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The Neanderthal Genome project and beyond

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Resum. No existeix actualment un consens per a una definició científica de la nostra pròpia espècie. Les anàlisi genòmiques de diferents poblacions humanes estan mostrant una variació interindividual més gran de l'esperada. Per tant, serà molt difícil, sinó impossible, posar un límit a aquesta variació només des dels estudis de les poblacions humanes contemporànies. Amb l'arribada de noves tecnologies d'ultraseqüenciació, ara som capaços de recuperar genomes complets d'espècies extingides, entre elles mamuts i neandertals. El genoma neandertal, recentment finalitzat, ens proporcionarà una referència evolutiva més propera a nosaltres en el temps, que ens ajudarà a descobrir quines variants genètiques estan compartides amb els neandertals i quines són exclusives dels humans moderns. Això ens permetrà generar una definició objectiva de la nostra espècie, si bé consistirà probablement en un complex llistat de variants genètiques en potser un centenar de gens.

Paraules clau: paleogenòmica · evolució humana · neandertal · ultraseqüenciació

Summary. A consensus regarding a scientific definition of our own species does not exist. Genomic analyses from different human populations show an inter-individual variation that is higher than previously expected. Therefore, it will be difficult, if not impossible, to put a limit on this variation from the study of contemporary populations exclusively. With the advent of new ultrasequencing technologies, we are now able to retrieve complete genomes from extinct species, such as mammoths and Neanderthals. The recently completed Neanderthal genome will provide us with a close external evolutionary reference, helping us to identify those genetic variants shared with Neanderthals and those present in modern humans alone. This, in turn, will allow us to generate an objective definition of our own species, although it will probably be based on a complex list of genetic variants in some one hundred genes.

Keywords: paleogenomics · human evolution · Neanderthal · ultrasequencing

Introduction

Ideas on human nature, whose meaning and definition has been debated by all the great philosophers since ancient times, including Aristotle, Descartes, Rousseau, Hobbes, Hume, and Sartre, have not been traditionally examined through the scientific method. More recently, from the field of morphology to that of cognitive science, repeated attempts have been made to discover features or unshared characteristics that would enable us to understand our own uniqueness. Nevertheless, the inherent limitations of the fossil record, as well as the fact of having as an evolutionary reference a species that is far away from us, the chimpanzee (our lineages diverged between 6 and 7 million years ago), have hampered this task. To define ourselves, we have frequently selected features or abilities that seem sublime or superior. It has been said that what makes us human is the capacity for symbolic thought, for language, for creating art, for philosophy, for being self-conscious, for trying to find God. Even so, no one would say that a person with mental deficits, or who is mute, or in a coma, is not human. It is difficult for us to understand that all these are aprioristic conceptions about what we wish would describe us as a species. But this does not necessarily coincide with reality.

An alternative approach that has been undertaken this year is to study current humanity from a genomic point of view. In this sense, ambitious genome projects have been launched, such as the 1000 Genomes Project, which intends to obtain the complete sequence of a thousand human genomes from different groups and geographical areas. This approach, however, seems equally destined to failure as a mechanism to define human characteristics, given the great inter-individual variation that exists within our species. With around 7000 million humans on the planet, we can find mutations in almost any gene, without its bearers losing their human condition. Moreover, some genomic studies have made evident extraordinary

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inter-individual variation associated with the duplications of whole chromosomal segments, a feature that, moreover, seems to be characteristic of the hominid lineage.

But now, at the beginning of the 21st century, we have at our disposal a novel factor that will allow us to establish an objective definition of being human: newly developed ultrasequencing (or massive sequencing) techniques form the basis for the project aimed at obtaining a draft of the Neanderthal genome. The Neanderthals (*Homo neanderthalensis*) were a human species that inhabited Eurasia some 400,000 to 30,000 years ago, becoming extinct following the arrival of our ancestors in Europe, from Africa, some 40,000 years ago [8]. The nature of their interactions with modern humans, the adaptive significance of their morphological features, and the actual reach of their cognitive capacities, are subjects of controversy among human evolution specialists.

