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NEANDERTALS: DECIPHERING OF THE EXTINCT SPECIES DNA STARTED

The discovery of the basis of genetic variation has opened inroads to understanding our history as a species. It has revealed the remarkable genetic similarity we share with other individuals as well as with our closest primate relatives. To understand what makes us unique, both as individuals and as a species, we need to consider the genome as a mosaic of discrete segments, each with its own unique history and relatedness to different contemporary and ancestral individuals.

Pääbo, NATURE 421, 409–412, 23 January, 2003

"… results illustrate the usefulness of retrieving direct genetic information from ancient remains for understanding recent human evolution."

Krause, Green, Pääbo, CURRENT BIOLOGY 17, 1–5, 6 November, 2007

ABSTRACT: *Last year marked the anniversary of the most famous discovery in 1856 of the Neandertal fossils in the Grotto now known as Feldhofer, located in Neander Valley near Düsseldorf, Germany. Over 140 years after the discovery, the first analysis of Neandertal mitochondrial DNA (mtDNA) was carried out. Although retrieval of ancient DNA samples from fossils and its analysis are technically challenging tasks due to frequent contamination and chemical degradation, the sequencing of the first million base pairs of Neandertal nuclear genome has been announced. This spectacular event started to yield insights into the nature of our closest relatives, which process could contribute to unravelling evolutionary pathways toward modern humans. Ever since that time molecular features which might confirm contribution of other extinct hominines to modern human evolution were exploited to deduce from fossils. Having retrieved data of Neandertal nuclear as well as mitochondrial sequences, scientists attempted to determine divergence time of modern humans and Neandertals, effective population size of Neandertals at the time of split or even the sequences responsible for particular features, for instance language and pigmentation, impossible to asses by other methods. Apart from analyzing past populations of* Homo, *the new DNA methodology enables also studies of other extinct species, especially those of the last Ice Age.*

KEY WORDS: *Neandertal – Modern human – aDNA – Evolution*

INTRODUCTION

It is a commonly accepted fact that *H. heidelbergensis* became a key species in the evolution of humans and Neanderthals, giving rise to *H. sapiens* in Africa and *H. neanderthalensis* in Europe (Klein 1999, Stringer 2002). A number of fossils move back the divergence time to more than 500 Kyr, for instance the first mandible of *H. heidelbergensis* found in 1907 by Otto Schoetensack (Schoetensack 1908) at the Mauer site in the basal sand and gravel complex of the Grafenrain near Heidelberg in Germany (Maurer 1) and Bodo cranium unearthed in 1976 at Bodo d'Ar, Ethiopia, in the Middle Awash Valley and dated to 600 Kyr (Stringer 1993).

Probably, long time will pass until unearthed yet remains release hidden details on recent evolutionary history of the genus *Homo*. Until recently, morphological features of fossils decided exclusively on evolutionary pathways of various ancestral populations, their migrations and presence on the Earth.

Only 23 years ago, the possibility of determining characteristic features became available, albeit not due to shape of skulls or other bones, but analysis of DNA molecules entrapped within remains. Revolutionary development of modern molecular techniques facilitated the insight, first into mitochondrial DNA (mtDNA), and very recently into nuclear DNA (nuDNA). A decade ago an informative fragment of Neandertal mitochondrial DNA has been analysed, and only a year ago the first million of nuclear DNA base pairs (bp) of our extinct cousin have been reported. Beside contemporary molecules, ancient DNA became significant and desirable, since it is the only possible source of information on features unachievable by other methods. All consequences of recent discoveries and continuous improvement of methodological applications are not easily predictable. However, it is obvious that comparison of genomes evolving through distinct periods of time, including those of extinct species, offers the most probable profile of structure and history of life, with points of its different forms divergence, established relationships and detailed ways of introgression, including genes involved e.g. in predisposition to diseases, behaviour and habits.

NEANDERTAL DNA SEQUENCES MIGHT ASSIST IN RECOGNITION OF THE SPECIES' HISTORY AND FEATURES

The discovery of several bones in the Feldhofer grotto, in the Neander Valley near Düsseldorf, Germany (Feldhofer 1) in August 1856, became a contribution to a generation of new disciplines in science, short after – *paleoanthropology* and quite recently, *molecular archaeology*. At the time and atmosphere of Darwin's new theory, a number of problems had arisen. First, and probably the most intriguing was the origin of our species. The second one required an explanation of reasons that led to extinction of species represented by human-like famous findings at Neander River Vally. The third but not last question to answer was the type of relations, if any, between both representatives of the genus *Homo*. Some hope that further clarification of the details will come from attempts of molecular studies, which offer methods to decipher precise basic information about species, especially extinct ones, through retrieval of ancient DNA (aDNA) from archaeological findings.

