimens would offer the opportunity to get insights into the long-time evolution of the Neandertal gene pool by comparison with other Neandertal sequences. As such, it could reveal possible drastic changes or long-time stability at the time of cohabitation and give insights into the Rapid Replacement/Multiregionalism debate. We therefore decided to retrieve the first sequence information from a Neandertal related to Oxygen Isotopic Stage 5 by analyzing the remains of the Scladina Child. It is true though two Neandertal bones predating the contact with modern humans in Europe (>50 and 100–110 ka BP from Warendorf-Neuwarendorf (Germany) and Krapina (Croatia) were already used for southern-blotting experiments (Scholz et al., 2000). However, the southern hybridization approach suffers from serious flaws and has been shown to be misleading (Geigl, 2001). Therefore, so far, the Scladina Child is the sole Neandertal specimen that has delivered authenticated DNA sequence information related to Oxygen Isotopic Stage 5 (Orlando et al., 2006).

2. Results

2.1. Sequence authenticity

We took advantage of previously reported Neandertal sequences to use primers that favour the amplification of Neandertal DNA. PCR was never successful when fragments larger than 173 bp were targeted as expected for ancient templates (Box 1). We amplified four fragments spanning 221 bp of the mtDNA HVR-I. Each PCR

Box 1

Ancient DNA: applications, pitfalls and experimental procedure

Ancient DNA technology offers the unique opportunity to recover genetic information from the past (for the last 500,000 years at best). As such, it opens the door for an incredibly wide range of applications. Of course, the prehistoric megafauna, with its innumerable bone and tooth remains preserved in caves, has represented a precious source of DNA so far (e.g. cave bears, woolly rhinos and giant elks in Hänni et al., 1994; Orlando et al., 2003; Hughes et al., 2006). But besides, ancient human individuals (Handt et al., 1996) as well as plant seeds (Jaenicke-Desprès et al., 2003) or pieces of wood (Deguilloux et al., 2002), microbes (Drancourt et al., 1998) and ancient viruses (Tumpey et al., 2005), coprolithes (Poinar et al., 2003) and even Pleistocene sediments (Willerslev et al., 2003) have also delivered nucleotidic sequences, providing valuable information as for the kinship among individuals inside necropoles (Kolman & Tuross, 2000), the tempo and mode of the domestication process (Zeder et al., 2006) or the consequences of global climate on biodiversity (eg. species richness: Willerslev et al., 2004; or population diversity: van Tuinen et al., 2004).

However, recovering ancient DNA is by no standard a straightforward process, mainly because of the chemical nature of ancient molecules in itself. DNA decay indeed starts the very moment an organism dies. Hydrolytic reactions contribute to extensive fragmentation of the DNA backbone (in molecules barely longer than 200 nucleotides), to local loss of sequence information (eg. through base elimination, such as depurination) and even to subtle changes in sequence information (e.g. Cytosine modified in Uracile as a result of a local deamination; Lindahl, 1993). These features considerably complicate the recovery of ancient DNA molecules and extensive laboratory work (including rigorous technical procedures as well as redundant experimental controls) is generally requested before any ancient DNA molecules can be authenticated (Gilbert et al., 2005).

Briefly, the extraction procedure is conducted in a laboratory specially devoted to ancient DNA work. It consists in an overpressurized lab where *(i)* work surfaces are bleached, *(ii)* DNA-free materials are used and *(iii)* no modern DNA is stored or manipulated ever. After a typical extraction procedure (sample powdering and decalcification followed by DNA purification and concentration), the total amount of ancient DNA molecules that can be retrieved is generally not sufficient for sequencing. Therefore, an amplification step by Polymerase Chain Reaction (PCR) is most of the times required. The PCR is a cycling-amplification process which restores minute traces of a chosen part of the genome into billions of copies. The specificity of the reaction is due to short oligonucleotides (DNA primers) that can act as probes for the targeted DNA locus. Whenever it is possible, the sequence of the primers is chosen to target specifically the DNA of interest. But given the very degraded nature of ancient DNA fragments, fresh DNA contaminants very often outcompete their ancient counterparts during the PCR process if primer annealing is possible. Therefore, contamination — and false positive amplification — is one of the most serious concern in palaeogenetics and scrupulous controls are requested to insure the bona-fide DNA was actually amplified (and not contaminant by-products).