The Neanderthal Genome project has not only provided the first genome of an extinct human species, but its results will also mark the philosophy of this century. Thanks to molecular biology, we have revealed a genome that was hidden in our past. The scientific knowledge provided by the project will force us to reconsider the meaning of the concept of humanity; it will define us as a species and help us to find, after so many thousands of years, our definite place in the natural world.

Paleogenomics

In the last three years, the study of remains of genetic material from the past, often referred to as ancient DNA and from which only a very modest volume of information has been retrieved [7], has fully entered the paleogenomic era. Paleogenomics is the study of the sequence, structure, and function of extinct genomes, both the nuclear genome, which includes the immense majority of the genetic message of an organism (with an average of 3200 million nucleotides in the case of humans), and the cytoplasmic one (likewise, approximately 16,500 nucleotides) [7], that is, the mitochondrial genome (mtDNA) and the chloroplastic genome (cpDNA, being present only in plants). Given the large number of copies of these cytoplasmic genomes compared to the nuclear genome (the empirical proportion is of 500–1000 to 1), for more than 20 years research carried out on ancient DNA had been based on the analysis, using the polymerase chain reaction (or PCR) of mtDNA or cpDNA, of extinct species, normally with phylogenetic or phylogeographic purposes. *A priori*, there are no determining factors—except those which derive from the greater difficulty associated with conservation—prohibiting the recovery of fragments from the nuclear genome; however, only in exceptionally preserved samples is it possible to access nuclear data.

In the year 2006, the first complete nuclear gene from an extinct species was recovered, by PCR; in this case, a single exon of a pigmentation gene of the woolly mammoth [18]. Subsequently, from Neanderthal samples, genes involved in physical external aspects, which are not conserved in the fossil record, have been recovered, such as those determining hair color (the gene MC1R), blood group (ABO), or cognitive capacities associated with language (FOXP2) [9–11]. These studies have contributed to create a closer and more real image of Neanderthals, through knowledge of their individual characteristics. In this way, we have not only advanced in our evolutionary understanding of this human species, but we have also contributed to personalizing the studied individuals, and in this way in humanizing them.

Despite this success, it is evident that with specific PCR-based approaches the recovery of large chromosomal regions cannot be expected. We know that the majority of DNA fragments conserved in a Neanderthal sample have an average length of only 50–70 nucleotides. In practice, that means that if we try to recover larger fragments, the probability of success will correlate directly with the percentage of conserved fragments of this length. Even if we try to recover, in a laboratory reaction, a short fragment of only 60 nucleotides, the results have a good chance of being negative, simply because genomic coverage of the ancient samples is very low and we might well be unsuccessful in finding the searched for chain in that particular PCR. In this sense, the recovery of a complete mitogenome of an extinct species (the moa, gigantic birds of New Zealand) in 2001 [1], marked, and still marks, the longest DNA length recovered using current technical procedures, since the consumption of extract DNA is very high in samples that are frequently unique and this procedure is excessively slow and painstaking. In other words, at the beginning of the 21st century, we are technologically stymied with regard to the possibilities of exploring ancient DNA in the genomic era, just as we have broken new ground with the sequencing of the human genome.

The new techniques of ultrasequencing

Nonetheless, at the end of the year 2005, a new technique appeared, ultrasequencing (massive production of DNA sequences), developed by the biotechnology firm Life Sciences [12] and referred to as 454 pyrosequencing. Originally, the 454 technology (GS FLX) produced around 250,000 sequences up to 200–250 nucleotides long in a single reaction. The improved version, released at the end of 2008 (the Titatium upgrade), yields fragments of up to 400 nucleotides. In parallel, other sequencing platforms have appeared in which the quantity of sequences generated per reaction is higher, but the fragment length is shorter, generally around 30 nucleotides. Among these, the Solexa platform, the so-called SOLID from Applied Biosystems, and a new one that sequences simple DNA chains without previous amplification, called Helicos, stand out. Obviously, we do not know which technology will prevail in the next two or three years, but they all have in common the possibility of generating millions of sequences per reaction, and therefore, to access extinct genomes, with the only limitations being purely economic ones. Through these technologies, paleogenetics is changing from a craft science to an industrial production one, and from being basically experimental to being mostly bioinformatical.