Until recently, the knowledge on genetic contribution of our extinct and closest relatives – the Neandertals – to modern human's genome was highly limited. Parallel methodological achievements in the mid-1980s, such as providing the analysis of DNA from remains representing historical molecules (Higuchi *et al.* 1984), as well as invention of polymerase chain reaction allowing for molecular cloning of particular fragment of template macromolecule (Saiki *et al.* 1985, Mullis, Faloona 1987), have altered somehow searching pathways in anthropology and archaeology. Although many positively false results have been published during the first decade of aDNA research, mostly due to contamination, the experience gained during successive templates' extraction from various kinds of ancient material, together with growing knowledge on DNA degradation processes, the first retrieval of DNA sequence isolated from extinct hominine remains succeeded (Krings *et al.* 1997). Sensitivity of DNA to degradation made sequences of this macromolecule readable post mortem probably not longer than 100–150 Kyr, however those molecules that persisted in permafrost regions are several hundred years old and intact enough to be amplified (Hebsgaard *et al.* 2005, Willerslev *et al.* 2007). Fortunately, most of the Neandertal fossils found are much younger than the established limit of age for DNA survival. Moreover, a number of remains had been uncovered for thousands of years in rather cold environment. Mitochondrial DNA (mtDNA) is a much more abundant molecule in the cell, and thus is more accessible than nuclear DNA (nuDNA) – therefore, its templates were deciphered earlier.

Researchers from Max Planck Institute of Anthropology, Leipzig, Germany succeeded in the sequencing of Neandertal mtDNA Hypervariable Region I (HVRI – positions from 16023 to 16400) (Krings *et al.* 1997). They established its primary structure and found that DNA analysed falls outside sequence variation of modern humans (difference range 1–24 positions). 27 positions were different along the 379 bp Neandertal sequence (range 20–34), while chimpanzee sequence differs in 55 positions (range 46–67). Altogether, their results did not confirm relations with modern humans, leaving little or no traces of interbreeding. This might support the view that *Homo sapiens* has arisen in Africa as a separate species and appeared in Europe gradually replacing Neandertals (e.g. Stringer 1993).

Calculation on the results suggested that probable time divergence between Neandertals' and modern humans' mtDNAs took place between 550 and 690 Kyr. Earlier searching for common ancestor of modern human mtDNAs divergence was determined as 120–150 Kyr (Cann *et al.* 1987, Vigilant *et al.* 1991) which was almost four times shorter. Computed data relies on the calibration point of the chimpanzee–human divergence as 4–5 Myr (Takahata 1995) which was recently corrected to 6–7 Myr (Kumar *et al.* 2005, Patterson *et al.* 2006). However, published results are in good agreement with archaeological and paleontological records which set the Neandertal/modern human split between 250 Kyr (Foley, Lahr 1997) and 500 Kyr (Stringer, Hublin 1999). It should be remembered that composition of particular population gene pool diverges well before the split into two separate outbreeding groups. Usually, gradual diverging is reflected by increasing level of polymorphism within the genome

of ancestral species (Nei 1987). Probably, this has been completed in *H. heidelbergensis* or even started in *Homo erectus*. However, until now such data on other ancestral hominine DNA is not available.

Three years later, in 2000, mtDNA of the second Neandertal, isolated from skeleton of an infant (~29 Kyr) found in Mezmaiskaya Cave in the northern Caucasus, has been analysed (Ovchinnikov *et al.* 2000). Following years brought further information on Neandertal mtDNA. The number of specimens unearthed throughout Europe and East Asia reached 13 (e.g. Schmitz *et al.* 2002, Caramelli *et al.* 2006, Lalueza-Fox *et al.* 2006, Beauval *et al.* 2005, Orlando *et al.* 2006, Serre *et al.* 2004, Krause *et al.* 2007a). Neandertal sequences already retrieved differ from any known archaic as well as modern humans and are recognizable with specific oligonucleotide PCR primers (Caramelli *et al.* 2003, Serre *et al.* 2004). Although it is clear that analysed sequences are human-like, the divergence observed at the level of mtDNA does not confirm inbreeding between *H. neanderthalensis* and *H. sapiens*. It should be underlined that subsequent and independent studies on separate specimens unearthed at different European and west Asian locations and different age provided similar, but not identical sequences from other Neandertals, supporting strongly their authenticity. Sequence differences between Neandertal specimens of 40 and 100 Kyr age reflect ingroup diversity – higher for older representatives (Orlando *et al.* 2006).