Note that PCR is often problematic when dealing with ancient templates. Molecules present in the soil as well as bone constituents *per se* (such as the protein Collagen type I) are coextracted with DNA molecules and act as *Taq* polymerase inhibitors, preventing any amplification (Scholz et al., 2000). Moreover, Maillard reactions may lead to covalent bond formation between DNA and sugar residues during the taphonomic process, resulting in templates unsuitable for Taq elongation. Lastly, artefactual mutations can be generated by PCR because some bases are turned into others in the course of the taphonomic process. The most prevalent of these DNA-damage induced errors leads to GC⇒AT mutations (Hofreiter et al., 2001[a]; Gilbert et al., 2006; Stiller et al., 2006). Consequently, no aDNA sequence can be deduced from a single amplification product but must be checked though multiple amplification replications to be trusted. Moreover, the cloning (and sequencing) of each PCR product is also highly recommended to pinpoint each artefactual mutations along the sequence. All these experimental procedures were respected for determining the DNA sequence of the Scladina Child. One supplemental precaution was undertaken: the DNA extract was treated with an enzyme (namely Uracile-DNA Glycosylase, UDG) to discard all DNA substrates carrying the artefactual Uraciles (Hofreiter et al., 2001[a]). Therefore, PCR products were generated starting from Uracile-free DNA templates and were highly unlikely to bear artefactual mutations.

product was cloned and the final sequence was deduced from the consensus of 61 clones. But given the age of the sample and material limitation, we managed to amplify the most 5' and 3' fragments only once; thus, the 39-first and 59-last nucleotides have been respectively deduced from the sequences of 8 and 12 clones from only one amplification product. These sites exhibit 5 substitutions never observed between the already published Neandertal sequences: 2 C⟹T, 2 G⟹A and one T⟹C. Though some polymorphism has already been observed in the same part of the HVR-I among other Neandertal sequences (eg. one A/C transversion and one C/T transition) and the DNA extract was treated with Uracil DNA-Glycosylase (to eliminate artefactual GC>AT substitutions, Box 1), we decided to discard the sequence information present in the 39-bp and 59-bp terminal parts of the Scladina sequence, leaving 123 bp of sequence information. Therefore, the final sequence of the Scladina Child consists only on nucleotide positions that have been recovered from at least two (up to four) independent PCR products. As a consequence, the final sequence can be considered as truly authentic and none of the polymorphic sites (with regards to other Neandertal sequences) can be regarded as artefactual mutations (i.e. DNA-damage induced errors, Box 1). Notably, none of these polymorphic sites has been found in any of the coworkers (Box 2) (nor in the *Homo sapiens* sequences found in some of the amplifications), which makes it highly unlikely that the Scladina sequence — obtained through combination of four overlapping PCR fragments — results from mosaic association of PCR contaminants, as it has been already suggested for other ancient sequences (Bandelt, 2005). Likewise, sequence differences between the Scladina sequence and other Neandertal sequences are not located in any of the sites demonstrated by Gilbert et al. (2003) as being highly affected by postmortem degradation in humans.

Furthermore, we are confident that the environmental conditions in Scladina Cave are particularly prone to biomolecule preservation (and therefore to DNA preservation). Cave bear bones from the same excavation layer as the Neandertal child, or from even older layers (former Layer 5, Loreille et al., 2001; Orlando et al., 2002) already delivered authentic ancient DNA. Moreover, 70–60 ka BP old nuclear DNA sequences were successfully amplified from woolly rhinoceroses from Scladina (Orlando et al., 2003). Lastly, carbon and nitrogen isotopic survey of one maxillary sample from the Scladina Neandertal has revealed an atomic C/N ratio typical of well-preserved collagen (Bocherens et al., 1999).

2.2. Comparison with available Neandertal sequences

The sequence of the Scladina Child has not been found among the 7161 human HVR-I sequences present in the HvrBase++. It appears more distantly related to the Cambridge Reference Sequence (1 insertion/deletion, 14 substitutions along 123 sites) than to the sequences from other Neandertal specimens (3–4 substitutions). Within the 123 bp of sequence information, only 1 substitution distinguished the previously reported Neandertal sequences. Therefore, the Scladina sequence reveals that the genetic diversity of Neandertals has been previously underestimated.