The new genomic projects devoted to extinct species are of the non-specific or metagenomic type. By metagenomic, we refer to the sequencing of a sample for which it has not been

possible to isolate the different organisms that comprise it. This would require that every sequence obtained is subsequently identified through alignments with the available genetic databases. Ancient bone samples not only contain the DNA, trapped in crystals of the hydroxyapatite matrix, of the individual when he or she was alive, but also great quantities of DNA from soil bacteria, fungi, etc., living in the sediments or that have colonized the bone. Ultrasequencing techniques generate massive quantities of sequences of a determinate ancient extract, without prior selection. Thus, in general, the process is extremely inefficient, with recovery percentages of 0.27 [17] to 4% [6] in samples from temperate latitudes such as Europe, and of up to 40–50% in samples conserved in the frozen soil of Siberia (mostly examined together with mammoth bones) [13]. However, the massive amounts of genetic data that are generated allow for significant parts of any genome to appear, even in unfavorable taphonomic conditions.

The Neanderthal Genome project

The Neanderthal Genome project is an ambitious scientific project led by Svante Pääbo, from the Max Planck Institute of Evolutionary Anthropology of Leipzig (Germany), and carried out in collaboration with the private company, 454 Life Sciences (USA), which currently belongs to Roche. The project began in July 2006, with the objective of generating a Neanderthal genomic draft within a deadline of two years. The achievement of this goal was publicly presented on 12 February 2009, Charles Darwin's 200th birthday [16].

The preliminary results of this project were published in *Nature* and in *Science* in the year 2006, from the data generated in a Neanderthal sample from the Croatian site of Vindija, labeled as Vi 33.16 [5,14,15]. In the *Nature* article, the data generated by 454 technology were shown, consisting of a total of 254,933 sequences. Of these, only 15,701 (6.2%) were human or similar sequences (as we would expect from the Neanderthals), while approximately 40,000 were sequences from bacteria, fungi, or other microorganisms. Some 200,000 sequences (79%) could not be related to an equivalent in the genetic databases, probably because they corresponded to bacteria not yet studied. Of the human sequences, 41 corresponded to mitochondrial DNA fragments, and 15,701 to almost a million nucleotides of the Neanderthal genome (i.e., close to 0.04% of the total genome). These sequences had an average length of ~60 nucleotides, ranging from 30 (shorter sequences were eliminated in the analysis) to 280 (the limit imposed by the 454 technique). From the total of 1 million nucleotides, 739,966 were identical in Neanderthals, modern humans, and chimpanzees, and 10,208 were identical in Neanderthals and humans, but different in chimpanzees. Finally, 422 nucleotides were unique in the human linage and 3447 in the Neanderthal lineage (of the latter, most corresponded to post-mortem chemical damages due to alterations in the original cytosines, a typical pattern observed in ancient DNA) [5,14].

Later, however, this work was criticized because there were several indications pointing to contamination of the extract with modern DNA, an accident that probably took place at the Life Sciences firm itself. Some researchers discovered, while analyzing the data, that the longest sequences (more than 100 nucleotides) gave genomic divergence times between Neanderthals and Cro-Magnons that were absurdly recent, while the shorter sequences gave dates close to 800,000 years (which would be consistent with the separation of close to half a million years, established from mitochondrial DNA). These results could only be explained if the longest sequences were recent contaminations and, therefore, had not had sufficient time to fragment as much as the original DNA [21]. Some researchers placed this possible contamination at >80% of the obtained sequences, although subsequent pyrosequencing reactions of the original extract to quantify the contaminating sequences of the mitochondrial DNA reduced it to 11% [6].

In spite of these methodological problems, seemingly inevitable with the rush to publish the first results, the completion of the Neanderthal mitogenome of Vindija 33.16 [6], with a provable contamination of just 0.3%, demonstrated the potential of a metagenomic approximation in the achievement of the Neanderthal genome.

Solving the problem of contamination

As we have seen, the main obstacle to paleogenetic studies in humans is the contamination of samples with modern human DNA, a complex and still poorly studied problem. Since the techniques used in the laboratory are extremely sensitive (in that reactions can initiate from a single DNA chain) and the original DNA may be chemically degraded, making it less responsive, the result can be the recovery of contaminating DNA rather than the endogenous DNA of interest.