WAITING FOR THE SEQUENCE OF NEANDERTAL NUCLEAR GENOME

When Matthias Krings with his colleagues (Krings *et al.* 1997) wrote that "nuclear genome of Neandertals seemed an unrealistic challenge because single-copy nuclear DNA sequences would be impossible to amplify from the extracts" they could not even imagine that less than 10 years later primary structure of nuclear Neandertal templates would become readable. However, a number of problems challenge enthusiasm if one wants to have an insight into the structure of historical sequences. The first, and probably the most difficult to overcome is low accessibility of Neandertal templates, not only due to the fact that nuclear ones are much scarcer than mitochondrial (1 versus hundreds or even thousands of copies), but mainly because of advanced degradation of macromolecules which shortens them dramatically and makes unavailable (Gilbert *et al.* 2005). The problem still awaits solution. Moreover, among those fragments which are long enough to give amplicons, other alterations of chemical structure, such as products of depurination, oxidation produced hydantoins and inhibitors of DNA polymerase can stop PCR. This problem has its recent solution in "molecular breeding" of DNA polymerases (DNA shuffling or directed evolution) – a process which produces enzymes passing over most of the lesions arisen post mortem (d'Abbadie *et al.* 2007). Thus, the second biggest problem to overcome is the reconstruction of authentic sequences. Experiencing for more than twenty years on aDNA molecules have enriched the knowledge on pathways and mechanisms of the molecule degradation. Main miscoding lesion and its frequency within ancient templates were confirmed recently, while retrieving nuclear Neandertal DNA – it is the cytosine deamination that constitutes for most of the changes altering primary DNA information. As was established earlier, double-stranded molecule surprisingly resistant, which is comparable with changes observed *in vitro* (Lindahl 1993), allow merely for only 1–2% C→U transitions (e.g. Hofreiter *et al.* 2001, Briggs *et al.* 2007) while susceptible single-stranded overhanging DNA exhibits majority of altered cytosine residues, amounting to ~70% (Briggs *et al.* 2007). It should be emphasized that the average length of single stranded overhanging ends is quite short and estimated to be 1.5–2 nucleotides. Single stranded nicks or gaps occur with the frequency of less than 2–3%, which means one over the length of 50 nucleotides on average.

Precise quantification of degradation-induced changes in aDNA templates is now possible due to equipments recently offered by Solexa, Applied Biosystems or Roche. The latter one proposes a system invented in laboratories of 454 Life Sciences, and named 454 sequencing. The equipment planned to release in 2008 will probably sequence 1 billion

bases (gb) within ~8 hours. Among many advantageous features of the method, such as the elimination of troublesome cloning, two are of spectacular significance – large number of fragments is sequenced simultaneously, which significantly shortens the time necessary to retrieve unknown sequence, and probably the most important one, i.e. separate amplification of complementary strands, which allows identification of miscoding lesions. Throughput of the most recent release, i.e. GS FLX (Roche) reaches 400,000 reads per run. It analyses fragments of 200–300 bases long with the highest possible accuracy, giving the yield of around 100 millions bases in less than 8 hours (http://www.454.com).

FIRST ATTEMPT TO SEQUENCE NUCLEAR DNA TEMPLATES OF *H. NEANDERTHALENSIS*

The nuclear genome is far scarcer than the mitochondrial one. Nevertheless, fall 2006 brought two publications presenting, for the first time ever, results of molecular analysis which started to decipher the Neandertal nuclear genome, information rewritten mostly after a number of more or less distant ancestors but in some details written *de novo* or introgressed from co-existing (?) hominines. At present, this approach is unique in uncovering pathways of evolutionary history of genus *Homo*. Until recently, our genome could only be compared to that of contemporarily living organisms including representatives of the closest related genus *Pan*, mammals and even invertebrates.