2.3. Phylogenetic analyses

Sequence comparisons were conducted with the 171 human HVR-I sequences used in Guttierez et al. (2002) as a representative subset of the overall human diversity. A supplemental dataset of 8 chimp haplotypes was used as outgroup. The selected models of molecular evolution accurately estimate nucleotide substitution parameters and takes into account rate heterogeneity among sites (Figure 1). When chimpanzee sequences are used as outgroups, all Neandertals cluster apart modern humans, in a monophyletic group with substantial bootstrap-support (67%). When chimpanzee sequences are excluded as in Schmitz et al. (2002), the separation between Neandertal and modern human lineages is supported by almost maximum bootstrap values (99%). Importantly, these results confirm previous claims of early divergence between Neandertal and modern human lineages and appear in sharp contrast to Gutierrez and colleagues's report of "no support for a branch separating the Neandertal cluster from the human sequences" (Guttierez et al., 2002).

2.4. Pairwise comparisons

Pairwise distances were estimated using the best phylogenetic model of nucleotide substitution and rate heterogeneity (HKY+G+I). According to the Rapid Replacement Model, the sequence from Scladina, being 70–60 ka BP closer to the MRCA

Box 2

Ancient or modern human DNA? The contamination case

The study of ancient DNA in human remains is plagued by problems caused by contamination of specimens since virtually all human fossils have been handled by human beings (before any DNA extraction is started). PCR experiments have clearly shown that human mtDNA sequences can be virtually retrieved from almost every ancient animal specimen. For instance, not less than 20 different modern human signatures could be retrieved from a 30,000-year-old bear tooth (Upper Cave, Zhoukoudian, China) using human-specific PCR primers (Hofreiter et al., 2001[b]). Monitoring human and dog mtDNA in dog bones and teeth from the Neolithic and medieval periods, Malmström et al. (2005) systematically found the presence of human DNA, often at levels exceeding the amount of authentic endogenous DNA. Likewise, dot blot experiments revealed the presence of human DNA in a large diversity of animal fossils (Nicholson et al., 2002). Genomic data from skeletal remains of a 40,000-year-old cave bear (Noonan et al., 2005) and a 27,000-year-old mammoth (Poinar et al., 2006) have more recently identified a significant presence of DNA of human origin.

Because the surface is the only part of the sample that is exposed to human contamination sources, it has been advocated to scrap the surface of human fossils or to bleach it with hypochlorite solution before DNA extraction to eliminate the false positive results such a contamination may lead to. Deliberate contamination of samples (by constant handling for 10 min at 30℃ to maximize sweating and therefore DNA transfer) have indeed revealed low penetration of contamination sequences inside fossils (Malmström et al., 2005). However, it is frequent to recover contaminant DNA on human samples, though scraped (Kolman & Tuross, 2000) or bleached (Kemp & Smith, 2005). Interestingly, Gilbert et al. (2005) have shown Havers canals and dental tubules offer appropriate circulation routes for exogenous DNA penetration in bones and teeth. All in all, the authenticity of ancient human sequences may be, most of the time, difficult (if possible) to demonstrate.

One way to circumvent contamination problems would be to excavate human specimens for ancient DNA analysis under strict clean and DNA-free conditions (Lalueza-Fox et al., 2006). Similarly, choosing the most freshly excavated specimens would lower the rate of contamination with modern human DNA. This strategy was followed for the genetic analysis of the Scladina Child. A molar tooth, excavated on 13 November, 2001, was selected for ancient DNA analysis. The tooth-root was sampled in sterile conditions using a power saw. The blade was sterilized in natrium hydroxide 10%; gloves, mobcap, and disposable coat were used. The sequences of all the co-workers that had potentially been in contact with the Neandertal sample was determined in order to trace any contamination from modern humans. Despite such precautions, some modern human sequences were found in the Scladina DNA extract (such was also the case in the very first ancient DNA study on Neandertals; Krings et al., 1997). But as all of these could be attributed to one of the coworkers, they could not be mistaken for Neandertal sequences. Moreover, we took advantage of Neandertal specific-primers (designed according to previously reported Neandertal sequences) to favour the amplification of Neandertal DNA by PCR. This procedure allowed to recover an authentic mtDNA sequence of the Scladina Child.

between Humans and Neandertals, should exhibit fewer substitutions than younger Neandertal sequences when compared to contemporary human sequences. According to minimum estimates of the HVR-I substitution rate, at least 3.9% substitution differences are indeed expected between sequences that are 70–60 ka BP distant (Excoffier & Yang, 1999). The observed pattern though, is the opposite: the pairwise distance distributions within humans, and between humans and Neandertals, become closer and overlap more extensively for younger Neandertals (age<42 ka BP, P<0.001) than for the Scladina specimen (Figure 2). The switch towards a modern human distribution is however not due to a closer proximity of Neandertals and Europeans after contact, as Europeans do not appear more closely related to Neandertals than humans from other continents are.