This is irrelevant when working with an extinct species, such as the mammoth, from which 80% of the genome has been recovered, as it is impossible that the sample has been contaminated with modern elephant DNA before it reached the laboratory, or even in the laboratory, if there was no previous work with the Proboscidea. In the case of human samples, however, the problem of contamination is severe, simply because it cannot be distinguished *a posteriori* and because it is difficult to know whether the anti-contamination precautions taken *a priori* have been truly effective. The latter include genotyping of the individuals involved in the excavation and the adoption of preventive measures, such as the use of laboratory clothing, sterile gloves, and facial masks, during the excavation. These are some of the measures that have been adopted in the excavation of the Neanderthal site of El Sidrón, in Asturias (Spain), where samples are not only extracted with anti-contamination measures, but are also immediately frozen and sent to the genetics laboratory (Fig. 1) [4]. In the mtDNA, where it is possible to easily distinguish endogenous sequences from contaminating ones, establishment of an anti-contamination protocol in the year 2005 resulted in near complete elimination of the problem. This is evidenced by the spectacularly low proportion of contaminating sequences, only 0.27%, determined in a recent analysis of the mitogenome contained in sample 1253 from El Sidrón.

Fig. 1. Method of excavation in the cave of El Sidrón (Asturias), following the anti-contamination protocol designed to eliminate the possibility of contamination of Neanderthal samples with modern human DNA. The samples are excavated by individuals wearing sterile suits and instruments and are frozen upon their collection, i.e., before they are sent to the molecular biology laboratories (in Barcelona and Leipzig) involved in the Neanderthal Genome project.

El Sidrón and the Neanderthal Genome project

On 20 March 2007, in a press conference at the National Museum of Natural Sciences of Madrid, the inclusion of the El Sidrón site in the Neanderthal Genome project, which until then had been based solely on the Vindija samples, was publicly announced.

El Sidrón is located in the Plioña municipality, in Asturias, in a Neanderthal site accidentally discovered by speleologists in 1994. It is not a cave where Neanderthals lived (or where intentional burials are found), but a deep karst system where, in a small gallery adjacent to the central cave, the remains of at least ten Neanderthal individuals accumulated, due to the collapse of a sinkhole on the surface [19]. The gallery, known as the Ossuary Gallery, is located some 220 m from the karst's entrance, and the possible entry route to the remains has been sealed since antiquity. This has allowed for conservation of the remains at an extremely constant and cold temperature, between 10ºC and 12ºC, which would help to explain the excellent conservation of the genetic material. New carbon-14 datings give values of around 49,000 years, and indicate that the accumulation of the ten individuals (six adults, two teenagers, one juvenile and one child) in the cavity was a synchronic event. In all likelihood, is was a family group that had been cannibalized, since most of the remains show cuts intended to slice muscle and tendon insertions, and sharp blows destined to break long bones and crania, to reach the bone marrow and the brain (Fig. 2) [19]. Curiously, both the Vindija material and the El Sidrón material show signs of cannibalism, which could account for their good genetic conservation, since a large part of the microbial action involved in the decomposition of bodies, which damages their DNA, was thereby eliminated.

Fig. 2. Fragment of an adult male femur, labeled as El Sidrón SD1253, extracted using the anti-contamination protocol. This sample is currently one of the best Neanderthal samples in terms of genetic conservation.

El Sidrón is being studied by a multidisciplinary Spanish team. The excavation is led by Javier Fortea (deceased on 1 October 2009), together with Marco de la Rasilla (both from the University of Oviedo); the paleontology study is headed by Antonio Rosas (from the National Museum of Natural Sciences of Madrid), and the genetic analyses by Carles Lalueza-Fox (from the Institute of Evolutionary Biology, Barcelona). Although in the samples of El Sidrón we initially worked to specifically recover mitochondrial DNA as well as nuclear genes of evolutionary interest, the possibility to collaborate with the group from the Max Planck Institute will allow us to take the site to a genomic scale of a magnitude unsuspected some years ago.