Neandertal Genome Project announced in July 2006 by molecular anthropologists from Max Planck Institute for Evolutionary Anthropology Dept. of Evolutionary Genetics, Leipzig, Germany (the same team which first analysed Neandertal mtDNA), opened a new era in studies of evolutionary pathways and mechanisms. Their approach tended to amplify and read by 'direct sequencing' a huge number of templates at the same time, after which sequenced templates are compared with reference sequence of *H. sapiens*, and then unmatched ones are discarded from the final sequence reconstruction process. Almost 94% of analysed templates have not provided any readable information on Neandertal sequence, of which 80% did not match any known human genome and 14% matched bacterial, fungal, viral etc. The approach resulted in retrieving the first million of base pairs localized at different chromosomes, constituting over three billion bp long genome (Green *et al.* 2006). The second approach, performed at the same time by the team from Lawrence Berkeley

National Laboratory, Berkeley, US, and in cooperation with the former one, is rather of metagenomic type. A traditional procedure (i.e. cloning) was applied to all available DNA from the sample, amplifying and then sequencing (by means of the classical method) only the templates trapped by human-like probes. Thus, only sequences of interest were recognized and retrieved. They targeted only 35 genes and succeeded in recovery of 29, written in not more than 62.5 Kb, which means that the method allows retrieving only regions of interest (Noonan *et al.* 2006). It seems that the choice between the two methods applied depends on the question being addressed: to sequence the full genome or search for one, exclusive gene. However, one might question searching only for one particular sequence, losing the other templates carrying invaluable information and enclosed in sparse specimens giving amplifiable templates.

STUDY OF NEANDERTAL aDNA: INFORMATION ON NEANDERTAL BIOLOGY AND RELATIONSHIP WITH MODERN HUMANS THAT CAN NEVER BE OBTAINED FROM BONES AND ASSOCIATED ARTEFACTS

Relations between two closely related hominines, i.e. Neandertals and modern humans, and possible interactions after the latter arrived in Europe ~40 Kyr (e.g. Tattersall, Schwartz 1999, Rougier *et al.* 2007), are among the most intriguing issues to be cleared up before our evolutionary history as a species is established. Studying modern human gene pool, as well as that of our extinct ancestors, might answer many questions on relation between the two groups. Although many sources suggested probable co-existence of the two groups for 8–10 Kyr (e.g. Harvati 2005), the recently published dating shortens it (Mellars 2006), at least in particular sites, to 2 Kyr. Extreme opinions even suggest that modern humans were mostly arriving at territories which Neandertals had left or had become locally extinct there (Finlayson 2004). During the conference "Neanderthals Revisited: New Approaches and Perspectives" organised at New York University (fall 2006) Chris Stringer said *"Modern humans may have had no direct effect on Neanderthal extinction. They actually walked into empty spaces where Neanderthals had already disappeared."*

A number of recent morphometric data and non–metric data confirm divergence of phenotypic features (e.g. Gunz, Harvati 2007) that separate Neandertals from modern humans, which suggest simply separate gene pools.
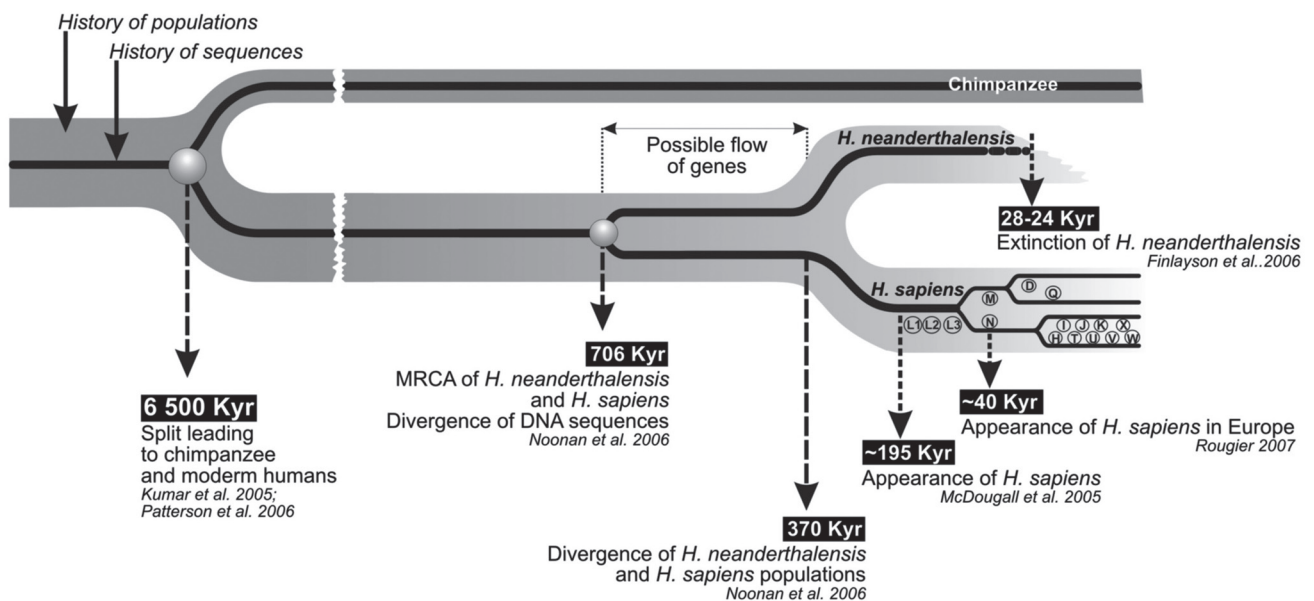