3. Discussion

The sequence extracted from the Scladina Child (Figure 3) has revealed unexpected levels of genetic diversity among Neandertals. Since its publication (Orlando et al., 2006), this discovery has been further supported.

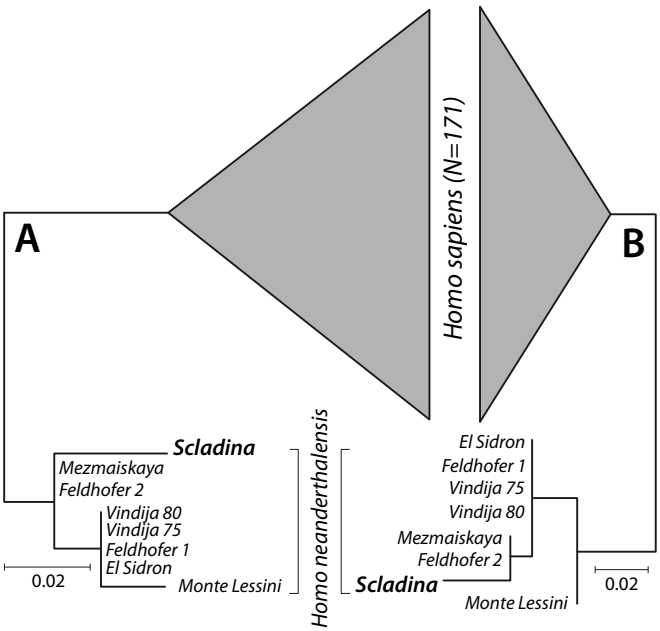

Figure 1: Phylogenetic trees showing the relationships between Neandertal and modern human lineages. (A) Rooted with 8 chimpanzee haplotypes (Maximum of Likelihood: HKY+G4). (B) Rooted according to the midpoint location (Maximum of Likelihood: HKY+G4+I). Phylogenetic trees have been reconstructed under the best model of molecular evolution (see Orlando et al., 2006). Note that the branching order among Neandertal lineages is still an open question and will require further information to be definitively solved.

Another Neandertal specimen (Monte Lessini, Italy, 50 ka BP) has been shown to carry another divergent mtDNA haplotype (exhibiting two supplemental polymorphic sites along the 123 bp in common with the Scladina haplotype; Caramelli et al., 2006). In spite of this genetic diversity, all Neandertal haplotypes cluster in a monophyletic group that diverged from the human lineage long before the coalescence time of the MRCA of all contemporary humans (Figure 1).

Pairwise mismatch distribution analyses have revealed a second unexpected pattern. Using an unbiased data set of 171 modern human sequences, pairwise distance uncorrected distributions between modern humans and between pairs of modern humans and Neandertal sequences were shown to largely overlap (Guttierez et al., 2002). This result was confirmed here using distances corrected for heterogeneity between rate of substitutions (Orlando et al., 2006, Figure 2). What the sequence from Scladina reveals is that this situation has been shaped between 100 and 42 ka BP (all the Neandertal sequences younger than 42 ka BP are significantly less distant to modern human than the Scladina sequence is; Figure 2).

Selective sweep as well as genetic drift could explain this pattern. The fact that human mtDNA could be subjected to selection has already been hypothesized (Hey, 1997; Hey & Harris, 1999). As some mtDNA lineages are preferentially associated with bioenergenetic disorders, the regional variation in mtDNA sequences of extant modern humans may have been possibly shaped by natural selection (Mishmar et al., 2003; Ruiz-Pesini et al., 2004). If more Neandertal-like haplotypes were selected among modern humans in response to similar environmental constraints (e.g. climatic conditions), the gap between modern humans and