After the genomic draft

In reality, the genomic draft of Vindija 33.16, at the date of this writing, in 2009, has a genomic coverage of 1×, which means that approximately 63% of the genome will have some sequence. To reach the figure of 3700 million Neanderthal nucleotides requires approximately 68,900 million sequences, most of which has been bacterial DNA [16]. Obviously, in the draft, there are many blank spaces and many genetic changes that have to be proven and confirmed by specific means, be it PCR or others that have yet to be developed. It has been calculated that to obtain a genomic draft with a rate of error of 1 every 10,000 nucleotides (more than acceptable in projects with living species), a genomic coverage of 12× would be necessary (in other words, that every nucleotide was represented, on average, by 12 different superimposed sequences) [6]. To achieve this level of reliability, another genomic draft in a very well preserved sample, such as the one from El Sidrón 1253, would have to be generated, and probably using a technique with greater data generation ability than 454. This second genomic draft will provide us with information on the genetic variations that may be polymorphic in Neanderthals and contribute to confirm the results generated in the first draft. That is, we will have gone from a single initial genome to an authentic study of genomic diversity in Neanderthals, something similar, albeit on a smaller scale, to what is being done with the genomic diversity of humans. We are lucky, as well, that two of the best Neanderthal sites, in terms of DNA conservation, Vindija in Croatia and El Sidrón in Spain, occupy not only a good part of the geographical range of Neanderthals but also cover different moments in time. While Vindija is quite recent, close to 40,000 years, El Sidrón clearly precedes the arrival of our ancestors in Europe. Therefore, by comparing results obtained from the two sites we could deduce the possible contributions of modern humans to the genome of the Vindija sample (if any of its ancestors was a hybrid of a Neanderthal and modern human).

When all the ultrasequencing work and bioinformatics analyses are finished, we will have a long list of genes whose variation will be distributed differently between the three species for which we have genomic data: chimpanzees, Neanderthals, and humans. Obviously, many genes will be identical among the three species, since close to 14,000 of the 24,000 genes that have been identified in our genome are the same in humans and chimpanzees. It has been estimated that between Neanderthals and humans there will be around one hundred functional changes, that is, modification of the amino acids in codifying regions.

We will also have genes that are identical in humans and Neanderthals and different in chimpanzees. The gene FOXP2, associated with cerebral areas involved in language and which is marked by two functional changes in the two species of the human lineage [1,3], is an example of this category of genes. Simply stated, it implies that the evolutionary changes associated with language must have taken place before separation of the two lineages, i.e., in the common ancestor. The gene of the blood group ABO is another interesting example. Characteriza-