FIGURE 1. Scheme of Neandertals' and modern humans' evolutionary history with population and sequence divergence times resulting from genetic and fossil data. Circled capitals represent main mtDNA haplogroups.

However, two recent findings complicated the above picture. The first resulted from dating of the Gorham's Cave, Gibraltar, site which Neandertals used for tens of thousands of years (Finlayson *et al.* 2006). Published data extend their occurrence there until 28–24 Kyr BP, which means several thousand years earlier than was previously thought. In turn, this makes contemporaneity of Neandertals with modern humans more probable; suggesting that they lived well after modern humans entered Europe, at least in some locations. The second finding describes near-complete remains of the boy at Lagar Velho in Portugal as Neandertal/modern human hybrid (Duarte *et al.* 1999). If confirmed, the skeleton probably reflects extensive admixture between population, and not only one interbreeding incident. This would suggest permanent mixing of both morphologically recognizable extant and closest extinct representatives of the genus *Homo*.

In context of only a few above-mentioned data, the long lasting debate between anthropologists on the relationship between the two hominines is understandable. Thus, insight into and comparison of nuclear gene pools of both species might be a solution, allowing hint of common heritable markers as DNA sequences flowed from ancestors. Not many publications on Neandertal DNA analysis have been published since that first by Krings *et al.* (1997), which is understandable due to both limited availability of Neandertal specimens and high degree of chemical degradation of historical templates. However, preliminary information derived from Neandertal nuclear sequence has thrown some light in the darkness of Paleolithic history of *H. sapiens*.

Only two papers have described the evolutionary event initiating separate histories of diverged species on the basis of nuclear DNA sequence (Noonan *et al.* 2006, Green *et al.* 2006). Average time to the most recent common ancestor (MRCA) of Neandertal and modern human reference sequences was calculated by Noonan *et al.* to 706 Kyr (468–1015) and by Green *et al.* to 516 Kyr (465–569), both assuming the average coalescence time for human and chimpanzee autosomes as 6.5 million years (Kumar *et al.* 2005, Patterson *et al.* 2006), which data are at first sight comparable and in wide range adherent to mtDNA analysis determined here to be 461,000–825,000 years. However, recalculation of both sets of data (Wall, Kim 2007) showed some inconsistencies, suggesting that shorter time to sequence divergence found by Green *et al.* could come mainly from contamination with contemporary molecules or elevated level of errors during sequencing procedure. Results of Noonan *et al.* fixed maximum likelihood for the split time of ancestral populations of Neandertals and modern humans to 370 Kyr (Europeans – 440 Kyr, East Asians – 390 Kyr, 290 Kyr Yoruba) which predates the oldest *H. sapiens* findings in Africa dated for 195 Kyr (e.g. McDougall *et al.* 2005).