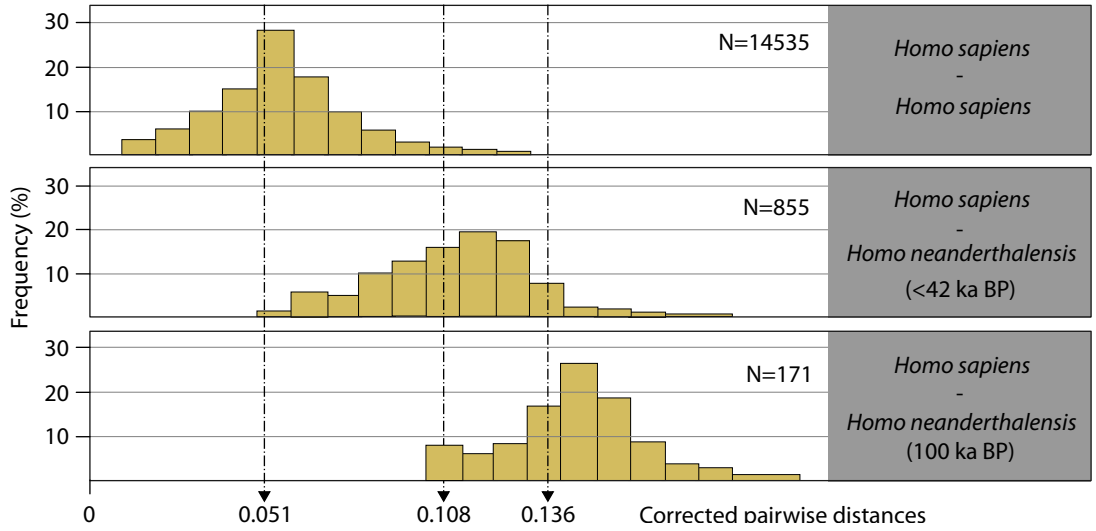

Figure 2: Pairwise mismatch distributions among modern humans or modern humans and Neandertals from different ages.

Figure 3: The sequence extracted from the Scladina Child has revealed unexpected levels of genetic diversity among Neandertals. Pictured left: at the Prehistory Service of the University of Liège, Dominique Bonjean (front) proceeds to the amputation of one rooth of the Scla 4A-13 molar while Ludovic Orlando prepares the tube that will collect the fragments (photographs Michel Toussaint, February 2002).

Neandertals would be reduced all the more than selection acted for a long time. Yet our data set reveals that the large majority of polymorphic sites might become homoplasic under a selective force. Indeed, only two sites have been fixed between the Neandertal and the modern human gene pools; at positions 16230 and between positions 16263 and 16264, Neandertals exhibit, respectively, A and G whereas modern humans display a gap and an A. By contrast, all polymorphic sites observed between Neandertal sequences are also polymorphic between modern humans. Any selected change on those sites might thus bring modern humans closer to Neandertals.

Genetic drift might alternatively have lead Neandertal and human mtDNA gene pools to become closer at the time Neandertals coexisted with premodern Europeans. Consider first how much the Neandertal mtDNA gene pool was diverse in the past. Now consider a putative demographic bottleneck that Neandertals experienced (for instance, following the competition with premodern humans in Europe till their extinction, or even earlier as a consequence of the global climatic changes related to the cold Oxygen Isotopic Stage 4 — 74–60 ka BP; Ambrose, 1998). As for any genetic drift event, less frequent haplotypes are the most probable to be lost; in populations the less frequent haplotypes are the youngest ones, because those have had less time than older ones to expand. Then, after the genetic drift event, the Neandertal haplotypes which are most likely conserved would be those that appeared first, that is the haplotypes more closely related to the MRCA between modern humans and Neandertals. After the drift, the old haplotypes are most likely to have become more frequent in Neandertal populations, which then in turn appeared genetically closer to modern human populations than older Neandertal populations. Interestingly, a recent survey of the regional distribution of SNPs within the complete human genome sequence is consistent with a demographic collapse in human demography at the time of the colonization of Europe (40 ka BP, Marth et al., 2003).

Though interesting, these results should be taken as very preliminary. Only 2 specimens older

than 50 ka BP have delivered some genetic information so far (Scladina and Monte Lessini) and for all but one of the other specimens (Vi-80), the available genetic information is still reduced. Any inference of the long-term changes in Neandertal genetic diversity remains still very speculative and additional information is required before drawing definitive conclusions.