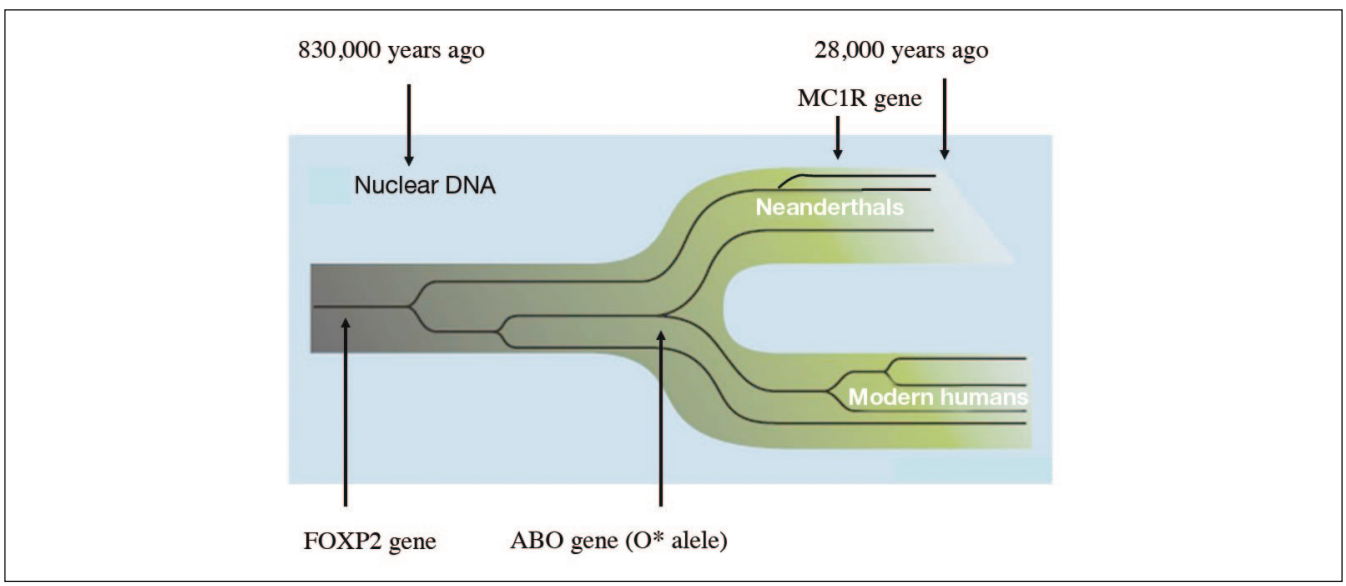

Fig. 3. Schematic representation of the phylogenetic tree of modern humans and Neanderthals, showing also the phylogeny of nuclear genes (the genetic tree). The three examples of nuclear genes, the language gene (FOXP2), the pigmentation gene (MC1R), and the blood group gene (ABO), occupy different positions in the diagram.

tion of this gene in both male samples of El Sidrón, 1253 and 1351c, revealed that these individuals were of blood group O, for the same reason—a deletion of a nucleotide in exon 6—as occurs in modern humans [12]. This is not surprising, but illustrates something that will be common in the Neanderthal genome as well: that fact that, for certain genes, some humans are more closely related to Neanderthals than to other humans (in this case, those with blood groups A, B, or AB).

A few of the genes will contain changes that are unique in the branch of the Neanderthals and thus may help us understand their particular evolutionary characteristics, some already discernible at the skeletal level. The gene MC1R, associated with pigmentation, shows variations that are unique to Neanderthals (such as a change in amino acid 307), although, curiously, some changes produce a phenotype similar to that seen in Europeans (redheaded individuals) through other variations in the same gene (Fig. 3) [2,10,20]. This suggests that other morphological features (such as the cerebral expansion observed in several branches of the human evolutionary tree) could be a consequence of processes of convergent evolution, due to genomic restrictions in the adaptation of hominids. That is, although mutations in the genome occur randomly, the distribution of such mutations does not, since their final effect in the phenotype is influenced by the function and structure of the genes and their possible interactions. The changes exclusive to Neanderthals must be proven individually, through functional studies such as the one carried out for MC1R [10]. Some of the genetic variations unparalleled in modern humans will require the implementation of animal models, basically through the "Neanderthalization" of mice (in other words, transgenic mice carrying Neanderthal genes), to understand their final phenotypic effect. These types of studies can require many years, since it has been calculated that there are some one hundred such functional changes exclusive to Neanderthals. Finally, we will have another group of genetic changes that will be particularly interesting: those which are common to Neanderthals and chimpanzees, but different (or unique) in modern humans. These variations, of which examples are as yet lacking, will be the ones that allow us to understand the final stretch of human evolution.

In conclusion, when the genomic drafts have been analyzed, the fact that we will have a new evolutionary reference, much closer to us than that of chimpanzees, will allow us to characterize those genetic variations exclusive to our species and those which we share with other human species of the past. By studying the genes exclusive to our lineage, we will understand the selective pressures that have acted upon our species since its African origin, some 200,000 years ago, and perhaps be able to define what it means to be human. In all likelihood, this definition will consider the complex list of genes related to immunity, metabolism, and physiology. But it also may be the case that there are only a very few changes associated with the development of cerebral areas or with readily interpretable cognitive functions. Surely, we like to think of ourselves as different, as was presumed when our species was the only one that we could study in so much detail, including at its most intimate level, the level of its DNA. But, in any case, this list will be objective, and as such will finally allow us to close in, from a scientific perspective, on the answer to the mystery of what it means to be human.

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