Noonan *et al.* showed also very low range of derived SNPs – 3%, that means single nucleotide polymorphism sites shared with modern humans. Results of their calculations of Neandertal nuDNA do not yet prove any gene introgression between populations, staying in accordance with earlier mtDNA data reflecting only single locus and maternal inheritance (Krings *et al.* 1997). Obviously, assessment of both populations interbreeding and gene flow is incomplete, thus awaiting recovery of more nuclear sequences, especially that on Y – chromosome, to confirm no admixture, which was estimated in the present study as zero. Even so, it should be considered that only one male Neandertal specimen from particular time and site delivering DNA cannot exclude introgression events. *Figure 1* shows possible divergence estimates since MRCA of modern humans and chimpanzee, resulting from genetic and fossil data.

NEANDERTALS HAD HUMAN-LIKE HYPOID BONE AND SHARED THE SAME ALLELE OF *FOXP2* GENE

For more than 30 years paleoanthropologists have been debating about the speech capabilities of Neandertals. It is postulated on the basis of anthropometric measurements of Neandertal's and modern human's skulls that probably their ancestor could have used a kind of speech (e.g. Granat *et al.* 2007). Considering genetic traits, it is obvious however that human speech required changes in a number of genes to result in advanced modification of vocal tract morphology (longer oral cavity and lower larynx) which is not present in other primates (Fitch 2000). Moreover, groups of genes responsible for neurological development, capacity for enhanced motor control over the articulators and that contributing to cognitive processing are likely to be involved in acquisition of spoken language (Pinker 1994).

Thus, it is obvious that genetic basis of language and speech are rather complex phenomena and some predict that even more than 500 genes may be involved. However, the role of the only gene known today, *FOXP2* (forkhead box P2), producing language impairments, was recently recognized (Lai *et al.* 2001). Inactivation of one copy of the gene results in deficits in orofacial movements and linguistic processing comparable to Broca's aphasia (Vargha-Khadem *et al.* 2005). At the time the gene was identified, the age of its modern variant was calculated for 200 Kyr on the basis of extant human sequences (Enard *et al.* 2002). It belongs to the sparse group of genes (5%) coding for highly conservative proteins in mammals (Enard *et al.* 2002). Analysis of *FOXP2* in different mammals showed only one sequence substitution arisen in chimps, gorilla and rhesus monkey since their common ancestor with mouse through 75 Myr period. Selection of further two changes in human lineage took only 6–7 Myr. Hunting for Neandertal allele resulted in conformation of two nonsynonymous substitutions (positions 911 and 977 in exon 7), the same as in modern humans (Krause *et al.* 2007b). Two changes in DNA reflect relocation of potential phosphorylation site from amino acid at position 303 (Thr→Asn) to 325 (Asn→Ser). Both residues, together with approx. 90 others amino acids, are involved in so called "forkhead domain". Relocation of phosphorylation site within quaternary structure of the domain of *FOXP2* effects in transcriptional regulation through swapping of domains, the unique structural feature of FOX proteins family, resulting in generation of changes whose nature is still unknown (Kops *et al.* 2002).

Identification of human-like allele in Neandertals moves previously calculated age of *FOXP2* (Enard *et al.* 2002) to the beginnings of the extinct hominines, if not further, reaching population ancestral to Neandertals and humans. These data clearly demonstrate the usefulness of direct typing of ancient DNA templates. Isolation of the templates from other extinct representatives of the genus *Homo,* such as *H. erectus* or at least *H. heidelbergensis*, would shed more light onto modern humans' evolution and could adjudicate the problem of *FOXP2*.

Long-lasting discussion on speech capabilities concerning any species, even ours, started many years ago when bulge of Broca's area was recognized in KNM-ER 1470 endocast (*H. habilis* or *H. rudolfiensis*) found in the 70s of the last century by Richard Leakey (Leakey 1973), although not in *A. afarensis*. Obviously, no macromutation made us speaking. Most likely, speaking as a complex phenotypic feature evolved slowly, in which process the brain evolution was the main factor. *FOXP2* mutations, leading to speech and language impairment, are exemplified in underactivation of Broca's area and activation of many regions on both hemispheres – as found in KE family (Lai *et al.* 2001).