4. Perspectives: Neandertal genomics

The Neandertal HVR-I sequences do suggest an early divergence of *Homo neanderthalensis* from *Homo sapiens* and suggest a rather weak (if any) admixture. But as the mtDNA is only maternally inherited, the mitochondrial evidence leave the possibility open that Neandertal father contributed to the modern nuclear gene pool. Recent technological breakthroughs have made it possible to recover nuclear DNA sequences from Neandertal specimens. Two-rounds multiplex-PCRs (Box 3) now allow the recovery of complete nuclear gene and therefore to relate genotype and phenotype for past individuals/species (Römpler et al., 2006). Furthermore, metagenomic screens and high-throughput sequencing technology now make it possible to recover large amounts of genomic information (Box 3). Such genomic data will soon furnish the opportunity to test our possible Neandertal inheritance through both parental lines (Green et al., 2006; Noonan et al., 2006). Moreover, comparative genomics of chimps, Neandertals and modern humans promise to provide the complete list of the functionally important genetic changes that gave rise to our species.

The first complete mtDNA genome from extinct species (two genera of New-Zealand moas) was published in 2001 (Cooper et al., 2001). In the following five years, two mammoth specimens have delivered complete mtDNA genomes (Krause et al., 2005; Rogaev et al., 2006). Now the first draft of the Neandertal genome may be achieved within the next two years (Green et al., 2006). Recent technological breakthroughs clearly represent the way forward and open the hunt for large scale DNA retrieval of other ancient hominids such as the late *Homo floresiensis* (Brown et al., 2004; Morwood et al., 2005). By doing so, ancient DNA will place the 'Rapid Replacement' *versus* 'Multiregionalism' debate in a broader context than the single Neandertal-*sapiens* admixture possibility and promises to give invaluable insights on our phylogenetic tree. Hopefully, the remains from the Scladina Child, as part of such projects, will keep on enlightening our own origins.

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Box 3

Technological advances and the dawn of Neandertal genomics

What is metagenomics?

Metagenomic DNA libraries are built in a two-step procedure (i.e. insertion, transformation). First, the DNA fragments from a sample are randomly inserted into plasmids (circular DNA of bacterial origin) thanks to DNA recombining technology. Then, each modified plasmid is integrated inside a bacteria (through a process called transformation). During cell divisions, the plasmid is transmitted from mother to daughter cells in bacterial colonies, leading to a clonal amplification of the plasmid. Since the sequence of the plasmid is known, the sequence of the inserted fragment can be determined. Finally, sequence comparison with already available annotated genomes and characterized sequences allow sequence identification. In addition to bona-fide sequences, other types of sequences — mainly from environmental bacteria — are characterized through this process (Noonan et al., 2005; Noonan et al., 2006).

High-throughput technology: the 454 sequencing system

Recent high-throughput technological breakthroughs have made the dream of sequencing the whole Neandertal genome a realistic objective. The strategy that delivered so far the most extensive sequence information relies on the 454 Life Science system. Briefly, each DNA fragment from a sample is attached to a bead. Beads are mixed and captured in the droplets of an emulsion where PCR amplification can occur. As a result, each bead is coated with ten million copies of the initial DNA fragment and can be deposited into one of the 1,6 million wells of a fibre-optic PicoTiterPlate for massive parallel pyrosequencing (Margulies et al., 2005). Most strikingly, the 454 technology is able to gather 25 million bases of sequence information every four hours which outcompetes by more than 60 times the current capacity of most automatic sequencing instruments. This sequencing system has already succeeded to recover 1-million base of the Neandertal genome from a 100-mg sample (Green et al., 2006).

Alternative strategies

One important drawback of both metagenomic library screening and 454 sequencing system is that most of the recovered sequences are environment contaminants. A selective (rather than random) might be more preferable. Using specific human DNA probes could help for fishing Neandertal genes in metagenomic libraries before sequencing, as proposed (and tested) by Noonan and colleagues (2006). Alternatively, two-round multiplex-PCRs could help for targeting genes under selection in modern humans (Römpler et al., 2006). Briefly, a first PCR amplification using large combinations of primers restores numerous DNA fragments to amplifiable levels. Each fragment separately is then amplified separately in a second step. This strategy has recently allowed the characterization of the whole mtDNA genome of the mammoth (Krause et al., 2006) and of the first complete coding sequence of a nuclear gene (Römpler et al., 2006).

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