PIGMENTATION IN NEANDERTALS

Skin and hair colour depends on the relative amounts of eumelanin (brown/black) to pheomelanin (yellow/red) pigments cumulated in melanosomes, whose number, size and distribution directly reflect the degree of pigmentation (Ha, Rees 2002, Roberts *et al.* 2006). The ratio of eumelanin/pheomelanin is controlled by melanocortin 1 receptor (MC1R), protein involved in regulation *via* cAMP pathway of three enzymes controlling melanocyte transcription (tyrosinase, tyrosinase-related protein 1 and DOPAchrom tautomerase) and availability of their substrates (tyrosine and cysteine) (De Luca *et al.* 1993). Assembly of MC1R into melanocyte membrane depends mainly on the allele that encodes the protein. Highly polymorphic *MC1R* gene is represented by over 80 known variants associated with a number of phenotypic features in humans, including the occurrence of red hair colour and fair skin (Valverde *et al.* 1995), susceptibility to skin cancer (Rees 2004) and mediation in analgesia mechanisms (Mogil *et al.* 2005). Loss of MC1R

function reflects impairment of G-protein coupling ability or reduced cell surface expression of particular variant (Beaumont *et al.* 2007).

Of alleles associated in modern humans with RHC (Red Hair Colour) no genotype has been identified in Neandertal nuclear DNA. Nevertheless, substitution A→G at nucleotide 919 was found resulting in Arg to Gly change at 307 position of polypeptide chain, never observed in modern humans (Gerstenblith *et al.* 2007). Alteration within the 4th intracellular loop of MC1R receptor protein, which forms amphipatic helix, where the 307 exchange is positioned, can alter its function (Santos *et al.* 2006). Although known variants of protein are altered in fox, cow and sheep to Lys, and in mouse to Met, they still regulate agonist-induced (α-MSH) intracellular cAMP level, and position 307 seems to resist mutations. Neandertal allele Arg307Gly transfected within plasmid DNA into COS-7 cells have expressed protein sensitive to α–MSH stimulation. However, synthesis of cAMP was limited, probably due to reduced receptor assembly into membrane (Beaumont *et al.* 2005) and/or altered efficacy of coupling with G-protein (Rees 2003). Significant reduction in Neandertal MC1R ability to produce high levels of cAMP and, in consequence, limitation of eumelanogenesis yield, suggests not only RHC phenotype variant in our closest extinct relatives, but strongly points out to convergent evolution of the trait which is common for both hominines, questioning possible introgression as well.

RANGE OF NEANDERTAL OCCURRENCE

Until recently, Neandertal mtDNA was sampled and identified at European and west Asians sites. Findings analysed recently (Krause *et al.* 2007a) have confirmed much eastern range of their dispersal. One of them is represented by partial skeleton of a ten-year-old child unearthed in the late 1930s in Teshik Tash, Uzbekistan, some 1,200 kilometres east of the Caspian Sea, which was known until now as the eastern border of Neandertal territory. Remains which have been uncovered at Okladnikov cave in the Altai Mountains, 2,000 kilometres farther east, represent the second set of analysed bones. Identification of Neandertal specific sequences, with only slight modifications, in specimens from Teshik Tash and Okladnikov confirmed their belonging to European and western Asian populations and the latter of analysed remains puts them well into southern Siberia. If their traces are found further east, molecular analysis may help to answer questions on Neandertal – *H. sapiens* or even Neandertal – *H. erectus* relations.

CONCLUSIONS

1. Although methods of ancient DNA analysis become more sophisticated and identification of primary miscoding lesions allows deciphering the code more precisely, two methodological problems still exist: lack of long enough templates, shortened mostly due to double and single breaks, as well as an excess of contaminating contemporary templates. Thus, if one succeeds in isolation and analysis of sequences, careful retrieval of data is recommended, especially when concluding on human evolution pathways.
2. Sequencing of Neandertal nuclear genome and comparison with other hominines is the most prominent task to realize and it may answer a number of questions on our own evolutionary pathways. Huge quantity of information from DNA sequences of extinct species, which might complete morphology based data, is still entrapped in unearthed remains; any kind of sample enriched in readable templates of DNA is of special interest and among them bones and teeth are the most valuable.
3. Identified traits like speech and pigmentation in Neandertals are important, but among yet unidentified genes, of at least equal importance, are e.g.:
   – *MCPH1*, microcephalin gene involved in regulation of brain size during development, positively selected since ~40 Kyr ago, probably after introgression from Neandertals as suggested by Evans *et al.* (2006) and

– *PDHA1*, pyruvate dehydrogenase A1 subunit which does not show evidence of human population expansion; contemporary haplotypes of *PDHA1* confirm first divergence approximately 1.8 Myr years ago and second ca. 200 Kyr years ago (Harris, Hey 1999)**.**

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